THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 44, pp. 32053–32064, November 2, 2007

The DNA Damage Response Mediator MDC1 Directly Interacts with the Anaphase-promoting Complex/Cyclosome*

Received for publication, July 18, 2007, and in revised form, September 6, 2007 Published, JBC Papers in Press, September 7, 2007, DOI 10.1074/jbc.M705890200

Gideon Coster[‡], Zvi Hayouka[§], Liron Argaman[‡], Carmit Strauss[‡], Assaf Friedler[§], Michael Brandeis[‡], and Michal Goldberg[‡1]

From the [‡]Department of Genetics, Alexander Silberman Institute of Life Sciences and [§]Department of Organic Chemistry, Hebrew University of Jerusalem, Jerusalem 91904, Israel

MDC1 (NFBD1), a mediator of the cellular response to DNA damage, plays an important role in checkpoint activation and DNA repair. Here we identified a cross-talk between the DNA damage response and cell cycle regulation. We discovered that MDC1 binds the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that controls the cell cycle. The interaction is direct and is mediated by the tandem BRCA1 C-terminal domains of MDC1 and the C terminus of the Cdc27 (APC3) subunit of the APC/C. It requires the phosphorylation of Cdc27 and is enhanced after induction of DNA damage. We show that the tandem BRCA1 C-terminal domains of MDC1, known to directly bind the phosphorylated form of histone H2AX (γ-H2AX), also bind the APC/C by the same mechanism, as phosphopeptides that correspond to the C termini of γ-H2AX and Cdc27 competed with each other for the binding to MDC1. Our results reveal a link between the cellular response to DNA damage and cell cycle regulation, suggesting that MDC1, known to have a role in checkpoint regulation, executes part of this role by binding the APC/C.

The cellular response to DNA damage is crucial for protecting the cells from genomic crisis. DNA damage in the cell activates the DNA damage response, which includes activation of cell cycle checkpoints, repair of the damage, transcriptional regulation and, if damage is excessive or unrepairable, activation of apoptosis. Proteins involved in the DNA damage response include sensors that detect the damage, transducer kinases that signal to downstream effectors, and mediators that mediate the signal from the transducer kinases to the effectors that execute the response itself (1).

MDC1 (also known as NFBD1) is a mediator of the DNA damage response, playing a role in the maintenance of BRCA1 and the Mre11·Rad50·Nbs1 complex at sites of damage, the activation of Chk1, BRCA1, and DNA-PKcs, and in DNA double-strand break repair. Furthermore, MDC1 is required for the establishment of the G_2/M and intra-S phase DNA damage checkpoints, suggesting a role in cell cycle regulation (2–7). Similar to other mediator proteins (*e.g.* BRCA1 and 53BP1), MDC1 is a large protein that contains two consecutive BRCA1 C-terminal (BRCT)[2] domains. In addition, MDC1 contains an N-terminal forkhead-associated (FHA) domain (1–5, 8, 9). FHA domains are phosphoprotein binding domains commonly found in signaling proteins (10). BRCT domains are found in many proteins that regulate the DNA damage response, and tandem BRCT domains (tBRCT domain) were demonstrated to be phosphoprotein binding modules (11–13). The consensus phospho binding sequence for the tBRCT domain of MDC1 was determined by peptide library screening as pSI(E/V/D)(Y/F) (pS is phosphoserine) (12). Recently, the tBRCT domain of MDC1 was found to interact directly with the phosphorylated form of histone H2AX (γ-H2AX), and the consensus for the binding of the tBRCT domain of MDC1 was further studied and determined as pS*X*(E/I/V)Y-COOH, suggesting that the phospho-residue should be four amino acid residues before the C terminus of the protein (14).

MDC1 functions as an adaptor protein, recruiting different proteins that have a role in the DNA damage response to the transducer kinases and to sites of DNA damage and, thus, facilitating the signal transduction after DNA damage. Therefore, it is likely that MDC1 interacts with many proteins to execute its role in the DNA damage response. The mechanism(s) by which MDC1 carries out its role is not fully understood. Here we report that the tBRCT domain of MDC1 interacts directly and in a phospho-dependent manner with the anaphase-promoting complex/cyclosome (APC/C). The APC/C is an E3 ubiquitin ligase composed of at least 12 subunits. It is required for cell cycle control and is active from early mitosis through late G_1. During this time the APC/C targets many critical regulators of the cell cycle for degradation and is essential for proper cell cycle progression (15–17). The APC/C acquires its substrate specificity via two co-activators, Cdc20 and Cdh1. Cdc20 is active during the metaphase-to-anaphase transition, whereas Cdh1 is active during later stages of mitosis and G_1 (18, 19). We further show that the interaction between MDC1 and the APC/C is mediated by the Cdc27 (APC3) subunit of the APC/C and enhanced upon DNA damage

* This research was supported by Israel Science Foundation (ISF) Grant 619/04 and Association for International Cancer Research (AICR) Grant 06-257 (to M. G.), by ISF Grant 598/02 and AICR Grant 05-012 (to M. B.), and by a Bikura grant from the ISF and a Human Frontier Science Program Career Development Award (to A. F.). 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.

[1] To whom correspondence should be addressed: Dept. of Genetics, Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel. Tel.: 972-2-6586452; Fax: 972-2-6586975; E-mail: goldbergm@vms.huji.ac.il.

[2] The abbreviations used are: BRCT, BRCA1 C-terminal; tBRCT, tandem BRCT; tBRCTm, K1936M mutated tBRCT; FHA, forkhead-associated; APC/C, anaphase-promoting complex/cyclosome; GST, glutathione *S*-transferase; HA, hemagglutinin; GFP, green fluorescent protein; IR, ionizing radiation; IP, immunoprecipitation; MS, mass spectroscopy.

induction. Our results propose a link between the cellular response to DNA damage and cell cycle regulation.

EXPERIMENTAL PROCEDURES

Plasmids—Glutathione *S*-transferase (GST)-FHA, GST-FHAm, and GST-tBRCT were previously described (2). GST-BRCT1, GST-BRCT2, and GST-linker were created by amplifying the relevant fragments by PCR using GST-tBRCT as a template. PCR fragments were cloned into pGEX-4T-3. GST-tBRCTm (K1936M mutant) was generated from GST-tBRCT using the QuikChange site-directed mutagenesis kit (Stratagene). Hemagglutinin (HA) MDC1 was generated from the KIAA0170 cDNA cloned into pcDNA3 that contains a double HA tag (pcDNA3-HA). HA-tBRCTm (K1936M mutant) and HA-ΔBRCT were generated from HA-MDC1 using the QuikChange site-directed mutagenesis kit. HA-FHA and HA-tBRCT were generated by subcloning the FHA or tBRCT domains from GST-FHA or GST-tBRCT, respectively, into pcDNA3-HA. GST-tBRCT-53BP1 was received from A. J. Doherty (20). Green fluorescent protein (GFP)-Cdc27 was a kind gift of C. Hoog (Karolinska Institute, Stockholm, Sweden). GFP-Cdc27-C and GFP-Cdc27ΔC, composed of amino acid residues 664–824 and 1–666, respectively, of Cdc27, were subcloned from GFP-Cdc27. S821A, S821D, T792A, S803A, and T814A mutants were generated from GFP-Cdc27 or GFP-Cdc27-C using the QuikChange site-directed mutagenesis kit. All vectors were sequenced to verify proper cloning.

Cell Culture, Extract Preparation, Protein Expression, and Purification—293T, MCF7, and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. Nuclear protein extracts were prepared according to Dignam *et al.* (21), and high salt protein extracts were prepared according to Li *et al.* (22). Extracts were prepared from undamaged cells, cells treated with different gray doses of ionizing radiation (IR; using Faxitron Cabinet X-Ray system, model RX-650, Faxitron X-Ray Corp.), cells treated with 50 J/m^2 of ultraviolet (UV) light, or 100 μg/ml of the radiomimetic drug phleomycin. For phosphatase studies extracts (~1 mg) were incubated with 400 units of λ-phosphatase (New England Biolabs) in the presence of 2 mM Mn^{2+}-only or 2 mM Mn^{2+} and 50 mM EDTA for 30 min at 30 °C before pulldown or immunoprecipitation (IP) assays were done. GST-fused recombinant proteins were expressed in *Escherichia coli* strain BL21 and purified on glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences).

Peptides—Peptides were synthesized and labeled as described (23). Phosphoserine was coupled manually to the peptides. Fmoc-Ser [PO(benzylester)OH]-OH (1.5 eq), hydroxybenzotriazole (1.5 eq), and diisopropylethylamine (1.5 eq) were dissolved in dibromomethane, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (1.5 eq) was added, and the solution was stirred for 15 min. The solution was added to the preloaded resin, warmed to 60 °C, and shaken overnight. The resin was washed with dibromomethane and loaded back onto the peptide synthesizer. Biotin or fluorescein labels were added to the N terminus of the peptides using the following procedures. For biotinylation, biotin (Sigma; 5 eq) was dissolved in dimethylformamide/Me_2SO 1:1. PyBop (5 eq) and diisopropylethylamine (10 eq) were added, the activated biotin solution was added to the preloaded resin, and the mixture was shaken overnight. The resin was washed with dimethylformamide/Me_2SO (1:1, 3 times) followed by dichloromethane (3 times) and methanol (3 times) and dried by vacuum. For fluorescein, peptides were labeled using 5′ (and 6′) carboxyfluorescein succinimidyl ester (Molecular Probes) as described (23).

Peptides used in this study were Cdc26 peptides (NNRSSQFGSLEF and NNRSSQFGpSLEF) and Cdc27 peptides (ADDTQLHAAESDEF and ADDTQLHAAEpSDEF). Biotinylated histone H2AX peptides (SGSTVGPKAPSGGKKATQASQEY and SGSTVGPKAPSGGKKATQApSQEY) were a kind gift of M. Stucki (University of Zurich, Zurich, Switzerland).

Antibodies—The commercial antibodies used in this study were monoclonal anti-Cdc27 (BD Transduction Laboratories) and APC7 (Santa Cruz Biotechnology) antibodies. Anti-MDC1 antibodies included rabbit and sheep anti-MDC1 directed against the FHA and tBRCT domains of MDC1 (2) and mouse anti-MDC1, clone MDC1–50 (Sigma-Aldrich). Rabbit anti-Cdc16 (1443, a gift of P. Hieter, University of British Columbia, Vancouver, Canada), monoclonal AF3.1 anti-Cdc27, and AR38 anti-Cdh1 (a gift from J. Gannon, Cancer Research UK, Clare Hall Laboratories, UK) antibodies were used. Also used as controls were sheep IgG antibodies (Sigma-Aldrich), mouse anti-Myc (9E10), and monoclonal anti-GST (Santa Cruz Biotechnology).

Immunoprecipitation, GST, and Peptide Pulldown Assays—GST pulldown assays were done with bacterially expressed and purified indicated GST fusions and glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences). IPs were done with the indicated antibodies and protein A- or G-Sepharose beads (Santa Cruz Biotechnology or Roche Applied Science). Nuclear protein extracts (0.5–1 mg) or high salt protein extracts (1–2 mg) were added to the IP or GST pulldown assays. Peptide pulldown assays were done using 350 pmol of biotinylated peptides and 20 μg of purified GST-tBRCT in the presence of 20 μl of streptavidin-coated Dynabeads M-280 (Dynal). Beads were washed extensively with wash buffer (20 mM HEPES, pH 7.4, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2% Triton X-100, 150 mM NaCl), and bound proteins were subjected to SDS-PAGE and Coomassie Blue staining or immunoblotting.

Identification of the Interaction between the tBRCT Domain of MDC1 and the APC/C—Proteins from 293T nuclear extracts that were retrieved by GST-tBRCT were separated by SDS-PAGE. Bound proteins were stained first with PageBlue (Fermentas), and positive bands were cut from the gel. Positive bands included bands that appeared in the GST-tBRCT-plus extract reaction but not with GST-tBRCT without extract or with GST plus extract. The gel was re-stained with silver, and additional bands were cut from the gel. Protein analysis and identification of the different bands were carried out at The Smoler Proteomics Center (Technion, Haifa, Israel). Briefly, all samples were digested by trypsin, analyzed by liquid chromatography-mass spectrometry (MS)/MS on ion-trap mass spectrometers (DECA/LCQ) and identified by Pep-Miner software (24) against human nucleotide data base.

Blot Overlay—Wild-type GFP-Cdc27-C or S821A was overexpressed in 293T cells, and protein extracts were prepared 1 h after exposure to 15 gray of IR. Equal amounts of extract were

TABLE 1
Identification of proteins that bind the tBRCT domain of MDC1 by mass spectometry

Peptide mass	Matching peptide	Protein	Observed M_r	Theoretical M_r
Da				*KDa*
2288.1	FSEQGGTPQNVATSSSLTAHLR	APC1	240	216
931.5	LILTGAESK	APC5	75	85
1856.9	LIEESCPQLANSVQIR	APC5	75	85
1632.8	QTAEETGLTPLETSR	APC6	70	71

either resolved by SDS-PAGE or used for GST pulldown experiments with GST-tBRCT. GFP was visualized directly in-gel (samples were incubated with sample buffer at 37 °C for 5 min before SDS-PAGE and visualized using either FUJIFILM FLA-3000 or a FUJI LAS-3000 imaging system), and separated proteins were then transferred to a nitrocellulose membrane and blocked overnight in 8% powder milk (Marvel) in TBST (25 mM Tris, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.1% Tween 20) at 4 °C. The membrane was then incubated for 1 h at room temperature with GST-tBRCT or GST-tBRCTm at a concentration of 10 μg/ml in 4% powder milk in TBST. The membrane was rinsed with TBST and incubated with antibodies directed against the tBRCT domain of MDC1. The membrane was then washed in TBST and incubated with a peroxidase-conjugated secondary antibody. Peroxidase activity was detected with the EZ-ECL chemiluminescence system (Biological Industries) and documented on a FUJI LAS-3000. GST pull down with λ phosphatase was performed as described above, with the exception that just before SDS-PAGE λ phosphatase (Upstate) was added to the glutathione beads and incubated for 10 min at 37 °C.

Phosphorylation Analysis by Mass Spectrometry—Wild-type GFP-Cdc27-C was overexpressed in 293T cells, and protein extracts were prepared 1 h after the addition of phleomycin (100 μg/ml). The extract was used in a GST pulldown experiment with GST-tBRCT to enrich the phosphorylated form of GFP-Cdc27-C. After extensive washing, the beads were split into two tubes, and λ-phosphatase was added to one sample. Both samples were incubated at 37 °C for 10 min, sample buffer was added, and samples were separated by SDS-PAGE. GFP was visualized directly in-gel, and both bands were excised for mass spectrometry analysis. Reduction, alkylation, and trypsinization steps were carried out in-gel as described (25). Peptides were extracted from the gel with 60% CH3CN, 1% CHOOH, evaporated to dryness, rehydrated with 1 μl of CH3CN, 1% CHOOH, and then diluted with 9 μl of 1% Formic acid. The peptide mixture was solid phase-extracted with a C18 resin-filled tip (ZipTip Millipore, Billerica, MA) and nanosprayed into a Q-TOF-2 MS system (Micromass) in 50% CH3CN, 1% CHOOH solution using a nanospray attachment (26). Data analysis was done using the Biolynx package (Micromass), and data base searches were performed with the Mascot package (Matrix Science). Peptides were positively identified by analysis of MS spectra and subsequent MS/MS spectra.

Anion Exchange Chromatography—Protein extract from HeLa S3 cells either untreated or treated with 10 gray and left to recover for 1 h, was diluted 1:7 with buffer A (50 mM Tris, pH 7.5, 1 mM dithiothreitol, and 100 mM NaCl) and loaded onto a Q-Sepharose FF column. Bound proteins were eluted with buffer B (as buffer A except 1 M NaCl) with a two-step gradient (60 and 100%). Collected fractions were concentrated by acetone precipitation and analyzed by SDS-PAGE and Western blotting.

RESULTS

The tBRCT Domain of MDC1 Interacts with the APC/C—To gain further knowledge about the functions of MDC1, we set out to identify proteins that interact with the tBRCT domain of MDC1. We used the tBRCT domain of MDC1 fused to GST (GST-tBRCT) as an affinity matrix to purify proteins that associate with MDC1. Several proteins were retrieved, among them proteins with relative molecular masses of ~240, ~75, and ~70 kDa. These bands did not appear in the controls, which included pulldown experiments of GST alone with extract and of purified GST-tBRCT without extract. Mass spectrometric analysis identified these proteins as APC1, APC5, and APC6 (Table 1), all of which are subunits of the APC/C (16). The apparent size, which was estimated by comparison to protein size marker, correlated well with actual molecular weights of these proteins (Table 1). Western blot analysis confirmed that GST-tBRCT efficiently retrieves the APC/C from protein extracts (Fig. 1*A*). Three different subunits of the APC/C, Cdc27, APC7, and Cdc16 (APC6), and one of the co-activators of the APC/C, Cdh1, which binds the APC/C from late-mitosis until the end of G_1 (16, 17), were retrieved by the tBRCT domain of MDC1 (Fig. 1*A* and data not shown). Notably, Cdh1 appears as two distinct bands in the input but only the faster migrating form is retrieved by the tBRCT domain of MDC1 (Fig. 1*A*). This is the unphosphorylated, active form of Cdh1 that binds the APC/C (27, 28). Thus, it seems that MDC1 interacts with active APC/C. These results were reproduced using extracts prepared from different cell lines (data not shown). The FHA domain of MDC1 or a mutated version of this domain fused to GST or GST alone did not retrieve the APC/C from protein extracts (Fig. 1*A*). In addition, the tBRCT domain of 53BP1, which, like MDC1, is a mediator of the DNA damage response, did not retrieve the APC/C from protein extracts (Fig. 2*A*), indicating that this interaction is specific to the tBRCT domain of MDC1.

To determine whether MDC1 interacts with the APC/C *in vivo*, we performed co-immunoprecipitation (co-IP) experiments. Polyclonal antibodies directed against different regions of MDC1 (the FHA and tBRCT domains) efficiently co-immunoprecipitated different subunits of the APC/C (Cdc27, Cdc16, and APC7) and the co-activator Cdh1 (Fig. 1*B* and data not shown). Notably, the antibodies directed against the FHA domain of MDC1 co-immunoprecipitated Cdc27 and Cdc16 more efficiently compared with the antibodies directed against its tBRCT domain (Fig. 1*B*, quantification). Because the tBRCT domain of MDC1 mediates the interaction with the APC/C and

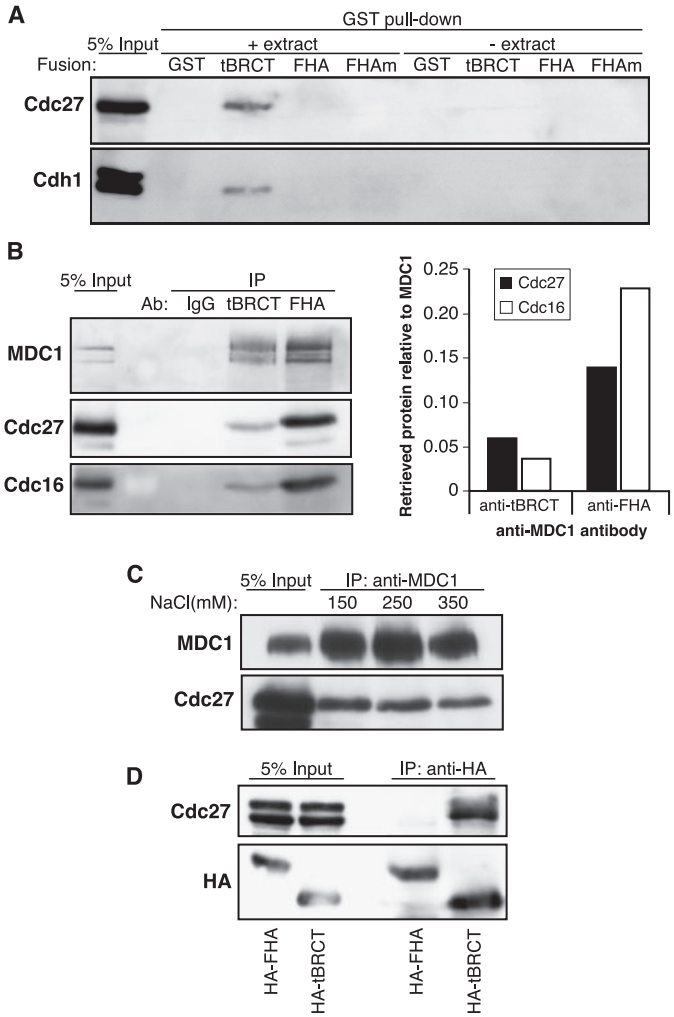

FIGURE 1. **MDC1 interacts with the APC/C.** *A*, GST-tBRCT retrieves Cdc27 and Cdh1 from extracts. GST pulldown assays were performed with GST-tBRCT, GST-FHA, GST-FHAm, or GST alone with or without nuclear extracts from 293T cells. *B*, subunits of the APC/C co-IP with MDC1. Antibodies directed against the FHA or tBRCT domains of MDC1 were used to IP MDC1 from protein extracts prepared from 293T cells. Control antibodies for the IP were species-matched sheep IgG. The lighter background seen near the input in the Cdc16 lane is caused by the 70-kDa marker. Western blot signals were measured and quantified. For each experiment band intensity of the IP was normalized to the corresponding input, giving percentage of retrieved protein. For Cdc16 and Cdc27 this value was normalized to the percentage of retrieved MDC1. *Ab*, antibody. *C*, the interaction between MDC1 and the APC/C is stable. MDC1 was immunoprecipitated from nuclear extracts and washed with indicated salt concentrations. *D*, the tBRCT domain of MDC1 mediates the interaction with the APC/C *in vivo*. Protein extracts were prepared from 293T cells overexpressing HA-FHA or HA-tBRCT. The HA-tagged proteins were immunoprecipitated using an anti-HA antibody. Bound proteins were separated and visualized by Western blotting using the indicated antibodies.

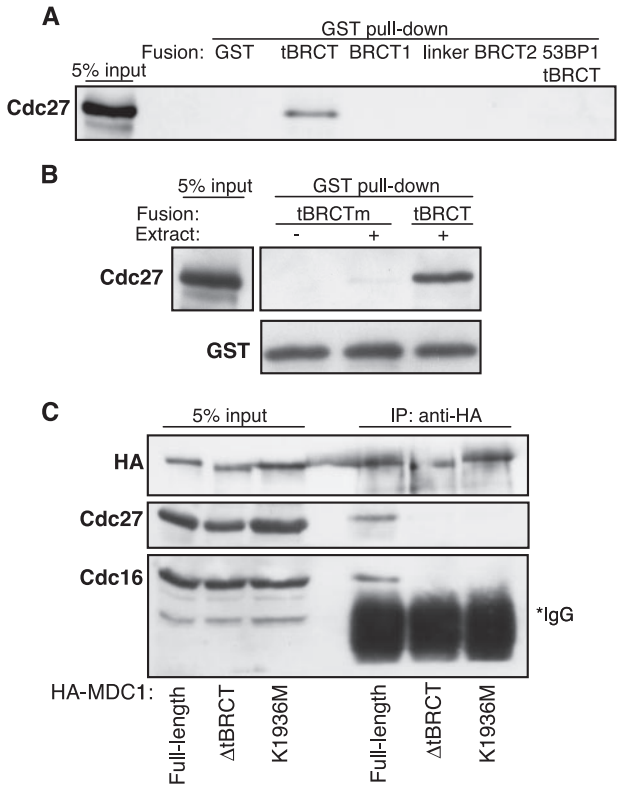

FIGURE 2. **The phosphate binding pocket of MDC1 is required for its binding to the APC/C.** *A*, the integrity of the tBRCT domain of MDC1 is required for the interaction with the APC/C. GST pulldown assays were performed with nuclear extracts from 293T cells and the tBRCT domain, the first (BRCT1) or second (BRCT2) BRCT repeat, or the linker region between them and in addition, with the tBRCT domain of 53BP1, all fused to GST. *B*, mutating the phosphate binding pocket of the tBRCT domain of MDC1 (K1936M, tBRCTm) abolishes its binding to the APC/C. GST pulldown assays were performed with GST-tBRCT or GST-tBRCTm together with nuclear extracts from 293T cells. Equal amounts of GST-tBRCT and GST-tBRCTm were bound to the beads, as determined from Western blotting against GST. *C*, the integrity of the tBRCT domain of MDC1 is required *in vivo* for the interaction with the APC/C. Protein extracts were prepared from 293T cells overexpressing HA-MDC1 (full-length, ΔtBRCT, or K1936M). The HA-tagged proteins were immunoprecipitated using an anti-HA antibody. The background of IgG antibody heavy chain is marked with an *asterisk*. Bound proteins were visualized by Western blotting using the indicated antibodies.

the antibodies used are polyclonal, it is likely that some of the anti-tBRCT antibodies interfere with the binding, whereas other antibodies in the sera do not, thus explaining the difference in co-IP efficiency. The interaction between MDC1 and the APC/C seems to be stable, since the APC/C stays bound to MDC1 at high salt concentrations (Fig. 1*C*).

To verify that the interaction between MDC1 and the APC/C is also mediated *in vivo* by the tBRCT domain of MDC1, we overexpressed HA-tagged versions of the FHA and the tBRCT domains of MDC1 (HA-FHA and HA-tBRCT, respectively) in cells. Antibodies directed against HA were used to IP the different domains of MDC1 from extracts, and as shown in Fig. 1*D*, Cdc27 was co-immunoprecipitated only from the extract prepared from cells overexpressing HA-tBRCT. To examine whether the tBRCT domain of MDC1 is the only domain that mediates this interaction, we overexpressed full-length HA-tagged MDC1 or a tBRCT domain-deleted HA-MDC1 (ΔtBRCT) and performed co-IP experiments using antibodies directed against HA. Although Cdc27 and Cdc16 were successfully co-immunoprecipitated from extracts prepared from cells overexpressing full-length HA-MDC1, they did not co-IP with the tBRCT-deleted HA-MDC1 (Fig. 2*C*), indicating that the tBRCT domain of MDC1 is crucial for the APC/C binding *in vivo*. Taken together, these results suggest that MDC1 interacts *in vivo* with the APC/C and that the tBRCT domain of MDC1 specifically and exclusively mediates the interaction.

The Integrity of the Phospho Binding Pocket of the tBRCT Domain of MDC1 Is Required for Its Interaction with the APC/C—The tBRCT domain contains two BRCT repeats and a short linker sequence between them. To examine whether the

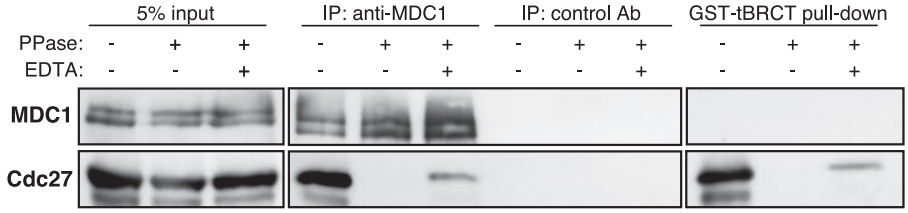

FIGURE 3. **The interaction between MDC1 and the APC/C is phospho-dependent.** Nuclear extracts prepared from 293T were untreated or pretreated with λ-phosphatase (*PPase*) alone or PPase with EDTA as an inhibitor and used for GST pulldown assays with GST-tBRCT or for co-IP of the APC/C using antibodies directed against MDC1. Control antibodies (*Ab*) were against Myc tag. Bound proteins were separated and visualized by Western blotting using the indicated antibodies.

entire tBRCT domain of MDC1 is required for the interaction with the APC/C or whether subparts of it are sufficient for mediating this interaction, we performed pulldown assays with the different components of the tBRCT domain of MDC1. Fig. 2*A* shows that only the full-length tBRCT domain retrieves Cdc27 from protein extracts.

The tBRCT domain is a phospho binding module (11, 12). We mutated one of the key residues of the phosphate binding pocket of the tBRCT domain of MDC1, lysine 1936 to methionine (K1936M, tBRCTm). This mutation is known to abolish the interaction between γ-H2AX and the tBRCT domain of MDC1 (14). GST-tBRCTm did not retrieve Cdc27 from nuclear extracts (Fig. 2*B*). Hence the phospho-binding ability of the tBRCT domain of MDC1 is required for the interaction.

We analyzed whether this mutation has the same effect on full-length MDC1 *in vivo*. Cdc27 and Cdc16 were not co-immunoprecipitated with antibodies directed against HA from extracts prepared from cells overexpressing an HA-tagged version of MDC1 with a point mutation in the tBRCT domain (K1936M), whereas they were co-immunoprecipitated using extracts prepared from cells overexpressing a wild-type version of HA-MDC1 (Fig. 2*C*). These results demonstrate that disrupting the phospho-specificity binding of the tBRCT domain of MDC1 eliminates the binding of the APC/C.

The Interaction between MDC1 and the APC/C Is Phospho-dependent—Because tBRCT domains are modules that bind proteins in a phospho-specific manner (11–13) and mutating the phosphate binding pocket of the tBRCT domain of MDC1 impaired the binding to the APC/C (Fig. 2, *B* and *C*), we analyzed if the interaction is phospho-dependent. Phosphatase treatment of protein extracts eliminated the ability of GST-tBRCT to retrieve the APC/C from extracts and of the APC/C to co-IP with MDC1. This effect was not observed when EDTA, which inhibits the phosphatase activity, was added (Fig. 3). These data reveal that the interaction between MDC1 and the APC/C depends on phosphorylation. It is likely that the phosphorylation event occurs on one of the subunits of the APC/C, since bacterially expressed recombinant GST-tBRCT that is not likely to be phosphorylated binds the APC/C in a phospho-dependent manner (Fig. 3).

A Phosphopeptide Corresponding to the C Terminus of Cdc27 Directly Binds the tBRCT Domain of MDC1—The interaction between MDC1 and the APC/C was mapped to the tBRCT domain of MDC1. We analyzed the protein sequences of different subunits of the APC/C using the NCBI human databases and found that two subunits, Cdc26 and Cdc27, contain the sequence S*X*EF-COOH, which resembles the consensus binding site of the tBRCT domain of MDC1 (12, 14). This suggests that MDC1 interacts with the APC/C through either the Cdc26 or Cdc27 subunits of the complex or through both proteins. Remarkably, this consensus sequence appears in the orthologs of Cdc26 and Cdc27 (Fig. 4*A*). In Cdc27 it appears from *Homo sapiens* to *Gallus gallus* (chicken), whereas in Cdc26 the putative phosphoserine that is crucial for the binding is not as conserved as it is in Cdc27 (Fig. 4*A*). MDC1 orthologs appear in organisms from *H. sapiens* to *Rattus norvegicus* (brown rat), whereas in *G. gallus* (chicken) there is a report of a short sequence similar to MDC1 containing a tBRCT domain (data not shown). Therefore, because of evolutionary conservation, Cdc27 seems to be a better candidate than Cdc26 for mediating the binding of the APC/C to MDC1.

To determine which subunit of the APC/C, Cdc26 or Cdc27, binds MDC1, we synthesized peptides corresponding to the 12 and 14 C-terminal amino acid residues of the proteins, respectively (see "Experimental Procedures"). The peptides contained a phosphoserine residue located four amino acid residues from the C terminus of the peptides (phosphopeptides) or a normal serine residue (unphosphorylated peptides) and were conjugated to biotin. The biotinylated phosphopeptides and their unphosphorylated derivatives were bound to streptavidin-coated magnetic beads and incubated with purified GST-tBRCT. Only the phosphopeptide corresponding to the C terminus of Cdc27 bound GST-tBRCT (Fig. 4*B*). The unphosphorylated derivate of that peptide or a phosphopeptide corresponding to the C terminus of Cdc26 did not retrieve the recombinant protein (Fig. 4*B*). These results suggest that Cdc27 is the APC/C subunit that when phosphorylated directly binds MDC1. Notably, none of the peptides retrieved the mutated form of the tBRCT domain of MDC1 (Fig. 4*B*) that is impaired in the ability to bind the APC/C (Fig. 2*B*), indicating that the intact phosphate binding pocket of the tBRCT domain of MDC1 is required for binding phosphorylated Cdc27.

To establish that Cdc27 is the APC/C subunit that mediates the interaction with MDC1, we performed a GST pulldown assay and demonstrated that the addition of the Cdc27 phosphopeptide competed with the interaction between the tBRCT domain of MDC1 and endogenous Cdc27 (Fig. 4*C*). As expected, the addition of the unphosphorylated Cdc27 peptide did not change the ability of GST-tBRCT to retrieve Cdc27 from extract as compared with control (no peptide added, Fig. 4*C*). This experiment offers further evidence for a direct interaction between the phosphorylated form of the C terminus of Cdc27 and MDC1 and implies that Ser-821 of Cdc27 is phosphorylated *in vivo*.

Cdc27 Is the APC/C Subunit That Directly Binds MDC1 in a Phospho-dependent Manner—Because MDC1 binds *in vitro* to a phosphopeptide corresponding to the C terminus of Cdc27, we assumed that Cdc27 mediates the binding of the APC/C to MDC1 *in vivo*. We analyzed whether the C terminus of Cdc27 interacts with the tBRCT domain of MDC1. We overexpressed

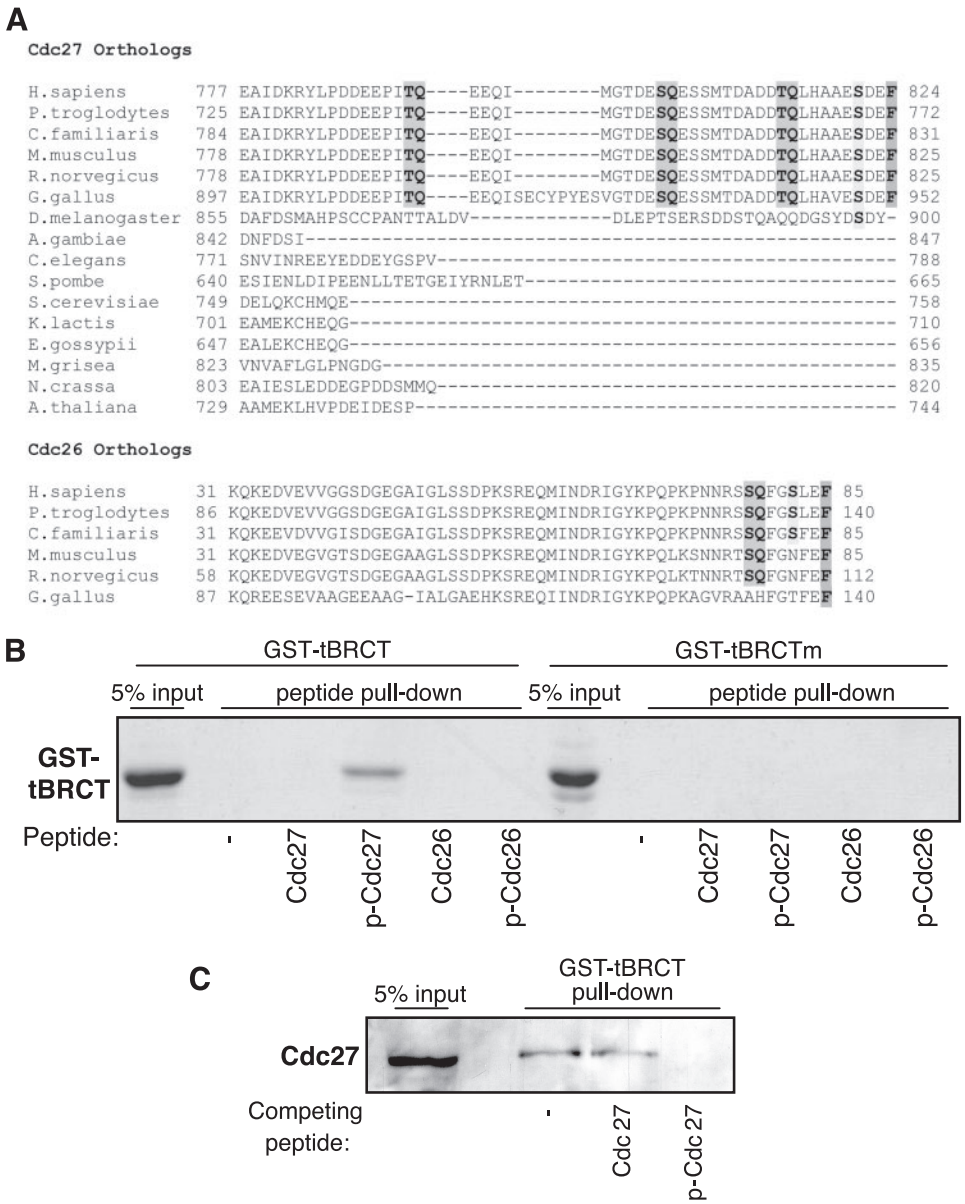

FIGURE 4. **A phosphopeptide corresponding to the C terminus of Cdc27 directly binds MDC1.** *A*, comparison of the C termini of the orthologs of Cdc26 and Cdc27. Alignments were obtained using the HomoloGene tool of NCBI. *Highlighted* are the conserved serine (*S*) and phenylalanine (*F*) residues of the consensus binding motif of MDC1 tBRCT domain and the putative consensus phosphorylation sites for the DNA damage transducer kinases (serine/threonine before glutamine ((S/T)Q)). *B*, the phosphopeptide that corresponds to the C terminus of Cdc27 directly binds the tBRCT domain of MDC1. Peptide pulldown assays were performed with phosphopeptides that correspond to the C terminus of Cdc27 or Cdc26 (p-Cdc27 and p-Cdc26, respectively) or with their unphosphorylated derivatives (Cdc27 and Cdc26, respectively) in the presence of an equal amount of GST-tBRCT or GST-tBRCTm. Bound proteins were visualized by Coomassie Blue staining. *C*, the phosphopeptide that corresponds to the C terminus of Cdc27 abolishes the interaction between MDC1 and the APC/C. GST pulldown assays were performed with GST-tBRCT and p-Cdc27 or its unphosphorylated derivate together with nuclear extracts from 293T cells. Bound proteins were visualized by Western blotting against Cdc27.

in cells full-length Cdc27, the C terminus of Cdc27 (amino acid residues 664–824; Cdc27-C), and Cdc27 that lacks its C terminus (amino acids residues 1–666; Cdc27ΔC), all fused to a GFP. GST-tBRCT retrieved GFP-Cdc27 and GFP-Cdc27-C, but not GFP-Cdc27ΔC, from extracts prepared from these cells (Fig. 5*A*). This result suggests that the C-terminal 160-amino acid residues of Cdc27 are required and sufficient for mediating the interaction with MDC1.

To determine whether the phosphorylation of the serine residue located four amino acid residues from the C terminus of Cdc27 (Ser-821) is required for the interaction with MDC1 *in vivo*, we mutated Ser-821 of full-length Cdc27 to an alanine residue (S821A). GST-tBRCT did not efficiently retrieve GFP-Cdc27 S821A from an extract prepared from cells overexpressing this protein (Fig. 5*B*), indicating that Ser-821 should be phosphorylated to bind MDC1. In addition, we mutated Ser-821 to the negatively charged aspartic acid (S821D) that in several cases was shown to mimic phosphorylation (28). GST-tBRCT partially retrieved GFP-Cdc27 S821D from the extract. Binding was weaker than the wild type but more efficient than the S821A mutation (Fig. 5*B*). Thus, the S821D mutation only partially mimics the phosphorylation event on serine 821 of Cdc27. Similar results were obtained when we mutated Ser-821 of GFP-Cdc27-C (data not shown). Taken together, these results demonstrate that phosphorylation of Ser-821 of Cdc27 is required for the interaction and that the 160 C-terminal amino acid residues of Cdc27 contain all the regulatory elements necessary for the binding to MDC1.

We further demonstrated that the tBRCT domain of MDC1 interacts directly and specifically with the C terminus of Cdc27 by a blot overlay assay using recombinant GST-tBRCT as a probe. Fig. 5*C* shows that GST-tBRCT successfully bound GFP-Cdc27-C in a specific and direct manner on a membrane containing a mixture of cellular proteins extracted from cells overexpressing GFP-Cdc27-C. As expected, GST-tBRCT did not bind the mutant GFP-Cdc27-C S821A (Fig. 5*C*). As an additional negative control, we used GST-tBRCTm as a probe. It did not bind either wild-type or S821A GFP-Cdc27-C (Fig. 5*C*). These results clearly demonstrate that the tBRCT domain of MDC1 directly binds the C terminus of Cdc27. In addition, the results suggest that the binding depends on phosphorylation of Cdc27, since both the intact phosphate binding pocket of MDC1 and Ser-821 of Cdc27 are crucial for this interaction (Fig. 2*B* and Fig. 5*B*).

Our results so far suggest that the tBRCT domain of MDC1 specifically binds a phosphorylated form of Cdc27 (Figs. 3, 4 (*B* and *C*), and 5, (*B* and *C*)). Although expression levels of GFP-Cdc27-C were apparently high (Fig. 5*C*, GFP), the signal

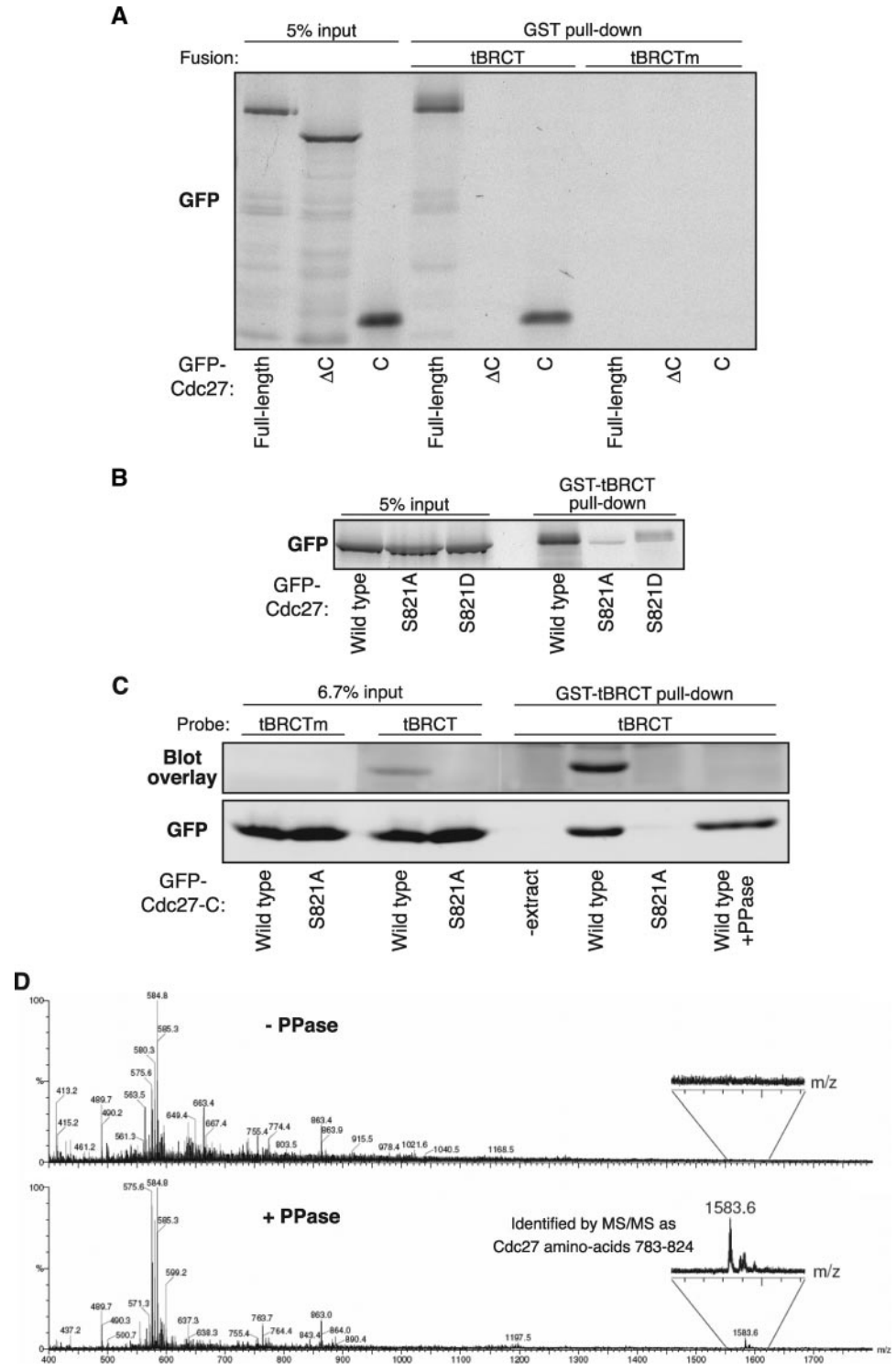

FIGURE 5. **The direct binding of MDC1 to Cdc27 requires phosphorylation of Ser-821 of Cdc27.** *A*, the tBRCT domain of MDC1 binds the C terminus of Cdc27. GFP-tagged versions of Cdc27 were overexpressed in 293T cells. The cells were irradiated with 15 gray and left for recovery for 1 h before protein extraction. The extracts were used for GST pulldown experiments with GST-tBRCT or GST-tBRCTm as a control. Bound proteins were separated, and GFP was directly visualized. *B*, the binding of the tBRCT domain of MDC1 to Cdc27 requires phosphorylation of serine 821 of Cdc27. GFP-tagged versions of Cdc27 were overexpressed in 293T cells. The ability of GST-tBRCT to retrieve these proteins from extracts was analyzed. Bound proteins were separated, and the GFP fusions were directly visualized. *C*, the tBRCT domain of MDC1 directly binds the C terminus of Cdc27 in a phospho-dependent manner. GFP-tagged versions of the C terminus of Cdc27 were overexpressed in 293T cells. The cells were irradiated with 15 gray and left for recovery for 1 h before protein extraction. The extracts were either resolved by SDS-PAGE or used for GST pulldown experiments with GST-tBRCT. Relative protein content was detected by direct visualization of GFP (*lower panel*). Subsequently, proteins were transferred to a membrane, and a blot overlay assay was performed with GST-tBRCT or GST-tBRCTm as probes. The probe was identified by an immunoblot with antibodies directed against the tBRCT domain of MDC1 (*upper panel*). Where indicated, proteins retrieved by GST pulldown were treated by λ phosphatase (*PPase*) just before SDS-PAGE. *D*, the C terminus of Cdc27 is phosphorylated *in vivo*. GFP-Cdc27-C was phospho-enriched by GST-tBRCT and was then untreated or treated with λ phosphatase and analyzed by mass spectrometry.

detected by the blot overlay was modest (Fig. 5*C*, *Blot overlay*). We hypothesized that this reflects the fact that most GFP-Cdc27-C in the extract is not phosphorylated on Ser-821 and, thus, not recognized by the GST-tBRCT probe. We reasoned that the blot overlay signal might be enhanced by first performing a GST pulldown with GST-tBRCT and an extract prepared from cells overexpressing GFP-Cdc27-C, thus enriching for the phosphorylated form of GFP-Cdc27-C. Indeed, the blot overlay signal was dramatically enhanced after a GST pulldown, whereas the levels of GFP fusion were similar to the input (Fig. 5*C*). We next sought to confirm that this enriched fraction is indeed a phosphorylated form of GFP-Cdc27-C. We performed the same GST pulldown as above but treated the beads with λ phosphatase just before loading the bound proteins onto the gel. Whereas direct GFP visualization confirms that GFP-Cdc27-C was retrieved by GST-tBRCT (Fig. 5*C*, *GFP*), phosphatase treatment completely abolished the blot overlay signal (Fig. 5*C*, *Blot overlay*), demonstrating that the direct interaction between MDC1 and Cdc27 strictly requires phosphorylation of Cdc27.

The C Terminus of Cdc27 Is Phosphorylated in Vivo—After revealing that a phosphopeptide corresponding to the C terminus of Cdc27 and the C terminus of Cdc27 overexpressed in cells binds the tBRCT domain of MDC1, we aimed to determine whether the C terminus of Cdc27 is phosphorylated *in vivo* by mass spectrometry. To obtain an enriched fraction of the phosphorylated form of GFP-Cdc27-C, we first performed a GST pulldown as described in Fig. 5*C*. This enriched fraction was either untreated or treated with λ-phosphatase before SDS-PAGE. Both samples were trypsinized in-gel and analyzed by electrospray ionization (ESI)-MS and ESI-MS/MS. Multiple peptides were positively identified in both samples with no apparent shift in expected size, indicating that they are not phosphorylated. Interestingly, comparison of the spectra of the untreated and the phosphatase-treated samples revealed that one peptide, corresponding to the 42 C-terminal amino acid residues of Cdc27, is absent from the untreated sample (Fig. 5*D*, mass-to-charge ratio (m/z) = 1583.6). The fact that this peptide could not be detected, whereas other peptides were identified, suggests that it is negatively or neutrally charged, as only positively charged species are detected in positive mode (29). Because the only difference between the two analyzed samples is phosphatase treatment, we conclude that the peptide is phosphorylated. Because the charge of the peptide in the phosphatase-treated sample is +3, the peptide should be phosphorylated at more than one site to lose its positive charge. Taken together, this result suggests that there is more than one phosphorylation site among the 42 C-terminal residues of Cdc27. This supports our mutagenesis analysis that indicates that Ser-821 is phosphorylated *in vivo*. Furthermore, this result raises the possibility that there is at least one more phosphorylation site in the C terminus of Cdc27.

The Interaction between MDC1 and the APC/C Is Enhanced by Ionizing Radiation—MDC1 plays a crucial role in the cellular response to DNA double-strand breaks (2–8, 14, 30–34). We tested whether the interaction between MDC1 and the APC/C is modulated after DNA damage induction. GST pulldown assays using GST-tBRCT and extracts prepared from untreated cells or from cells induced with IR or UV light revealed that IR, which causes DNA double-strand breaks but not UV treatment, enhanced the interaction between MDC1 and different subunits of the APC/C (Fig. 6*A*). The enhanced interaction between MDC1 and the APC/C after IR was also observed *in vivo* while performing co-IP experiments using antibodies directed against MDC1 (Fig. 6*A*), although less pronounced

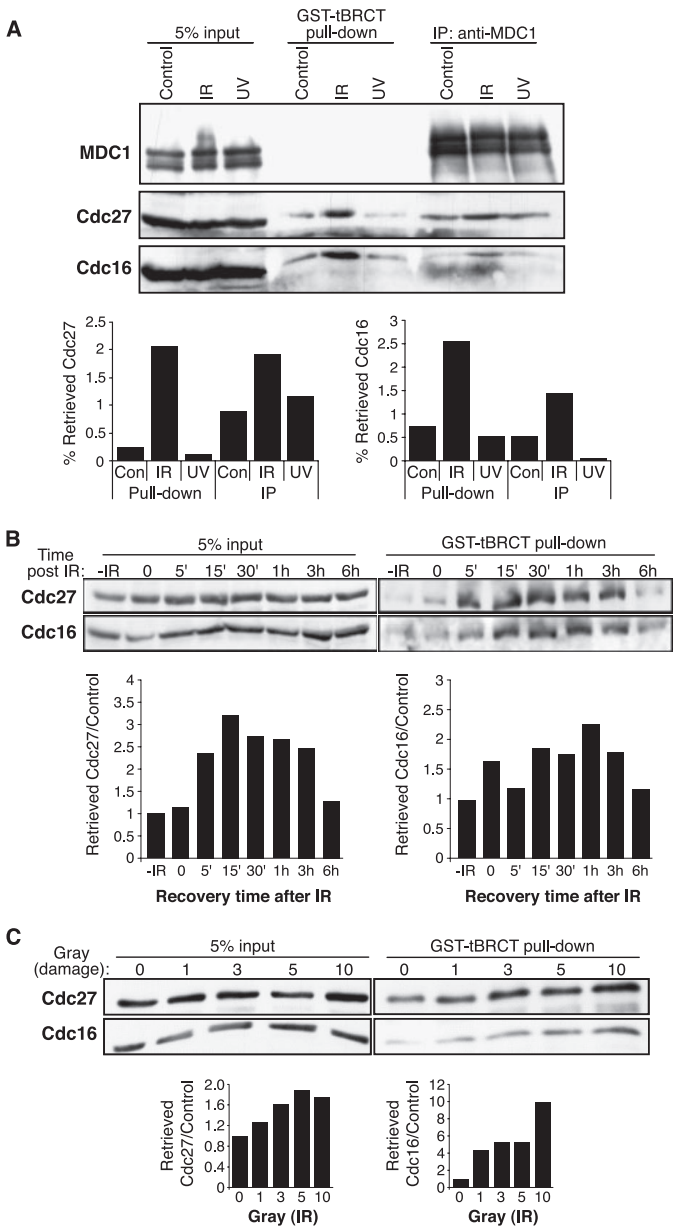

FIGURE 6. **The interaction between MDC1 and the APC/C is enhanced after IR.** *A*, the interaction between MDC1 and the APC/C is increased after IR but not UV. 293T cells were untreated, treated with IR (10 Gray) or UV (50 J/m^2), and left for 1 h to recover. GST pulldown assays with GST-tBRCT or IP experiments using anti-MDC1 antibodies were performed using protein extracts prepared from these cells. *B*, rapid enhancement of the interaction between MDC1 and the APC/C after damage induction. GST pulldown assays were performed with GST-tBRCT together with nuclear extracts from 293T cells untreated or treated with 15 gray of IR and left to recover for the indicated times. *C*, dose-dependent enhancement of the interaction between MDC1 and the APC/C. GST pulldown assays were performed with GST-tBRCT together with nuclear extracts from 293T cells untreated or treated with increasing doses of IR. Bound proteins were visualized by Western blotting against the indicating antibodies. Quantification of Western blot signals were done as in Fig. 1*B* except that in *B* and *C* they where normalized to the control.

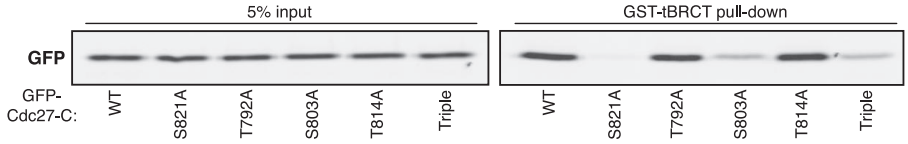

FIGURE 7. **Phosphorylation of serine 803 of Cdc27 is required for the binding between MDC1 and the APC/C.** Mutated versions of GFP-Cdc27-C were overexpressed in 293T cells. The ability of GST-tBRCT to retrieve these proteins from extracts was analyzed. Bound proteins were separated, and the GFP fusions were directly visualized. The triple mutant is T792A, S803A, and T814A. *WT*, wild type.

compared with the GST pull-down experiments. This difference may reflect additional biological factors that play a role in regulating the interaction between MDC1 and the APC/C. The enhancement in the binding between MDC1 and the APC/C after induction of IR occurs very rapidly (Fig. 6*B*). The enhanced interaction happens as DNA damage is induced and is seen also in extracts prepared from cells harvested just after irradiation (Fig. 6*B*, time point 0). This enhancement in the interaction remains for up to about 1 h after damage induction and then slowly decreases back to the level in untreated cells level at about 6 h after DNA damage induction (Fig. 6*B*). To further analyze the dependence of the interaction on IR, cells were untreated or treated with increasing doses of IR, and nuclear extracts were prepared from these cells 1 h after irradiation. GST pulldown assays with these extracts revealed that in addition to the enhancement of the interaction due to DNA damage induction, binding is further increased with higher doses of irradiation (Fig. 6*C*). We, thus, conclude that the interaction between MDC1 and the APC/C occurs in untreated cells and is enhanced in cells induced with DNA damage in a fast and dose-dependent manner. We cannot exclude the possibility that the interaction between MDC1 and the APC/C in untreated cells reflects endogenous DNA damage.

Serine 803 of Cdc27 Might Regulate the Interaction between MDC1 and the APC/C—The C terminus of Cdc27 contains, in addition to a consensus binding site for the tBRCT domain of MDC1, three putative phosphorylation sites for the DNA damage transducer kinases ATM, ATR, and DNA-PKcs. These putative phosphorylation sites, Thr-792 (TQE), Ser-803 (SQE), and Thr-814 (TQ), are conserved (Fig. 4*A*). Because the interaction between the APC/C and MDC1 is enhanced after IR (Fig. 6) and mass spectrometry analysis suggests that there are at least two phosphorylation sites on the C terminus of Cdc27 (Fig. 5*D*), we decided to examine whether these serine/threonine residues are important for the interaction between the APC/C and MDC1. We mutated these residues to alanine in GFP-Cdc27-C and examined the ability of the tBRCT domain of MDC1 to retrieve these mutated forms. Interestingly, mutating Ser-803 impaired the binding of Cdc27 to MDC1, although not as much as the S821A mutation (Fig. 7). Mutating Thr-792 or Thr-814 alone did not seem to have an effect. Furthermore, a triple mutant (T792A, S803A, and T814A) had a similar effect on the binding as the single Ser-803 mutant (Fig. 7). This result suggests that Ser-803 of Cdc27 is phosphorylated and has a role in regulating or mediating the interaction between MDC1 and the APC/C. We conclude that Ser-821 is crucial for the interaction itself and propose that Ser-803 might carry a regulatory role, maybe affecting the phosphorylation of Ser-821.

MDC1 Co-purifies with the APC/C—DNA damage induces MDC1 to form foci at sites of damage where it probably interacts with many proteins involve in the DNA damage response (2–4, 8). Because the APC/C does not appear in these foci (data not shown), we analyzed whether MDC1 and the APC/C may form a complex. Separation of protein extract by gel filtration chromatography revealed that MDC1 and the APC/C are eluted in the same fractions (data not shown). The resolution of the separation by gel filtration chromatography was too low. We, therefore, used an anion exchange column at conditions known to elute the APC/C (35). We separated proteins prepared from IR-treated cells and revealed that MDC1 co-elutes with the APC/C (Fig. 8*A*). Strikingly, the APC/C (represented by Cdc27) appears in all fractions in which MDC1 was eluted (fractions 15–20, Fig. 8*A*). These results support a notion of a cellular complex between MDC1 and the APC/C. Similar results were also obtained when we used non-treated cells (data not shown). We cannot rule out the possibility that both proteins simply elute at the same conditions. It is worth mentioning that only part of γ-H2AX co-eluted with MDC1 and with the APC/C (fractions 18–20), whereas the majority of the protein was eluted at higher salt concentrations (fractions 21–27, Fig. 8*A*). This result suggests that although after DNA damage induction, MDC1 and γ-H2AX co-localize to the same nuclear foci (2, 4) and directly interact (14), this interaction is not exclusive, and they also appear in separate complexes.

Cdc27 and γ-H2AX Bind the Same Site on the tBRCT Domain of MDC1—The only established direct interaction involving the tBRCT domain of MDC1 is with γ-H2AX (14, 36). In this study we have established that the APC/C directly binds MDC1. Because the APC/C and γ-H2AX contain similar binding sites to the tBRCT domain of MDC1 (Fig. 4 and Ref. 14), the same mutation in MDC1 (K1936M) abolishes the interaction with both the APC/C and γ-H2AX (Fig. 2, *B* and *C*, and Ref. 14), and because MDC1 co-fractionates with the APC/C and γ-H2AX (Fig. 8*A*), we aimed to study the interplay between MDC1, the APC/C, and γ-H2AX. We analyzed whether the same region in MDC1 binds the APC/C and γ-H2AX using competition experiments. We demonstrated that a phosphopeptide that corresponds to the C terminus of γ-H2AX competes with the binding of the APC/C to MDC1. We performed a GST pulldown assay and demonstrated that the addition of the γ-H2AX peptide competed with the interaction between the tBRCT domain of MDC1 and endogenous Cdc27 (Fig. 8*B*) and Cdc16 (data not shown), indicating that the peptide can displace the entire APC/C. As expected, the unphosphorylated derivate of that peptide did not change the ability of GST-tBRCT to retrieve Cdc27 from extract as compared with control (no peptide added, Fig. 8*B*). We further demonstrated that γ-H2AX and Cdc27 bind the same site on MDC1 using a peptide pulldown assay. Biotinylated γ-H2AX peptide bound to streptavidin-coated magnetic beads retrieved purified GST-tBRCT (Fig. 8*C*). The addition of non-biotinylated competing

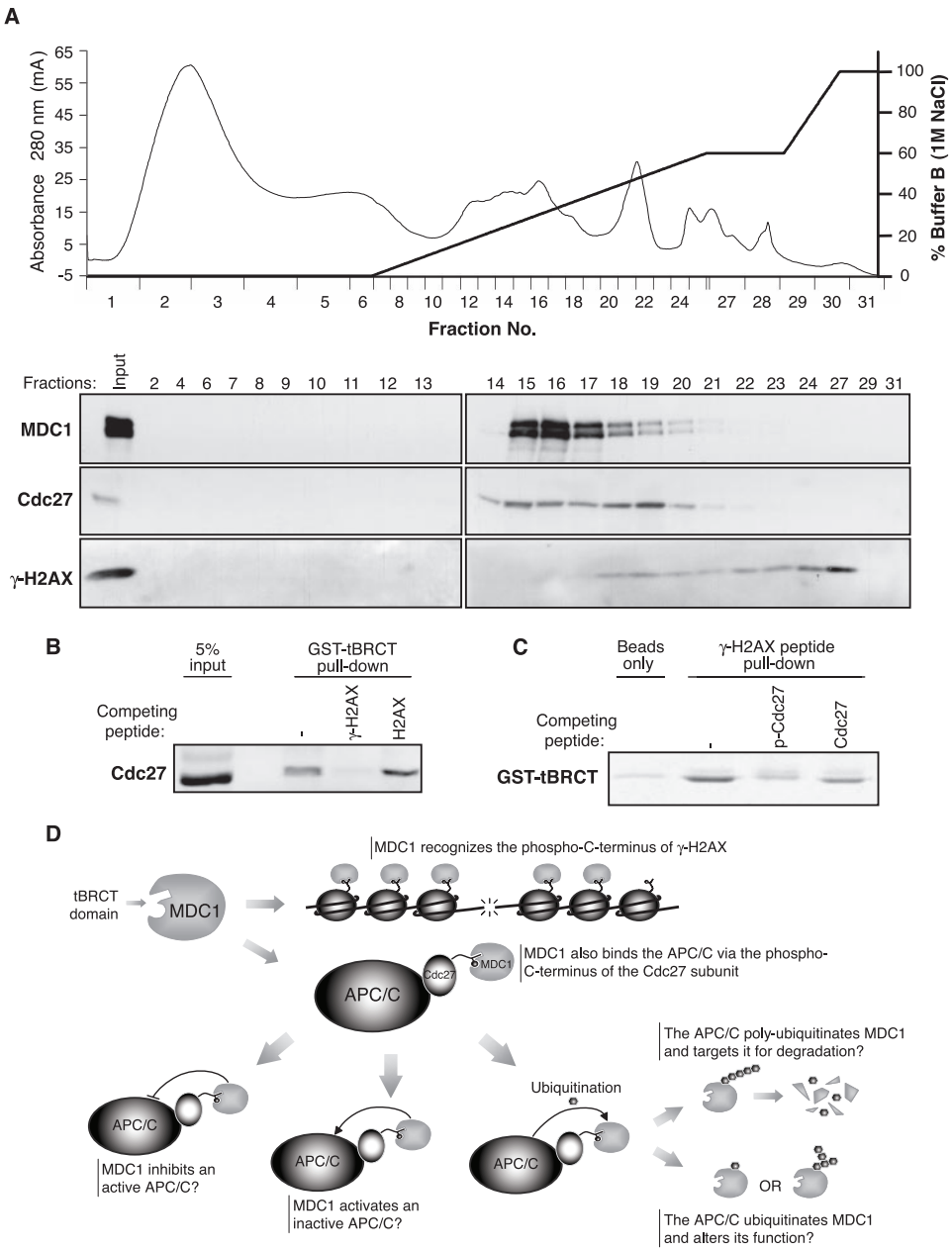

FIGURE 8. **Cdc27 and γ-H2AX bind the same site on MDC1.** *A*, MDC1 co-purifies with both Cdc27 and γ-H2AX on an anion exchange column. Protein extract from HeLa S3 IR-treated cells was loaded onto a Q-Sepharose FF column. Bound proteins were eluted with a two-step NaCl gradient. Chromatogram depicts detected absorbance at 280 nm as a general indicator of protein content (*upper panel*). Salt gradient is superimposed on chromatogram (*upper panel*, *bold*). Collected fractions were concentrated by acetone precipitation and analyzed by Western blotting using the indicated antibodies (*lower panel*). *B*, the phosphopeptide that corresponds to the C terminus of γ-H2AX abolishes the interaction between MDC1 and the APC/C. GST pulldown assays were performed with GST-tBRCT and γ-H2AX peptide or its unphosphorylated derivate together with protein extracts from HeLa S3 cells, irradiated with 15 gray, and left for recovery for 1 h before protein extraction. Bound proteins were visualized by Western blotting against Cdc27. *C*, the phosphopeptide corresponding to the C terminus of Cdc27 competes with the binding of γ-H2AX peptide to MDC1. Peptide pulldown assays were performed with streptavidin beads, the biotinylated γ-H2AX peptide, and GST-tBRCT. Phospho-Cdc27 peptide or its unphosphorylated derivate were added to the reaction where indicated. Bound proteins were visualized by Coomassie Blue staining. *D*, a model for the interactions involving the tBRCT domain of MDC1 and the APC/C or γ-H2AX with postulated functions. See "Discussion" for further details.

Cdc27 phosphopeptide but not of its unphosphorylated derivate abolished the ability of γ-H2AX to bind GST-tBRCT (Fig. 8*C*). Taken together, these results imply that Cdc27 and γ-H2AX bind the same site on the tBRCT domain of MDC1 and suggest that they may compete with each other for the binding.

DISCUSSION

In this study we identified a direct interaction between the DNA damage mediator MDC1 and the APC/C, a major regulator of the cell cycle.

A Novel Connection between the DNA Damage Response and Cell Cycle Regulation—The connection between MDC1 and the APC/C suggests a new role for the APC/C in the DNA damage response and/or a role for MDC1 in cell cycle regulation. We address the various possibilities that arise and present all relevant existing knowledge that support or contradict these hypotheses.

The interaction between MDC1 and the APC/C is enhanced after DNA damage induction (Fig. 6), suggesting a role in regulating the DNA damage response. There are several options for the mechanism by which the interaction regulates the DNA damage response (Fig. 8*D*). (*a*) The APC/C regulates MDC1: (i) MDC1 is a classical APC/C substrate, and thus, the APC/C ubiquitinates and targets MDC1 for degradation by the proteasome similar to its function on many cell cycle regulators (15–17). APC/C substrates contain specific destruction signals that are recognized by the co-activators Cdc20 and Cdh1. These sequences have been shown to be important for the interaction between substrates and the APC/C and for subsequent proteasomal degradation. Although it has been shown that the APC/C can bind substrates by itself, binding of substrates through its coactivators is required for processive ubiquitination that leads to degradation (15). We demonstrated that MDC1 binds the Cdc27 subunit of the APC/C. Disrupting this interaction with specific peptides results in dislocation of the entire APC/C complex from MDC1 (Figs. 4*C* and 8*B* and data not shown), suggesting that MDC1 does not interact directly with Cdc20 or Cdh1. In addition, the protein level of MDC1 appears to be stable after DNA damage induction (2, 4), whereas the interaction with the APC/C is enhanced (Fig. 6). Overall, these results suggest that MDC1 is not a classical substrate of the APC/C. (ii) The APC/C

ubiquitinates MDC1 and regulates its function. E3 ubiquitin ligases may attach ubiquitin molecule(s) on the target proteins, resulting in mono-ubiquitination or poly-ubiquitination events that do not target the protein for degradation (*e.g.* via lysine 63 of ubiquitin, resulting in post-translational modifications that regulate the function, localization and structure of the protein (37)). Because all known substrates of the APC/C to date are degraded by the proteasome (16), it is not likely that this is the result of the interaction between the APC/C and MDC1. However, we cannot exclude the possibility that we have documented the first non-classical APC/C substrate. (*b*) MDC1 regulates the APC/C. (i) The binding of MDC1 to the APC/C inhibits the APC/C and restrains mitosis entry. MDC1 is required for an intact G_2/M checkpoint (3, 4). DNA damage during G_2 might lead to the inhibition of the APC/C by MDC1, thus preventing degradation of substrates and blocking cells from entering mitosis (38, 39). If this is the outcome of the interaction between MDC1 and the APC/C, we would expect MDC1 to bind all of the APC/C in the cell and inhibit it. Otherwise, the inhibition would not be efficient. (ii) The binding of MDC1 to the APC/C activates the APC/C, resulting in cell cycle arrest. This activation may be through the recruitment of a substrate, yet to be found, by MDC1 to the APC/C or by activation of the APC/C at cell cycle phases when it is not active. At the end of G_1 the APC/C becomes inactive, thus allowing the accumulation of essential cell cycle regulators and proper progression into S and G_2 phases (15–17). Activation of the APC/C during S or G_2 phases may result in the degradation of these regulators, thus preventing their accumulation. This would lead to cell cycle arrest and allow time for the cell to repair the damaged DNA and prevent replication or segregation of damaged DNA. We favor this possibility and postulate that after DNA damage induction MDC1 binds the APC/C and activates it. This will result in the degradation of several of the substrates of the APC/C before the cell cycle time point in which they are usually degraded. This hypothesis is supported by the fact that the APC/C is irregularly activated during S-phase in response to DNA damage (40).

An involvement of the APC/C in the DNA damage response has been suggested in the past. DNA damage activates the binding of Cdh1 to the APC/C and induces the activity of the complex during S phase, when it is usually inactive. In addition, DT-40 cells lacking Cdh1 fail to maintain the DNA damage-induced G_2/M checkpoint (40). These data suggest that the APC/C and its co-activator Cdh1 have a role in the cellular response to DNA damage but how this role is carried out is not known. The results presented here imply that MDC1 may be the missing link to the function of the APC/C in the DNA damage response. MDC1 retrieves Cdh1 from extracts (Fig. 1*A*), but we could not detect Cdc20 in the pulldown experiments (data not shown), strengthening our observation that MDC1 interacts with the APC/C after DNA damage induction, when Cdh1 binds and activates it. In addition, studies in the filamentous fungus *Aspergillus nidulans* demonstrated genetic interactions (epistatic and synergistic) between the APC/C subunit APC1 and Rad50, a member of the Mre11·Rad50·Nbs1 complex, at the S-phase checkpoint and in response to DNA damage (41). In higher organisms, where MDC1 exists, MDC1 binds the Mre11·Rad50·Nbs1 complex and regulates its localization at sites of damage (2, 4). The genetic interactions seen in the fungus (37) suggest that the cross-talk between the DNA damage response and cell cycle regulation is conserved in evolution and evolved further in organisms containing MDC1.

The Interplay between the Binding of γ-H2AX and the APC/C to MDC1—Previous works identified γ-H2AX as a direct binding partner of the tBRCT domain of MDC1 (14, 34). This interaction plays a major role in the mammalian response to DNA damage, regulates the phosphorylation of histone H2AX, and is required for normal radio-resistance and efficient accumulation of the DNA damage response proteins at the sites of damaged DNA (14). Here we find that the tBRCT domain of MDC1 also binds the APC/C, an interaction that is augmented after DNA damage induction (Fig. 6). Furthermore, we demonstrated that γ-H2AX and Cdc27 bind the same site on MDC1 (Fig. 8, *B* and *C*). Therefore, we suggest that after DNA damage induction, both histone H2AX and Cdc27 are phosphorylated at their C termini and capable of binding MDC1. Reinforcement for this notion was obtained from the anion exchange chromatography (Fig. 8*A*) that revealed possible complexes containing these proteins. We propose that part of MDC1 binds γ-H2AX and localizes to sites of DNA damage, whereas some MDC1 binds the APC/C (Fig. 8*D*). Our results suggest that after DNA damage induction the fraction of MDC1 that binds γ-H2AX will be located at nuclear foci, whereas the fraction of MDC1 that binds Cdc27 will not. Therefore, after DNA damage induction we did not expect to find Cdc27 or other subunits of the APC/C at nuclear foci. Indeed, when we performed immunofluorescence studies to analyze the localization of the APC/C after DNA damage induction, we could not detect re-localization of the complex to sites of damage (data not shown). There may be additional proteins that bind the tBRCT domain of MDC1, and the interplay of the binding of different proteins to this domain probably provides a fine-tuned mechanism for regulation of MDC1.

The Appearance of an Elongated Cdc27 in Vertebrate—The tBRCT domain of MDC1 binds to a phosphorylated consensus binding sequence located preferably at the C terminus of the protein (12, 14). The APC/C consists of at least 12 subunits (15), of which two subunits, Cdc26 and Cdc27, contain this consensus binding sequence. Only Cdc27, which binds MDC1, has the consensus binding sequence in all organisms in which MDC1 exists (Fig. 4*A* and data not shown). It is noteworthy to mention that there is an elongated C terminus addition in Cdc27 in all organisms in which MDC1 exists, which may suggest an additional role for this extended region. It is possible that the addition of the consensus binding site in the C terminus of Cdc27 evolved in parallel to the appearance of MDC1, thus allowing a new mode of regulation of the APC/C by MDC1. The consensus binding sequence in Cdc27 appears in an elongated sequence of about 30 amino acid residues (Fig. 4*A*). Notably, this elongated sequence, conserved in organisms that contain MDC1, consists of three putative phosphorylation sites for the DNA damage transducer kinases ATM, ATR, and DNA-PKcs (TQE, SQE, and TQ). We demonstrated that the putative phosphorylation site that includes Ser-803 probably regulates the interaction between MDC1 and Cdc27 (Fig. 7). This phospho-

rylation event may trigger the phosphorylation of Ser-821 of Cdc27 that is required for the interaction with MDC1 by an additional kinase yet to be found.

In summary, our study demonstrates a novel link between the DNA damage response and cell cycle regulation. The direct interaction between MDC1 and APC/C suggests that one might regulate the other. Further studies will indicate whether this interaction defines a mechanism of a DNA damage checkpoint or a more general cell cycle-related regulatory pathway.

Acknowledgments—We thank members of our laboratories for helpful discussions, V. A. Smits, B. Kerem, and O. Feine for critical reading of the manuscript and M. Lebendiker from the Protein Purification Laboratory, Hebrew University, for discussions and technical help. We thank A. J. Doherty, J. Gannon, P. Hieter, C. Hoog, and M. Stucki for reagents and O. Moshel for mass spectrometry analysis.

REFERENCES

1. Motoyama, N., and Naka, K. (2004) *Curr. Opin. Genet. Dev.* **14,** 11–16
2. Goldberg, M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J., and Jackson, S. P. (2003) *Nature* **421,** 952–956
3. Lou, Z., Chini, C. C., Minter-Dykhouse, K., and Chen, J. (2003) *J. Biol. Chem.* **278,** 13599–13602
4. Stewart, G. S., Wang, B., Bignell, C. R., Taylor, A. M., and Elledge, S. J. (2003) *Nature* **421,** 961–966
5. Lou, Z., Chen, B. P., Asaithamby, A., Minter-Dykhouse, K., Chen, D. J., and Chen, J. (2004) *J. Biol. Chem.* **279,** 46359–46362
6. Lukas, C., Melander, F., Stucki, M., Falck, J., Bekker-Jensen, S., Goldberg, M., Lerenthal, Y., Jackson, S. P., Bartek, J., and Lukas, J. (2004) *EMBO J.* **23,** 2674–2683
7. Zhang, J., Ma, Z., Treszezamsky, A., and Powell, S. N. (2005) *Nat. Struct. Mol. Biol.* **12,** 902–909
8. Xu, X., and Stern, D. F. (2003) *J. Biol. Chem.* **278,** 8795–8803
9. Shang, Y. L., Bodero, A. J., and Chen, P. L. (2003) *J. Biol. Chem.* **278,** 6323–6329
10. Durocher, D., and Jackson, S. P. (2002) *FEBS Lett.* **513,** 58–66
11. Manke, I. A., Lowery, D. M., Nguyen, A., and Yaffe, M. B. (2003) *Science* **302,** 636–639
12. Rodriguez, M., Yu, X., Chen, J., and Songyang, Z. (2003) *J. Biol. Chem.* **278,** 52914–52918
13. Yu, X., Chini, C. C., He, M., Mer, G., and Chen, J. (2003) *Science* **302,** 639–642
14. Stucki, M., Clapperton, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., and Jackson, S. P. (2005) *Cell* **123,** 1213–1226
15. Peters, J. M. (2006) *Nat. Rev. Mol. Cell Biol.* **7,** 644–656
16. Castro, A., Bernis, C., Vigneron, S., Labbe, J. C., and Lorca, T. (2005) *Oncogene* **24,** 314–325
17. Wasch, R., and Engelbert, D. (2005) *Oncogene* **24,** 1–10
18. Geley, S., Kramer, E., Gieffers, C., Gannon, J., Peters, J. M., and Hunt, T. (2001) *J. Cell Biol.* **153,** 137–148
19. Hagting, A., Den Elzen, N., Vodermaier, H. C., Waizenegger, I. C., Peters, J. M., and Pines, J. (2002) *J. Cell Biol.* **157,** 1125–1137
20. Iwabuchi, K., Basu, B. P., Kysela, B., Kurihara, T., Shibata, M., Guan, D., Cao, Y., Hamada, T., Imamura, K., Jeggo, P. A., Date, T., and Doherty, A. J. (2003) *J. Biol. Chem.* **278,** 36487–36495
21. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
22. Li, J., Williams, B. L., Haire, L. F., Goldberg, M., Wilker, E., Durocher, D., Yaffe, M. B., Jackson, S. P., and Smerdon, S. J. (2002) *Mol. Cell* **9,** 1045–1054
23. Hayouka, Z., Rosenbluh, J., Levin, A., Loya, S., Lebendiker, M., Veprintsev, D., Kotler, M., Hizi, A., Loyter, A., and Friedler, A. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 8316–8321
24. Beer, I., Barnea, E., Ziv, T., and Admon, A. (2004) *Proteomics* **4,** 950–960
25. Rosenfeld, J., Capdevielle, J., Guillemot, J. C., and Ferrara, P. (1992) *Anal. Biochem.* **203,** 173–179
26. Wilm, M., and Mann, M. (1996) *Anal. Chem.* **68,** 1–8
27. Listovsky, T., Zor, A., Laronne, A., and Brandeis, M. (2000) *Exp. Cell Res.* **255,** 184–191
28. Lukas, C., Sorensen, C. S., Kramer, E., Santoni-Rugiu, E., Lindeneg, C., Peters, J. M., Bartek, J., and Lukas, J. (1999) *Nature* **401,** 815–818
29. Mann, M., Ong, S. E., Gronborg, M., Steen, H., Jensen, O. N., and Pandey, A. (2002) *Trends Biotechnol.* **20,** 261–268
30. Mochan, T. A., Venere, M., DiTullio, R. A., Jr., and Halazonetis, T. D. (2003) *Cancer Res.* **63,** 8586–8591
31. Lou, Z., Minter-Dykhouse, K., Wu, X., and Chen, J. (2003) *Nature* **421,** 957–961
32. Peng, A., and Chen, P. L. (2003) *J. Biol. Chem.* **278,** 8873–8876
33. Ozaki, T., Nagase, T., Ichimiya, S., Seki, N., Ohiri, M., Nomura, N., Takada, N., Sakiyama, S., Weber, B. L., and Nakagawara, A. (2000) *DNA Cell Biol.* **19,** 475–485
34. Xu, X., and Stern, D. F. (2003) *FASEB J.* **17,** 1842–1848
35. Miller, J. J., Summers, M. K., Hansen, D. V., Nachury, M. V., Lehman, N. L., Loktev, A., and Jackson, P. K. (2006) *Genes Dev.* **20,** 2410–2420
36. Lee, M. S., Edwards, R. A., Thede, G. L., and Glover, J. N. (2005) *J. Biol. Chem.* **280,** 32053–32056
37. Passmore, L. A., and Barford, D. (2004) *Biochem. J.* **379,** 513–525
38. den Elzen, N., and Pines, J. (2001) *J. Cell Biol.* **153,** 121–136
39. Zur, A., and Brandeis, M. (2002) *EMBO J.* **21,** 4500–4510
40. Sudo, T., Ota, Y., Kotani, S., Nakao, M., Takami, Y., Takeda, S., and Saya, H. (2001) *EMBO J.* **20,** 6499–6508
41. Malavazi, I., Lima, J. F., von Zeska Kress Fagundes, M. R., Efimov, V. P., de Souza Goldman, M. H., and Goldman, G. H. (2005) *Mol. Microbiol.* **57,** 222–237