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Detection of c-Abl kinase-promoted phosphorylation of Rad51 by specific antibodies reveals that Y54 phosphorylation is dependent on that of Y315

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ABSTRACT

Rad51 plays a crucial role in homologous recombination and recombinational DNA repair. Its activity is regulated by phosphorylation by the c-Abl kinase. Either Tyr54 or Tyr315 have been reported as the target of phosphorylation but the interconnection between their phosphorylation is not known. We prepared two specific antibodies that selectively detected the Tyr54 or Tyr315 phosphorylation site of Rad51. By co-transfection of HeLa cells with c-Abl and Rad51, we clearly showed that both Tyr54 and Tyr315 of Rad51 are phosphorylated by c-Abl. Furthermore, we showed that the phosphorylation of Tyr315 stimulates that of Tyr54, which indicates that the phosphorylation of Rad51 by the c-Abl kinase is a sequential process.

Structured summary:
MINT-7034009: *cABL* (uniprotkb:P00519) *physically interacts* (MI:0218) with *RAD51* (uniprotkb:Q06609) by *pull down* (MI:0096)

1. Introduction

Rad51 protein is involved in homologous recombination (HR) and repair of DNA double-strand breaks (DSBs) produced by exposure to ionizing radiation [1,2]. Rad51 activity is based on pairing and strand exchange between homologous DNAs. In response to radiation-induced DNA damage, Rad51 forms distinct subnuclear complexes called foci [3–7]. Although the molecular regulation of Rad51 levels and activity has not yet been completely defined, the Abl family of tyrosine kinases appears to play a significant role [8–10].

c-Abl is a tyrosine kinase involved in DNA repair signal transduction [11–14]. Several studies have shown that c-Abl interacts with and phosphorylates Rad51 on tyrosine in response to DNA damage in an ATM-dependent manner [15]. In spite of the importance of Rad51 phosphorylation in recombinase activity, the interconnection between the phosphorylation site(s) is (are) not clearly determined. In one previous study, the authors described a single phosphorylation of Rad51 on the Tyr54 residue [16] while other authors provided evidence of Tyr315 phosphorylation by c-Abl, which negatively affected Rad51–DNA binding or positively affected Rad51 activity by enhancing its association with Rad52 [17]. It remains unclear whether c-Abl can phosphorylate a single or both tyrosines in a simultaneous or in a sequential manner.

Immunoblot analysis is commonly used for protein analysis but the study of protein post-translational modifications by this technique is largely limited by antibody specificity and the sensitivity of detection. To overcome these difficulties, we prepared highly specific anti-phospho-Y54 and anti-phospho-Y315 antibodies. Using these and secondary antibodies coupled to fluorophores, we obtained a strong tool for the detection of Rad51 phosphorylation sites. We provide evidence that c-Abl can phosphorylate both Tyr54 and Tyr315 residues of Rad51 in a sequential manner, Tyr315 first followed by Tyr54. SH2 domain of c-Abl recognizes the phosphorylated Tyr315 site which is required for Tyr54 phosphorylation. We also propose an explanation of the apparent discrepancies in the results on which tyrosine residues in Rad51 are phosphorylated by c-Abl.

2. Materials and methods

2.1. Generation of phosphospecific antibodies by affinity chromatography

Two peptides containing Rad51 tyrosine phosphorylation sites Tyr54 and Tyr315, respectively, were designed, synthesized,

Abbreviations: HR, homologous recombination; BSA, bovine serum albumin; pBSA, phosphotyrosineBSA; pY54 or pY315, phosphotyrosine 54 or 315

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conjugated to ovalbumin and used to immunize rabbits (NeoMPS) (Fig. 1). Sera were applied to a Sulfolink (Pierce) affinity column coupled to the immunizing phosphopeptide. Retained antibodies were eluted and applied to an affinity column coupled to the corresponding non-phosphopeptide and the antibodies of interest were collected in the flow-through. Anti-phospho-Y54 (anti-pY54) antibodies were thus purified by two sequential immune-affinity columns coupled to either the phospho- or the non-phosphopeptide. The purification of the anti-phospho-Y315 (anti-pY315) antibodies required one additional step using an affinity column conjugated to the Y54 phosphopeptide. After each purification step, the antibody specificity was evaluated by peptide microarrays containing increasing concentrations of phosphopeptides (pY54, pY301 and pY315) or non-phosphopeptides (Y54, Y301 and Y315). Bovine serum albumin (BSA) and phosphotyrosineBSA (phosphoBSA) were added to the microarray as a protein control.

2.2. Plasmid construction

Flag-tagged wild-type or kinase-dead (KD) c-Abl, or HA-tagged Rad51 eukaryotic expression vectors, driven by a human elongation factor-1 promoter (the tag sequences were located upstream of the coding sequences) have been described previously [18]. Tyr54, Tyr301 and Tyr315 of human Rad51 were mutated to phenylalanine, using a PCR strategy.

2.3. Transfection and cell lyses

HeLa cells were a kind gift from Dr. Pitard (Nantes, France). Cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 5% antibiotics in a humified atmosphere with 5% CO_2. HeLa cells were transiently transfected (FuGENE6 reagent) separately or co-transfected with plasmids carrying wild-type or mutated Rad51 and c-Abl. Twenty-four hours after transfection, cells were washed with ice-cold PBS and lysed in cold EBC Buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM β-mercaptoethanol, 50 mM NaF and 1 mM Na_3VO_4) plus a protease inhibitor cocktail (Sigma) [17]. After incubation for 20 min on ice, cells were scraped from plates, sonicated and centrifuged at 13 000 rpm for 10 min at 4 °C. Protein concentration was determined by the Bradford method (Bio-Rad).

2.4. Immunoblot analyses

Equal amounts of cellular lysate proteins were separated by electrophoresis on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Amersham). Membranes were blocked for 1 h in 0.1% TBS-Tween20 containing 5% BSA or 2% Amersham blocking agent. Primary antibodies were mouse anti-Rad51, anti-c-Abl, anti-tubulin or rabbit anti-pY54, anti-pY315, or anti-HA. Membranes were washed three times with 0.1% TBS-Tween20 for 10 min and incubated for 1 h with anti-mouse Alexa-Fluor700-conjugated or anti-rabbit Alexa-Fluor800-conjugated secondary antibodies (Molecular Probes). Membranes were scanned at 700 nm and 800 nm, respectively, using the Odyssey infrared imaging system (Li-cor, Biosciences). Images were treated and proteins were quantified with the Odyssey software. Amounts of Rad51 and phospho-Rad51 on the blot were normalized to the amount of tubulin and the ratio of phospho-Rad51 to total Rad51 was determined from three independent experiments.

2.5. GST pull-down analysis

Cell lysates equivalent to 2×10^6 HeLa cells were incubated with 100 µl of a 50% slurry of glutathione-agarose beads (GE Healthcare) for 2 h at 4 °C with end-over-end mixing. After centrifugation, precleared lysates were transferred to a clean microcentrifuge tube. Ten micrograms of GST-Abl SH2 domain (Marligen) was added to each tube and the mixture was incubated at 4 °C for 2 h with end-over-end mixing. Beads were washed four times with lysis buffer (20 mM Tris, pH 8, 200 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM PMSF) as previously described in [19]. The beads were mixed and boiled directly into an equal volume of

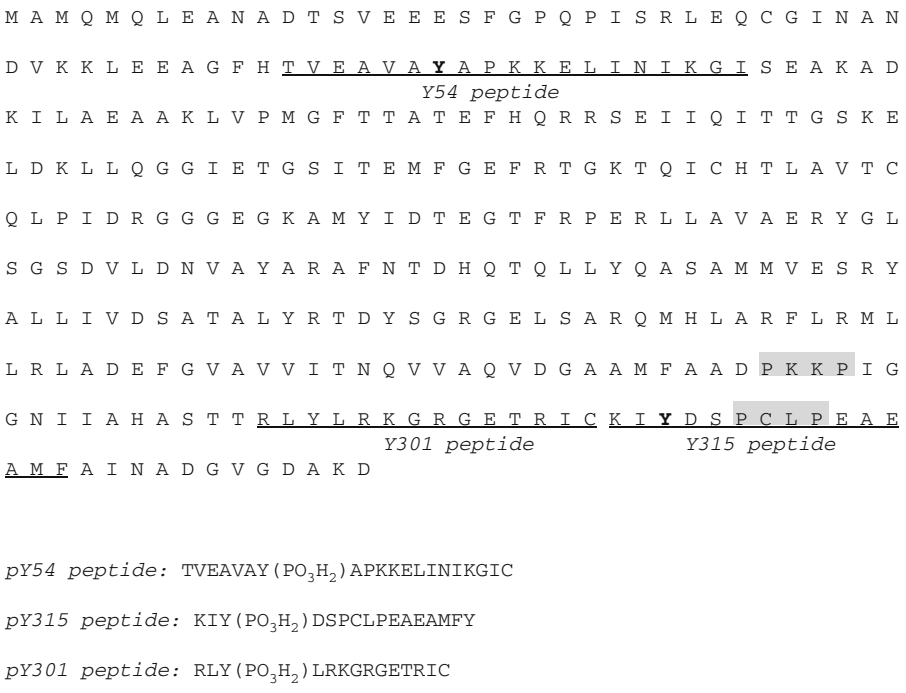

Fig. 1. Rad51 and antigenic peptide sequences. Y54 and Y315 peptides used to generate phosphospecific antibodies are underlined. The PXXP and phosphoYXXP sequence motifs recognized by c-Abl are indicated by a grey background. The sequence of the Y301 peptide used as a negative control is also shown.

2× SDS–PAGE sample buffer. The proteins were resolved on a 10% SDS–PAGE gel and analyzed by Western blotting with anti-GST (Sigma) and anti-pY315 antibodies.

3. Results

3.1. Validation of the phospho-specific antibodies

The sera obtained from rabbits immunized with a phospho-Tyr54 or a phospho-Tyr315 peptide (see Fig. 1 for peptide sequences) contain different antibodies. Phospho-specific antibodies were isolated through sequential phospho- and dephospho-affinity chromatography from crude sera (see Section 2).

Using peptide microarrays spotted with the immunizing and the corresponding dephosphopeptides, we followed and estimated the anti-pY54 and anti-pY315 antibody specificity after each step of purification (Fig. 2). pBSA which presents a large quantity of phosphotyrosine per protein mole and thus enables the potential cross-reactivity of antibodies to be detected and a pY301 phosphopeptide was also spotted on the microarrays and used as negative controls.

The anti-pY54 specific antibody was isolated by two affinity chromatography steps. After the first step on a pY54-coupled affinity column, the eluted product was mainly specific to the pY54 peptide but it also recognized the non-phosphorylated Y54 peptide (Fig. 2A). The eluted antibodies were then applied to a Y54-dephosphopeptide affinity column. The pY54 specific antibodies do not bind to this column and are collected in the flow-through. Fig. 2A clearly shows that the obtained antibodies are highly specific to the pY54 site.

Unlike anti-pY54 antibody purification, three affinity chromatography steps were required to isolate the anti-pY315 specific antibodies. After the first step of purification on a pY315-coupled column, the eluted antibodies recognized preferentially the pY315 peptide but also the other phosphorylated peptides, as well as pBSA and the Y315 peptide. These antibodies were then applied to a Y315-dephosphopeptide affinity column. This second step of purification eliminated the Y315 peptide cross-recognition, as shown in Fig. 2B. A final third step using a pY54 peptide conjugated column was necessary to eliminate the phosphotyrosine-recognizing antibodies. Finally, the flow-through containing the anti-pY315 antibodies presents high specificity to the pY315 peptide, as shown in Fig. 2B. Fig. 2 shows that our antibodies recognize neither pBSA nor the pY301 peptide which confirms the high specificity of these antibodies.

3.2. c-Abl kinase can phosphorylate Tyr54 and Tyr315 residues of Rad51 in cells

Firstly, we transiently expressed wild-type or mutated HA-tagged Rad51 in HeLa cells. In the mutated protein the tyrosine residue at positions 54, 301 or 315 was replaced with a phenylalanine (Rad51-Y54F, Rad51-Y301F and Rad51-Y315F mutants, respectively). Cells were lysed and the expression level of exogenous Rad51 was quantified by immunoblotting with anti-Rad51 and anti-HA antibodies in order to ensure that the intracellular quantity of transfected Rad51 was equivalent between the wild-type and the mutated forms. Fig. 3 clearly shows that the expression levels of the exogenous wild-type Rad51 and the exogenous mutated Rad51 proteins are comparable. Transfection of wild-type or mutated Rad51 protein is constant, equivalent and reproducible.

Secondly, we transiently co-expressed HA-tagged Rad51 and Flag-tagged c-Abl in HeLa cells. An anti-c-Abl antibody was used to confirm the efficiency and reproducibility of c-Abl transfection. The level of c-Abl protein expression was analyzed by immunoblotting of cellular extracts co-transfected with wild-type and mutated Rad51. Fig. 3B shows that c-Abl expression is equivalent when co-transfected with wild-type and mutated Rad51.

Fig. 3B also shows that the expression level of the exogenous Rad51 protein is comparable between wild-type and mutated Rad51 when co-transfected with c-Abl.

After confirming the equal expression of exogenous proteins in co-transfected cells, we analyzed the level of Rad51 phosphorylation by immunoblotting with our purified anti-pY315 and anti-pY54 antibodies. The amount of total Rad51, as well as the amount of Rad51 phosphorylated on Tyr54 and Tyr315 was quantified by Western blot analysis in cell extracts co-expressing c-Abl and wild-type or mutated Rad51 (Y54F or Y315F). Anti-Rad51, anti-pY54 and anti-pY315 antibodies, respectively, were used. Another Rad51 mutant, Y301F, was used as a negative control to confirm the specificity of antibodies. From these quantifications, the phospho-Rad51/total Rad51 ratio was determined in order to estimate

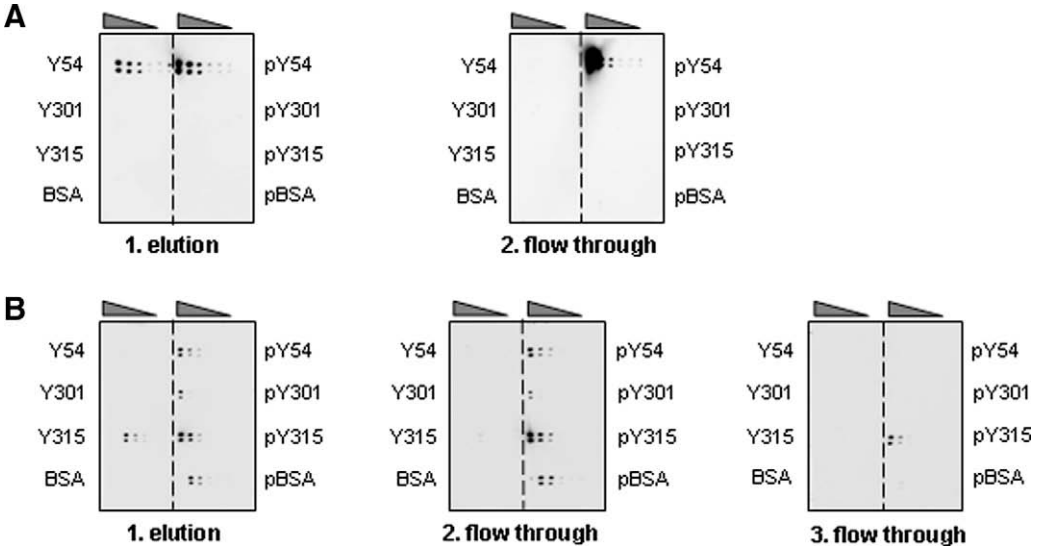

Fig. 2. Confirmation of anti-phosphoY54 (anti-pY54) and anti-phosphoY315 (anti-pY315) antibody specificity by dot blotting. Antibodies were purified by two or three steps of affinity chromatography. Anti-pY54 (A) and anti-pY315 (B) antibody specificity was evaluated after each step of purification by using phospho- (pY54, pY301, pY315 and phosphoBSA) or non-phosphopeptides (Y54, Y301, Y315 and BSA). The concentration of each peptide and of the BSA protein ranged from 3 pg to 6 ng.

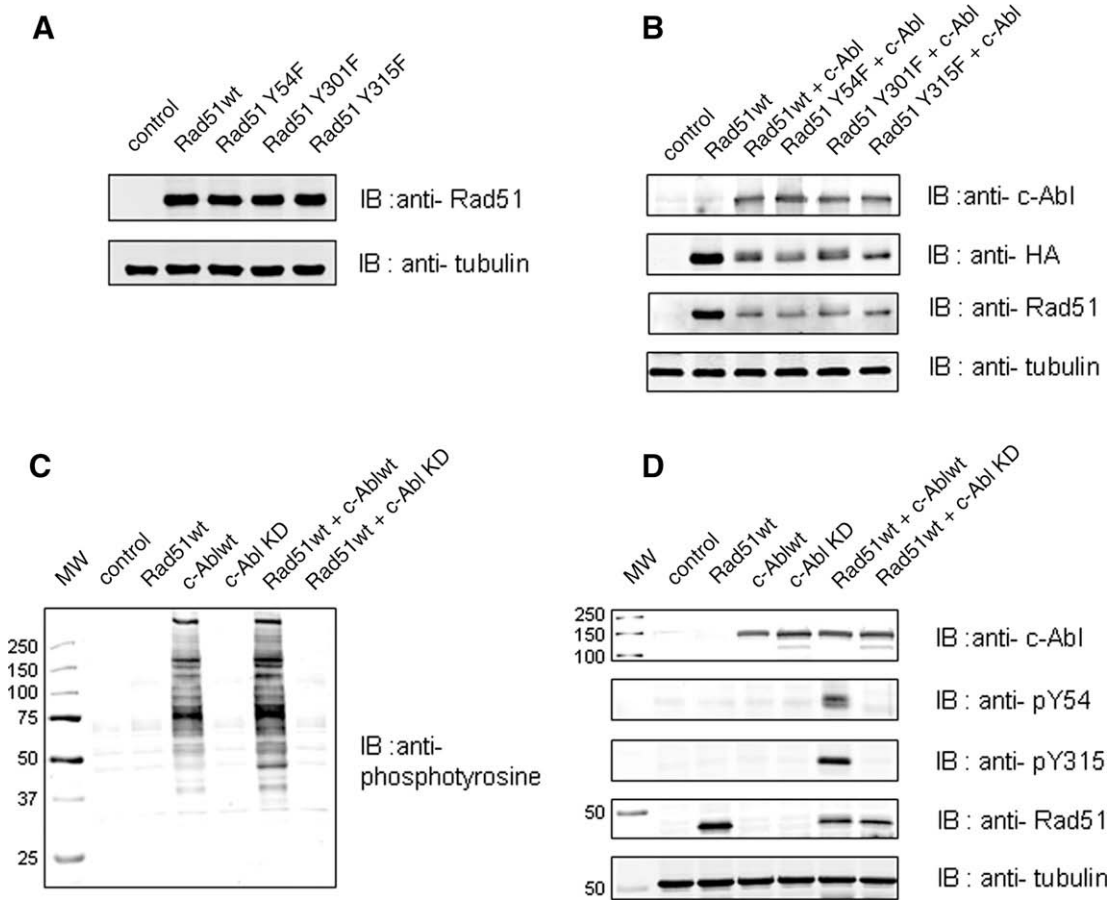

Fig. 3. Rad51 and c-Abl expression in transfected HeLa cells. HeLa cells were transfected with Rad51 or co-transfected with c-Abl and Rad51 and lysed 24 h post-transfection. Whole-cell extracts were resolved by SDS–10% PAGE and analyzed by Western blot. (A) Cells were transfected with wild-type (wt) or mutated Rad51 (Y54F, Y301F or Y315F). (B) Co-transfected cell extracts were analyzed by Western blot using antibody, as indicated. (C and D) Cells were transfected or co-transfected with Rad51 wt, active c-Abl (c-Abl wt) or dead-kinase c-Abl (c-Abl KD). Cell extracts were analyzed by Western blot using antibody, as indicated. The control lane corresponds to untransfected cell extract. Tubulin was used as a loading control.

the level of phosphorylation on tyrosine 315 (bar graph of Fig. 4A) and tyrosine 54 (bar graph of Fig. 4B).

Fig. 4A shows the phosphorylation of Tyr315 and indicates that the level of this phosphorylation is similar between the wild-type and the control Rad51-Y301F mutant. Moreover, the Tyr315F mutation of Rad51 eliminates Y315 phosphorylation since no significant phosphorylation is observed. These results confirmed the intracellular phosphorylation of Rad51 on Tyr315 by c-Abl (Fig. 4A).

The same analysis was carried out using the anti-pY54 antibody. Fig. 4B shows that Rad51wt is phosphorylated on Tyr54. For the first time, we provide direct evidence that c-Abl can phosphorylate Rad51 on Tyr54 in cells. A comparable level of Tyr54 phosphorylation is observed with the Rad51-Y301F control mutant.

3.3. Interconnection between Y54 and Y315 phosphorylation by c-Abl

By using Rad51 mutants, we confirmed that c-Abl can phosphorylate Rad51 at Tyr54 and Tyr315. No phosphorylation of Rad51 was observed when inactive c-Abl was expressed, which confirms that Rad51 phosphorylation is induced by c-Abl (Fig. 3C and D).

As shown in Fig. 4A, Tyr54 phosphorylation is absent when Rad51 is mutated as Y54F, which confirms the ability of c-Abl to phosphorylate Rad51 on Tyr54 in cells.

An unexpected result was obtained with Rad51-Y315F. This mutation induced a significant decrease in Tyr54 phosphorylation with a Rad51pY54/Rad51 ratio of less than 20% compared to wild-type Rad51 (Fig. 4B, lane Y315F). No effect on the level of Tyr54 phosphorylation was observed when Tyr301 was mutated to Phe. These results show that Tyr54 phosphorylation is dependent on Tyr315 phosphorylation.

A GST pull-down assay was performed to investigate the interaction between the SH2 domain of c-Abl and phospho-Rad51. The results presented in Fig. 5 demonstrate a unique band revealed by the anti-pY315 antibody which confirms the interaction between the SH2 domain of c-Abl and Rad51 phosphorylated on Tyr315.

4. Discussion

In order to study the phosphorylation of Rad51 we prepared specific anti-phospho-Rad51 antibodies. Tyrosine phosphorylation is specific to multicellular organisms and plays a crucial role in signal transduction which is frequently related to cancer. Its detection is difficult since it constitutes less than 0.1% of total intracellular protein phosphorylation [20]. Anti-phosphotyrosine antibodies are commercially available but they cannot distinguish between the different phosphorylation sites of the same protein. Moreover, a large number of false positive interactions can be detected. Using affinity chromatography, we have obtained high affinity antibodies

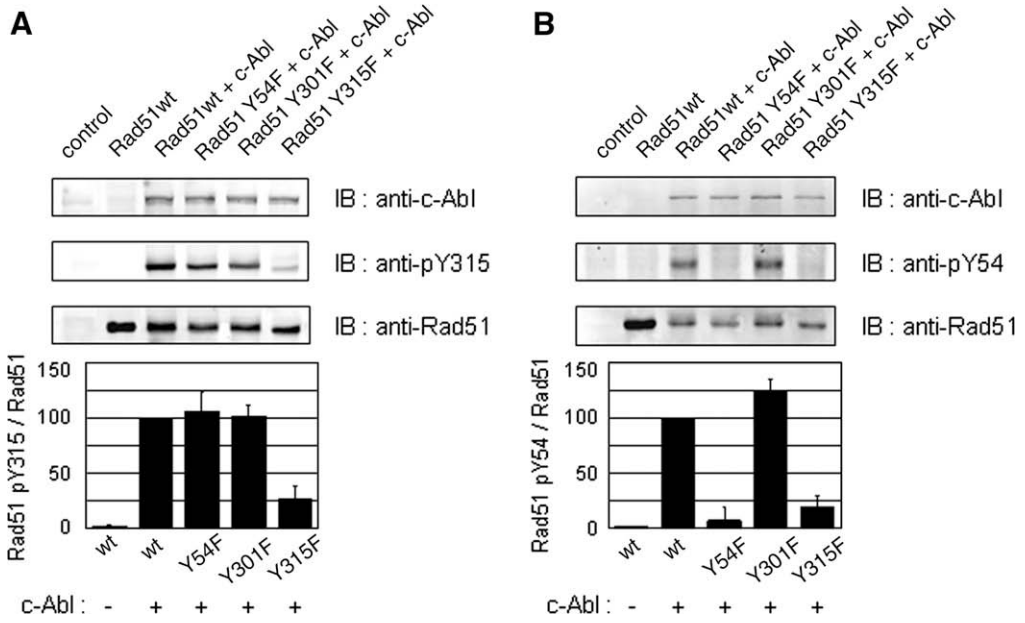

Fig. 4. Intracellular phosphorylation of Tyr54 is dependent on Rad51 Tyr315 phosphorylation. HeLa cells were transfected with Rad51 wt or co-transfected with c-Abl and wild-type (wt) or mutated (Y54F, Y301F or Y315F) Rad51 and lysed after 24 h. Expression of phosphorylated Rad51 was detected in total cell lysates by Western analysis using either anti-pY315 (A) or anti-pY54 (B) antibodies and was compared to total Rad51. The bar graphs indicate the ratio of Rad51 phosphorylated at Y315 (A) and at Y54 (B) to Rad51 from quantification of the blotting data (expressed as a percentage compared to Rad51wt). Results represent the average and S.D. of three independent experiments. Error bars indicate S.D.

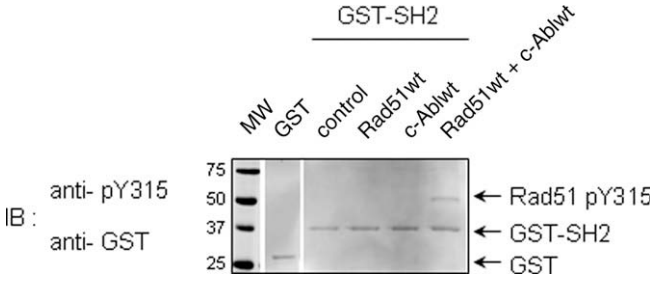

Fig. 5. Interaction of SH2 domain of c-Abl kinase with phosphorylated Rad51. HeLa cells were transfected or co-transfected with Rad51 wt and c-Abl wt and lysed after 24 h. Lysates were incubated with glutathione-beads and GST-SH2 peptide domain for 2 h. The pull-down products were detected by Western blot with anti-pY315 followed by anti-GST antibody.

specific for each site of Rad51 tyrosine phosphorylation. This immunoblotting approach allowed us to establish a high-performance method for the detection of Rad51 phosphorylation sites.

In HeLa cells co-transfected with c-Abl and Rad51, we have shown that Rad51 is phosphorylated on tyrosine 54 and tyrosine 315 by c-Abl. Indeed, the replacement of the tyrosine residue by a phenylalanine residue decreases the level of Rad51 phosphorylation. Moreover, we found that the Y315F mutation eliminates both Tyr54 and Tyr315 phosphorylation. There are two possible explanations for the effect of the Y315F mutation on Tyr54 phosphorylation. This mutation could induce important structural modifications in the Rad51 protein, which could inhibit the access of c-Abl to Rad51. Another possibility is that the phosphorylation of Tyr315 by c-Abl stimulates the phosphorylation of the Tyr54 site by the same kinase.

The first hypothesis seems unlikely. As reported by Takizawa et al. [21], the substitution of tyrosine 315 by a phenylalanine does not affect the structure or the function of Rad51. Indeed, the Rad51-Y315F mutant exhibited DNA-binding and strand-exchange activities similar to those of the wild-type protein (Y315F was used as a positive control mutant). The Y315F mutation only affected the ability of the substituted residue to be phosphorylated.

Furthermore, the second hypothesis is supported by the fact that c-Abl fulfils its biological function through domain interactions with other cellular components. Two binding domains, an SH3 and an SH2 domain, which are located at the N terminus of c-Abl, can bind peptides carrying PXXP and (p)YXXP motifs, respectively [14,22]. There are two PXXP motifs (amino acids 283–286 and amino acids 318–321) and one potential (p)YXXP motif (amino acids 315–318) in the Rad51 sequence (Fig. 1). The phosphorylation of Tyr315 reveals one new (p)YXXP recognition motif. Indeed we show for the first time that c-Abl SH2 domain recognizes and interacts selectively with the pY315 Rad51 form. This c-Abl/phospho-Rad51 interaction could permit Tyr54 phosphorylation, explaining its dependence on the phosphorylation of Tyr315.

The phosphorylation of Tyr315 was not observed by mass spectrometry analysis of Rad51 phosphorylation in vitro. Only the phosphorylation of Tyr54 was detected [16].

In this assay the Rad51 protein was digested by trypsin into smaller peptides. The absence of detection of Tyr315 phosphorylation could be explained by the length (28 amino acids) and negative charge (pH_i 4.03) of the Tyr315-containing fragment which could interfere with its detection by mass spectroscopy [20]. The Tyr54-containing fragment is shorter (17 amino acids) and less negatively charged (pH_i 4.83) than the Tyr315-containing one. These length and charge differences make it difficult to obtain an evaporated fragment containing Tyr315, which may explain the apparent absence of Tyr315 phosphorylation by c-Abl of the Rad51 protein. This also shows that our results are not contradictory with those proposed by Yuan et al.

Successive phosphorylation of Y315 followed by Y54 is more than likely. The biological significance of this double phosphorylation of Rad51 remains unclear. It has been reported that the Tyr54-containing N-terminal region of Rad51 directly interacts with DNA [23,24], while the phosphorylation of Tyr315 which is located near

the subunit interface could affect polymerization of Rad51 [25]. This sequential phosphorylation may thus modulate Rad51 affinity for the damaged DNA.

Because our specific antibodies have been generated against peptides, the recognition of their epitopes may be less efficient in native conditions such as in immunoprecipitation or immunofluorescence approaches. By contrast, our antibodies can be used in immunoblotting conditions. Thus, they allowed us to differentially detect the phosphorylation states of Rad51 and will help understand the physiological role of each phosphorylation.

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