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The plant Rad50–Mre11 protein complex

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Abstract The Rad50–Mre11–Xrs2/Nbs1 protein complex plays critical roles in cellular processes involving DNA ends. This complex is implicated in DNA recombination and replication, meiosis, telomere maintenance and cellular DNA damage responses. The Rad50 and Mre11 proteins are essential for viability in animals, although not in yeast. We have prepared antibodies to the Rad50 protein of the model plant *Arabidopsis thaliana* which recognize a 175 kDa protein in wild-type *Arabidopsis* protein extracts. Furthermore, we report here demonstration of the existence of the Rad50–Mre11 complex by co-immunoprecipitation of the Rad50 and Mre11 proteins from the plant cell extracts.

Key words: Rad50; Mre11; Protein complex; Plants; DNA repair; Recombination

1. Introduction

DNA double-strand breaks (DSBs) occur in living cells exposed to ionizing radiation or genotoxic chemicals. They are also produced during replication, meiosis, mating-type switching in yeast and V(D)J recombination in animal cells [1–3]. Two general pathways exist to repair DSBs and maintain genome integrity: non-homologous end-joining (NHEJ) that directly rejoins DSBs and homologous recombination (HR) which utilizes a sister chromatid or homologous chromosome as a template for DNA resynthesis and rejoining (reviews by [4–7]).

NHEJ is the major mechanism for the repair of DSBs in mammalian and plant cells whereas yeast cells rely preferentially on homology-based recombinational DNA repair. In *Saccharomyces cerevisiae*, several proteins including Rad50, Mre11 and Xrs2 are known to participate in NHEJ and processing of DSBs (reviewed by [6]). Rad50 and Mre11 sequences are well conserved and homologs of the yeast proteins have been identified in many species [8]. In mammals the functional equivalent of Xrs2 is p95 or NBS1, a protein implicated in the Nijmegen breakage syndrome [9,10]. It has been shown that the three proteins (Rad50, Mre11 and Xrs2 or Nbs1) physically interact and that the complex has nuclease activities and is implicated in recombination (both HR and NHEJ and the initiation of meiotic recombination), telomere maintenance, DNA damage detection and checkpoint signalling (discussed by [11–13]). Structural biochemistry and interaction architecture of the Rad50–Mre11 complex has recently been described [14–17].

Rad50, which is a member of the structural maintenance of chromosome (SMC) proteins family, consists of bipartite N- and C-terminal ATPase segments separated by two long heptad repeat regions. An antiparallel assembly of two Rad50 molecules creates two ATP binding:catalytic domains (Rad50 cds) joined by a coiled-coil. This three-dimensional structure is similar to that of ABC ATPase transporters. Mre11 binds as a dimer between the catalytic domains of Rad50 bringing the nuclease activities of Mre11 in close proximity of the ATPase and DNA-binding activities of Rad50. Nbs1 directs the nuclear localization of the complex and the radiation-induced focus formation [9,18].

The null mouse *rad50* mutant is inviable both in cultured ES cells and in developing mouse embryos [19]. This cell lethal phenotype has also been seen in mouse [20] and chicken [21] *mre11* mutants. This cell lethality has greatly complicated in vivo studies of the role of the Rad50–Mre11–Nbs1 complex in vertebrates. This is not so in plants, the *Arabidopsis thaliana rad50* mutant is viable [22], nor in *Caenorhabditis elegans* in which the *mre11* null mutant is viable [23]. Thus, in addition to the intrinsic interest of understanding DNA metabolism phenomena in the plant kingdom, studies of the Rad50–Mre11 complex in plants may also help in the understanding of these phenomena in vertebrates.

Plant homologs of both *MRE11* [24] and *RAD50* [22] have been identified, although no plant *XRS2* nor *NBS1* homolog is currently known. The predicted *Arabidopsis* Rad50 protein conserves the central coiled-coil conformation domain as well as Walker A and Walker B characteristics of the ATP-binding site. An *Arabidopsis rad50* mutant plant exhibits similar phenotypes to those of the yeast *rad50* mutant: sterility, hypersensitivity to MMS [22], hyper-Rec phenotype [25] and defects in telomere maintenance [26].

We have prepared antisera against the *A. thaliana* Rad50 and Mre11 proteins and show here the existence of a Mre11–Rad50 complex by co-immunoprecipitation of plant protein extracts.

2. Materials and methods

2.1. Growth of plants and cell suspensions

A. thaliana plants (Wassilewskija (ws) ecotype) were grown in a greenhouse under standard conditions. Seedlings carrying the *rad50* insertional mutation were selected on Murashige and Skoog medium (Sigma #M5519; St Quentin Fallavier, France) containing 50 mg l^{-1} kanamycin. Homozygous and heterozygous plants for the RAD50 allele were distinguished by phenotypic and PCR analysis. Cell suspensions were derived from wild-type and from homozygous (*rad50*/*rad50*) and heterozygous (*rad50*/*RAD50*) plants using standard tech-

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niques as described previously by Gallego et al. [22]. The *A. thaliana* cell suspension (T87) was established by Axelos et al. [27]. Cells were grown in Gamborg's B5 medium (Sigma #G5893), supplemented with 30 g l^{-1} sucrose and hormones as previously described [22] on a rotating platform (120 rpm) at 22°C with 16 h light/8 h dark. Cells in liquid culture were subcultured at weekly intervals.

2.2. Protein extraction

Frozen plants or cell culture tissues were ground in liquid N_2 with mortar and pestle. The powder was homogenized in a buffer containing 20 mM HEPES, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, pH 7.6, with freshly added protease inhibitor cocktail (Sigma #P9599), 0.1% NP-40 and 2 mM β-mercaptoethanol. NaCl concentration was adjusted to 420 mM with 5 M NaCl. Homogenates were then centrifuged at 20 000 × *g* for 30 min at 4°C. Supernatants were filtered through miracloth (100 μ mesh). Protein concentrations in the extracts were determined by using the Bio-Rad protein assay reagent kit.

2.3. Antibody production, immunoblot analysis and immunoprecipitation

The rabbit antiserum 98 was raised against a recombinant protein comprising amino acids 707–1256 of *A. thaliana* Rad50 fused to a His-tag and expressed in *Escherichia coli* BLR(DE3)pLysS (Novagen). The recombinant protein was insoluble so it was purified by excision from a 7.5% SDS–PAGE gel. The rabbit antiserum 304 was raised against a mix of two synthetic peptides covering amino acids 562–577 and 624–639 of *Arabidopsis* Rad50. Affinity-purified anti-Mre11 rabbit antibodies were generously provided by Drs H. Offenberg and C. Heyting (Laboratory of Genetics, Wageningen Agricultural University, Wageningen, The Netherlands). It was used at a 1:50 dilution.

Protein extracts from different plant tissues or suspension cells were separated in 7.5% SDS–PAGE gels. Proteins were transferred to nitrocellulose membranes (Hybond™ ECL™, Amersham Pharmacia Biotech). Membranes were blocked for 1 h in 10% non-fat dried milk in PBS-T (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, Tween 20 0.1%, pH 7.4), washed in PBS-T and incubated for 2 h in a 1:1000 dilution of antiserum 304 in 10% non-fat dried milk in PBS-T. Blots were washed in PBS-T and incubated for 2 h in 1:30 000 dilution of goat anti-rabbit HRP-conjugated secondary antibody (Bio-Rad) in 10% non-fat dried milk in PBS-T. Blots were washed in PBS-T and antibody-bound proteins were visualized with the ECL Western blotting detection system (Amersham Pharmacia Biotech). All steps were performed at room temperature.

For immunoprecipitations, total protein content from *A. thaliana* cell suspension (T87) and *A. thaliana* flower buds (1 mg protein per assay) were incubated in 1 ml PBS-T with 4 ml of Rad50 antiserum (98 or 304) or 15 ml of affinity-purified anti-Mre11 antibodies for 1 h at 4°C. Pre-immune antisera were used as negative controls. 20 μl of protein A-agarose beads (Santa Cruz Biotechnology) was added and incubation was continued overnight at 4°C with mixing. Beads were then collected by centrifugation at 1000 × *g* for 5 min at 4°C. Pellets were washed six times in PBS-T each time repeating the centrifugation step. After final wash, pellets were resuspended in 40 μl of 1× electrophoresis sample buffer (Tris–HCl 62.5 mM, pH 6.8, glycerol 25%, SDS 2%, β-mercaptoethanol 700 mM, bromophenol blue 0.1%), boiled for 3 min and proteins present in the immune complexes were separated in 7.5% SDS–PAGE gels. They were transferred to nitrocellulose and blots were revealed with Rad50 antiserum (304) or affinity-purified anti-Mre11 antibodies as described above.

3. Results and discussion

3.1. Expression of the RAD50 gene in Arabidopsis

Expression of the *Arabidopsis* Rad50 was analyzed in different plant tissues and suspension cells of the ws ecotype. In wild-type plants and cells, two major products were detected with the antiserum 304 (Fig. 1). The upper band corresponds to a protein of 175 kDa, in good agreement with the theoretical molecular weight calculated from the amino acid sequence of *Arabidopsis* Rad50 (152 719 Da). Absence of this product in the homozygous RAD50 mutant (Fig. 1, lanes 5 and 8) confirms that the 175 kDa protein is the *Arabidopsis* Rad50 protein. The lower band of approximately 110 kDa is present in all tissues tested without differences in its accumulation levels. As it is present in wild-type extracts, the 110 kDa band cannot derive from the rad50 mutant locus (Fig. 1, lanes 3 and 4). We therefore assume that the 110 kDa protein is a non-specific signal or a protein with common epitopes to Rad50 protein.

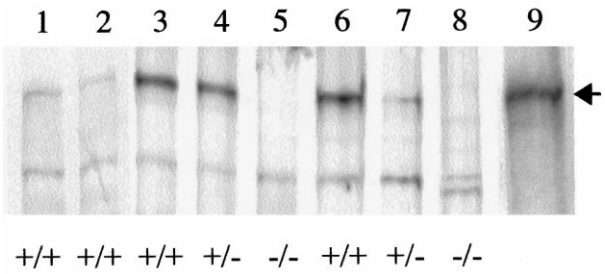

Fig. 1. Western blot analysis of Rad50 expression in different *Arabidopsis* tissues and suspension cells. The blot was incubated with antiserum 304 at a 1:1000 dilution. Lane 1, young rosette leaves grown for 2 weeks; lane 2, leaves of plants grown for 5 weeks; lanes 3–5, flower buds; lanes 6–8, ws suspension cells; lane 9, T87 suspension cells. +/+, wild-type plants or cells; +/−, plants or cells heterozygous for the *rad50* mutation; −/−, plants or cells homozygous for the *rad50* mutation. For each lane, 50 μg of total protein was loaded in the gel. The 175 kDa Rad50 band is indicated by an arrow. ws, Wassilewskija ecotype.

The expression level of Rad50 is 2–3-fold higher in flower buds (Fig. 1, lane 3) and 3–4-fold higher in suspension cells (Fig. 1, lane 6) than in leaves (Fig. 1, lane 6). This expression pattern is similar to that we have previously reported for Rad50 mRNA by Northern analysis, which showed higher Rad50 expression in suspension cells or plant tissues containing a high proportion of cells undergoing division [22]. Whereas in leaves (Fig. 1, lanes 1 and 2), where mitotic activity is limited, Rad50 accumulation is low, independent of the plant developmental stage. The strongest expression level is observed in T87 suspension cells (Fig. 1, lane 9) and these were chosen as the first source of material for immunoprecipitation experiments.

3.2. Rad50/Mre11 interaction

Anti-Rad50 sera 304 and 98 were tested to precipitate *Arabidopsis* Rad50 and any associated proteins from the T87 suspension cell protein extract. Only antiserum 98 could immunoprecipitate the protein Rad50, antiserum 304 was raised against synthetic peptides and does not recognize the native Rad50 protein (data not shown). When cell extracts were immunoprecipitated with anti-Mre11 antibody, Rad50 was detected in the immunoprecipitated material by antiserum 304 by Western blot. Control immunoprecipitation with the Mre11 pre-immune serum confirmed that the Rad50 and Mre11 proteins in the precipitate were specific to the anti-Mre11 antibodies. When the immune complexes were analyzed in Western blot with the anti-Mre11 antibodies, a band of approximately 83 kDa is detected (Fig. 2a). The same band is observed in total protein extracts from T87 suspension cells. Molecular weight of this product is in good agreement with the theoretical molecular weight calculated for the Mre11 protein (80 290 Da). Thus the anti-Mre11 antibody precipitates Rad50 protein from wild-type plant cell extracts. We were unable to carry out the inverse experiment as the

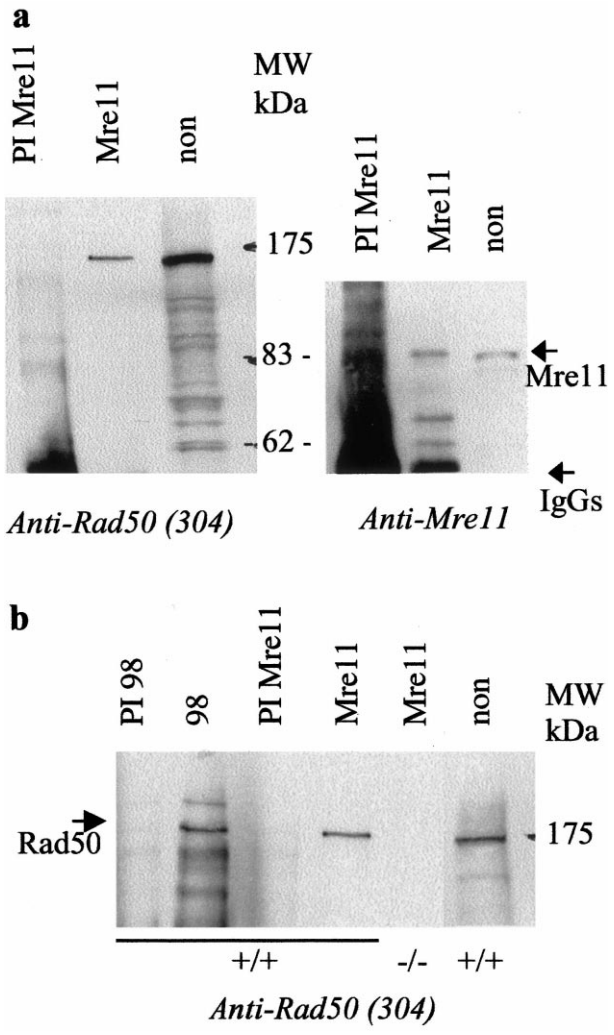

Fig. 2. Co-immunoprecipitation of Rad50 and Mre11 in a: T87 suspension cells and b: *Arabidopsis* flowers buds. Immunoprecipitates from T87 suspension cells and from flower buds of wild-type plants (+/+) and plants homozygous for the mutation of RAD50 (−/−) were analyzed in Western blot with the anti-Rad50 antiserum (304) and the anti-Mre11 antibody. Sera used in the immunoprecipitation experiments are indicated above each panel. Pre-immune sera (PI) were used as controls. Mre11, affinity-purified anti-Mre11 antibody; I98, antiserum 98 anti-Rad50; non, protein extracts analyzed in Western blot without immunoprecipitation experiments before. IgGs, heavy chain of gammaglobulins.

HRP-conjugated secondary antibody generated a background signal that masked the blot between 55 and 100 kDa where the Mre11 protein would be found.

The Rad50/Mre11 interaction was also visualized in protein extracts from flower buds (Fig. 2b). Rad50 could be precipitated both by anti-Rad50 (serum 98) and anti-Mre11 antibodies. As expected, in the *rad50* mutant no band was detected for the Rad50 product.

We thus conclude that the Rad50 and Mre11 proteins physically interact in plants as has been demonstrated in the other organisms [16,17,28–30]. This result, together with the similar phenotypes observed for the *Arabidopsis rad50* mutant compared to yeast *rad50* mutant – sterility and hypersensitivity to MMS treatment [22], stimulation of homologous recombination [25], deficiency in maintenance of telomere length [26] – suggest a high conservation of recombination and DNA repair mechanisms between plants and microorganisms. Furthermore, identification of a human functional homolog (Nbs1) for the *S. cerevisiae* Xrs2 protein [9,10] makes probable the existence of such a homolog in plants. Analysis of the *Arabidopsis* genome databases for sequence homologies with *XRS2* or *NBS1* was unfruitful, although this is perhaps not unexpected given the low sequence conservation of *XRS2* and *NBS1*. Using the antisera described here, we are currently working on the identification of a possible *Arabidopsis* Xrs2/Nbs1 homolog.

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