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pH- and Salt-Dependent Self-Assembly of Human Rad51 Protein Analyzed as Fluorescence Resonance Energy Transfer between Labeled Proteins

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Human HsRad51 protein assembles on a DNA molecule through cooperative binding and forms a long filament for homologous recombination. We have characterized the self-assembly of HsRad51 by measuring the fluorescence resonance energy transfer from the fluorescein-labeled protein to the rhodamine-labeled protein. Self-assembly quickly reached equilibrium and can be described by the head-to-tail polymerization of monomers, like that of its procaryotic homologue, RecA. It depended strongly on pH and was inhibited by high salt concentrations, indicating that ionic interactions between negatively and positively charged aminoacid residues are important. By contrast, neither ATP nor ADP significantly affected the reaction.

Key words: fluorescence resonance energy transfer, homologous recombination, protein self-assembly, Rad51 protein.

Abbreviations: FITC, fluorescein 5-isothiocyanate; RITC, rhodamine B 5-isothiocyanate; FRET, fluorescence resonance energy transfer.

The protein Rad51 is one of a widely distributed group of eukaryotic homologues of the procaryote RecA protein (*1*). They are found in species as different as yeast and man (*2*), and are involved in homologous recombination and recombinational repair (*3*). Rad51 interacts with the breast cancer suppressors BRCA1 and BRCA2 (*4–6*), and with another tumor suppressor, p53 (*7*). It is thus important for DNA repair and defense against tumor formation. It is also required for normal development of the mouse embryo (*8*, *9*). Mammalian cells lacking Rad51 undergo apoptosis.

Rad51, like *Escherichia coli* RecA, catalyzes the exchange of strands between homologous DNA molecules *in vitro* in the presence of ATP, although Rad51 catalyses the reaction less efficiently than RecA and requires other proteins for optimal activity (*10–17*). Rad51 forms a filamentous complex with DNA; the structure of this complex is similar to that of the RecA-DNA complex (*18*, *19*). Rad51 monomers form a helical filament (about 6 subunits per turn) around the DNA. Both Rad51 and RecA also undergo self-assembly to form helical filaments in the absence of DNA (*20–22*). The filaments of Rad51 and RecA are stretched by the nucleotide cofactor, ATP, increasing the pitch of the helix (*21*, *22*). This similarity between RecA and Rad51 suggests that filament formation is important for their activities. RecA mutants that cannot undergo self-assembly cannot catalyze strand exchange (*23*). Furthermore, determination of the self-assembly of the protein is necessary for analysis of its interaction with DNA (*24*)

We have characterized this self-assembly of human HsRad51 by measuring the fluorescence resonance energy transfer (FRET) from fluorescein-labeled HsRad51 to rhodamine-labeled HsRad51. We examined the effects of pH and salt to clarify the nature of the contact between the subunits, and the effects of ATP and ADP.

MATERIALS AND METHODS

Purification of Recombinant HsRad51 Protein—HsRad51 was produced in *E. coli* strain BL21(DE3) harboring pET-hsRad51 (kind gift from Dr. A. Shinohara). The bacteria were grown with ampicillin (0.1 mg/ml) at 37°C in LB medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration: 0.5 mM) was added when the turbidity at 600 nm reached 0.3–0.6, and the bacteria were incubated for a further 10–12 h at 25°C to induce Rad51 production. The bacteria were then harvested by centrifugation and suspended in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 25% sucrose.

The cells were processed twice in a French Press at 20,000 psi. All subsequent purification procedures were performed at 4°C. Polyethylenimine (Sigma) (0.1% final) was added to the resulting cell extract and the precipitate was removed by centrifugation at 30,000 ×*g* for 30 min. Ammonium sulfate (33% saturation final) was added to the supernatant under continuous stirring. The pellet was collected by centrifugation at 30,000 ×*g* for 30 min, dissolved in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl and 10% glycerol, and then loaded onto a Q-Sepharose (Amersham Pharmacia Biotech) column. HsRad51 was eluted with a linear gradient of NaCl (50–600 mM). The fractions containing HsRad51 were collected and loaded

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onto a hydroxyapatite column (Bio-Gel HTP, BioRad). HsRad51 was eluted with a linear gradient of sodium phosphate (0–500 mM), pH 7.0, in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10% glycerol. HsRad51 was precipitated by adding ammonium sulfate (50% saturation). The pellet was collected by centrifugation, dissolved in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM DTT and 10% glycerol, and then dialyzed against 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 50% glycerol. The resulting protein was stored at –80°C. It was over 95% pure, as judged on SDS–polyacrylamide gel electrophoresis.

Preparation of Fluorescein- and Rhodamin-Labeled HsRad51—HsRad51 was labeled with fluorescein or rhodamine by reaction with FITC or RITC (Sigma) (*25*). FITC or RITC was dissolved (20 mM) in *N,N*-dimethylformamide, and then added (5 μM final) to HsRad51 (2–3 μM) in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM EDTA. The mixture was incubated for 1 h at 25°C in the dark. Excess FITC or RITC was removed by dialysis against the same buffer for 3 h at 4°C.

Fluorescence Measurements—Fluorescence was measured with a FR-7500 spectrofluorometer (Jasco). Emission spectra were measured (bandwidths: 3 nm for the experiments at pH 7.4, and 5 nm for those at other pHs) with the selective excitation of fluorescein at 450 nm (band width: 3 nm). The spectra were measured more than twice to ensure the absence of photobleaching and averaged to increase the signal/noise ratio. The spectra were corrected for background and Raman scattering by subtracting the buffer signal. All experiments were performed at 25°C using Hepes/NaOH buffer for the experiments at pH 7.4, PIPES/NaOH for pH 6.5 and 6.0, and sodium acetate for pH 5.5, 5.0, and 4.5. The buffer concentrations were 50 mM, and the solutions usually contained 50 mM NaCl and 1 mM $MgCl_2$.

Molecular Sieve Chromatography—Unmodified and modified HsRad51 (20 μg in 20 μl) were chromatographed on Sephadex G-75 (Amersham Pharmacia) (6 cm × 0.4 cm column) in either 50 mM PIPES-NaOH, pH 6.0, 50 mM NaCl, and 1mM $MgCl_2$ (panel A), or 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 1 mM $MgCl_2$ (panel B) to examine their self-assembly. Elution drops (52 ± 1 μl for the low salt buffer and 55 ± 1 μl for the high salt buffer) were collected and their protein contents determined (Bio-Rad Protein Assay). Aliquots (20 μl) of each fraction were added to 150 μl of 1/5 diluted Protein Assay solution, and then the absorption at 595 nm was measured.

Other Materials—Fluorescein-labeled oligo(dT), 36 bases long, was obtained from Genset and used without further purification. Fluorescein was attached to the 5′ end of the oligonucleotide. ATP and ADP were from Sigma, and their stock solutions were brought to pH 7 with NaOH.

RESULTS

Detection of HsRad51 Self-Assembly by FRET—To avoid inactivation of the protein on labeling, we performed the modification with a low concentration of organic solvent (*N,N*-dimethylformamide) and limited the modification to about one fluorescent probe attached to one protein subunit on average. The degree of modification was verified by absorption spectra of fluorescein-labeled and rhodamine-labeled HsRad51. The labeled proteins actively bound to DNA and underwent self-assembly. The binding of DNA to modified and unmodified proteins was studied using fluorescein-labeled oligo$(dT)_{36}$ as a DNA. The binding of the rhodamine-labeled protein was analyzed by FRET from the fluorescein probe of the oligonucleotide to the rhodamine label of the protein, while the binding of DNA to the unmodified protein was monitored as the change in fluorescence anisotropy of fluorescein-labeled oligo$(dT)_{36}$ (*26*). The titration of fluorescein-labeled oligo$(dT)_{36}$ by the labeled and unlabeled HsRad51 was very similar, with a binding stoichiometry of about 6 bases of DNA/protein monomer (not shown). The self-assembly of labeled and unlabeled HsRad51 was examined by molecular sieve chromatography. Both modified and unmodified HsRad51 were eluted in the void volume with low salt and low pH (50 mM PIPES-NaOH, pH 6.0, 50 mM NaCl, and 1mM $MgCl_2$) (Fig. 1A), indicating that they underwent self-assembly to form oligomers. By contrast, both proteins were eluted mainly as monomers with high salt and neutral pH (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 1 mM $MgCl_2$) (Fig. 1B). The labeling had no effect on self-assembly.

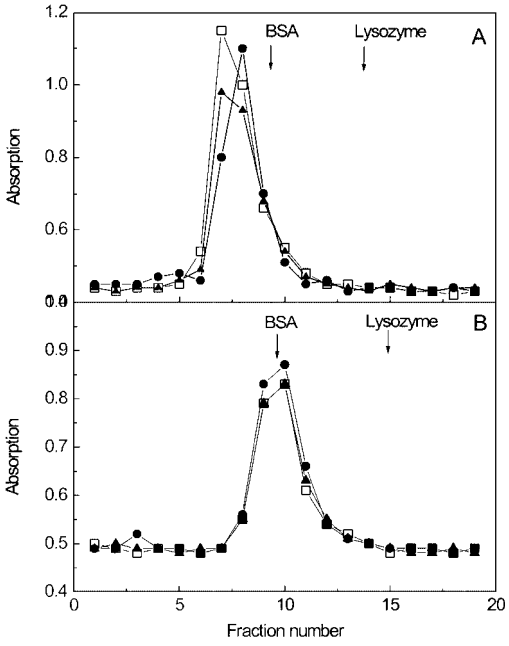

Fig. 1. **Self-assembly of modified and unmodified HsRad51.** The elution patterns of fluorescein-labeled (closed circles), rhodamine-labeled (closed triangles), and unlabeled (open squares) HsRad51 from Sephadex G-75 with low (panel A) and high salt (panel B) are shown, plus the positions of the BSA (66 kDa) and lysozyme (14 kDa) elution peaks.

The fluorescence emission of a mixture of 8 μM fluorescein-labeled HsRad51 and 8 μM rhodamine-labeled HsRad51 differed from that of 8 μM fluorescein-labeled HsRad51 alone, although rhodamine-labeled HsRad51 alone did not significantly fluoresce under our experimental conditions (Fig. 2). The mixture gave a less intense emission peak at 520 nm (the fluorescence of fluorescein) than the fluorescein-labeled protein alone, and a second peak at 575 nm (the fluorescence of rhodamine).

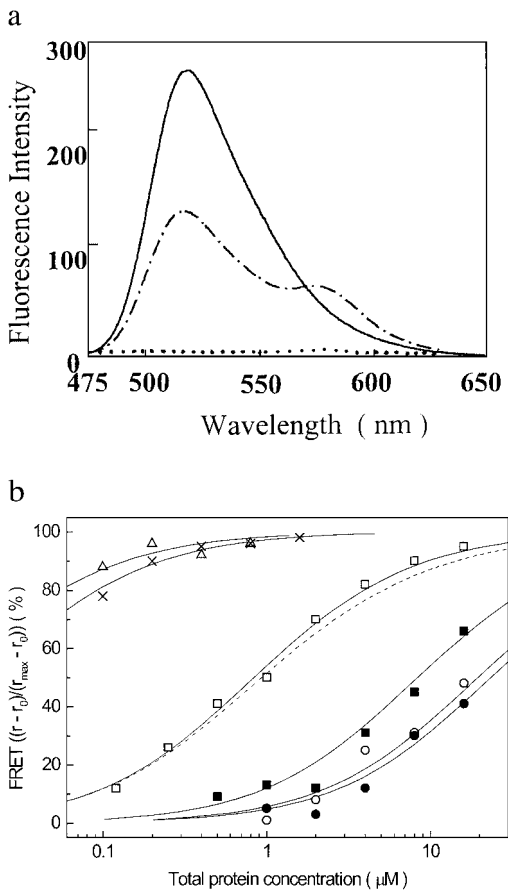

Fig. 2. **Protein concentration and pH dependent self-assembly of HsRad51.** Panel A: The emission spectra of 8 μM fluorescein-labeled HsRad51 with (broken line) and without (continuous line) 8 μM rhodamine-labeled HsRad51, plus the spectrum of 8 μM rhodamine-labeled HsRad51 alone (dots) at pH 5.5. Panel B: The fractions of the fluorescein-labeled protein in contact with the rhodamine-labeled protein were computed from the FRET ($r = F_{575}/F_{520}$), as described in the text, with various concentrations of HsRad51 at pH 7.4 (solid circles), pH 6.5 (open circles), pH 6.0 (solid squares), pH 5.5 (open square), pH 5.0 (×), and pH 4.5 (open trianbles). Theoretical curves were computed with $\alpha = 1$ and $K_1 = 50$, 30, 0.7, 0.07, 0.03, and 0.025×10^6 $M^{-1}$for the data at pH 4.5, 5, 5.5, 6, 6.5, and 7, respectively. For the data at pH5.5, the curve with $\alpha = 0.8$ is also computed (broken line)

There was clear FRET from the fluorescein-labeled HsRad51 to the rhodamine-labeled HsRad51, reflecting their interaction. The FRET appeared immediately after they were mixed and remained unchanged for 20 minutes, indicating that the association occurred quickly. The FRET, judged as the ratio of the fluorescence intensities at 575 nm and 520 nm (F_{575}/F_{520}), became lower when samples were diluted with buffer (Fig. 2B). This dependence of FRET on the protein concentration further indicates that the FRET is linked to the self-assembly of HsRad51. FRET decreased quickly after dilution, indicating that the oligomers also dissociated rapidly.

This dependence of FRET on the protein concentration was quantified using a head-to-tail polymerization model (*24*), because the Rad51 filament, like the RecA one, is polar (*27*):

$$A_1 \overset{K_1}{\leftrightarrow} A_2 \overset{K_2}{\leftrightarrow} A_3 \overset{K_3}{\leftrightarrow} A_4 \ldots A_{n-1} \overset{K_n}{\leftrightarrow} A_n \overset{K_{n+1}}{\leftrightarrow} A_{n+1} \ldots$$

where A_n is the n-mer of HsRad51 and K_n is the association constant for the binding of a monomer to an n-mer to form $(n+1)$-mer HsRad51. $K_n = K_{n-1} * \alpha^{n-1}$, with α being cooperativity. Self-assembly can occur through contact between two protein subunits that are labeled with the same fluorescence probe. This does not promote any FRET and could complicate analysis. We mixed a 4-fold excess of the rhodamine-labeled protein with the fluorescein-labeled protein to minimize this effect. We also assumed that the efficiency of FRET was not affected by the binding of a second rhodamine-labeled protein. The FRET between the subunits that were not direct neighbors was neglected because the distance between them should be greater than 6 nm according to the structure of the helical filament of Rad51 indicated by electron microcopy and small-angle-neutron-scattering (*18*, *19*, *22*).

The fraction of the fluorescein-labeled protein in contact with the rhodamine-labeled protein was determined from the ratio of the intensity of the rhodamine signal at 575 nm (F_{575}) to that of fluorescein at 520 nm (F_{520}), with the relationship:

$$\text{Fraction of protein in contact} = (r - r_0)/(r_{\max} - r_0),$$

where $r = F_{575}/F_{520}$, r_0 is this ratio in the absence of the rhodamine-labeled protein, and $r_{\max}$ is the value expected with a saturating amount of the rhodamine-labeled protein. Since the fluorescein fluorescence depended upon pH, $r_{\max}$ were estimated for each pH by extrapolation of 1/r *vs.* 1/[protein] plots. The FRET was estimated more accurately from the ratios than from the changes in intensity of either fluorescein (F_{520}) or rhodamine (F_{575}) fluorescence. All the experiments were repeated at least twice and the results were averaged. The precision was better than 10%.

The head-to-tail polymerization model predicts that the fraction of protein in contact is $\Sigma\,[K_1 * \alpha^{i-1} * [A_i] * (i + 1)]/(1 + \Sigma\,[K_1 * \alpha^{i-1} * [A_i] * (i + 1)])$ at a total protein concentration of $(1 + \Sigma\,[K_1 * \alpha^{i-1} * [A_i] * (i + 1)]) * [A_1]$, with $[A_1]$ being the concentration of monomer. The simulation analysis showed that the cooperativity, α, has little effect on the theoretical curves. This probably reflects the fact that the FRET occurs with the formation of dimers, the first step of self-assembly. However, weak cooperativity ($\alpha = 0.8$) can still be detected, if it is present (Fig. 2B). The intrinsic association constant K_1 is linked to the concentration of protein required for half effect and can be accurately determined. The experimental data obtained at pH 5.5 fitted this model very nicely, with $K_1 = 0.7\ (\pm 0.2) \times 10^6$ M^{-1} and $\alpha = 1\ (\pm 0.2)$ (Fig. 2B). The ratio of rhodamine-labeled protein/fluorescein-labeled protein did not significantly affect the result (not shown).

Effects of pH and Salt on HsRad51 Self-Assembly—The degree of FRET, expressed as the F_{575}/F_{520} ratio, increased with the protein concentration, but did not reach a plateau even at 20 μM HsRad51 at pH 7.4 (Fig. 2B). By contrast, the value was very high at pH 4.5, even with low protein concentrations, and almost plateaued at 1 μM. The pH affected the association constant, but all the data could be fitted nicely by the head-to-tail self-

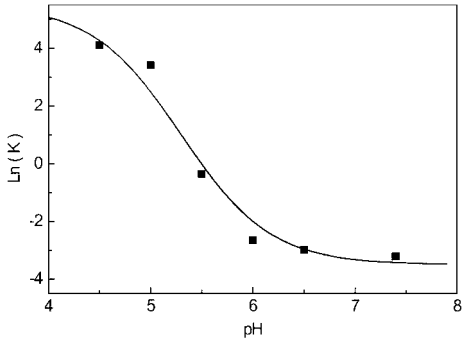

Fig. 3. **Effect of pH on HsRad51self-assembly.** The association constants for HsRad51 self-assembly were determined at various pHs, and analyzed assuming that the protonation of one residue of protein increases the association constant.

assembly model, with $\alpha = 1$ (Fig. 2B). The association constant changed markedly between pH 5 and 6 (Figs. 2B and 3). This large change over a narrow pH range indicates that the protonation of a chemical group is important for the reaction. The results can be analyzed (Fig. 3) by assuming that the protonation of one residue with $pK_a = 5.3$ increases the association constant K_1 from 2.5×10^4 to 5.0×10^7 M^{-1}.

The self-assembly of HsRad51 was also affected by the salt concentration (Fig. 4). The association constant decreased markedly (10-fold) when the NaCl concentration was increased from 50 to 150 mM (Fig. 4A), indicating that an ionic interaction between the subunits is involved in oligomer formation. However, a further increase in the NaCl concentration from 150 to 500 mM had less effect on self-assembly (2-fold decrease). The ionic interactions may be offset by hydrophobic interactions, as in RecA. Some hydrophobic interactions may be involved in the contact between subunits. The effects of salt at pH 5.5 (Fig. 4A) and pH 6.5 were similar (Fig. 4B).

Effects of ATP and ADP on HsRad51 Self-Assembly—Adding ATP or ADP to the mixture of fluorescein- and rhodamine-labeled HsRad51 did not affect the FRET at either pH 6.5 or pH 5.5 (not shown). The results were also similar with a high salt concentration (0.5 M NaCl). The thermodynamic parameters of self-assembly of the HsRad51 protein were not significantly affected by the binding of ATP or ADP.

DISCUSSION

We have used FRET measurements to analyze the self-assembly of HsRad51 and to demonstrate its head-to-tail organization. We have precisely determined the equilibrium constants for the self-assembly of Rad51, This determination is required for analysis of the real DNA binding character of Rad51 because the self-assembly affects the DNA binding, as demonstrated for RecA (*24*). Without analyzing the self-assembly, it would be impossible to determine whether the effects of ATP and ADP on the DNA binding to Rad51 (*28*) are due to the change in self-assembly or to their direct effect on DNA binding. Our observation that the nucleotides do not affect the Rad51 self-assembly association constant therefore indicates that these nucleotides directly affect the binding of DNA to Rad51.

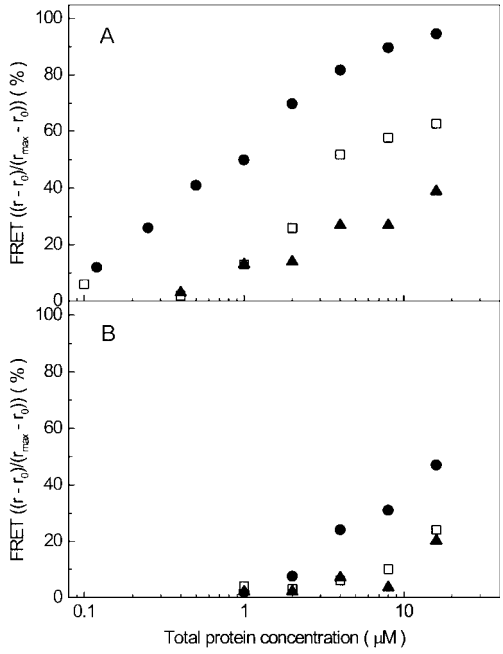

Fig. 4. **Effect of salt on HsRad51 self-assembly.** The changes in FRET ($r = F_{575}/F_{520}$, see the text) with the HsRad51 concentration were measured in the presence of 0.05 M (solid circles), 0.15 M (open squares), and 0.5 M (solid triangles) NaCl at pH 5.5 (panel A) and 6.5 (panel B).

Our results also indicate that both ionic and hydrophobic interactions between the subunits contribute to the self-assembly of Rad51, as they do to that of RecA (*24*, *29*). Crystallographic and mutation analyses of RecA have shown that the RecA filament is formed through contact between the positively charged N-terminal part of one molecule and the cluster of negatively charged residues around residue 125 of another (*30*, *31*). We have therefore looked for similarity between the N-terminal parts of HsRad51 and RecA. But HsRad51 has no cluster of positively charged residues in its N-terminal segment and there is no apparent similarity between their sequences (*2*). Furthermore, the N-terminal part of Rad51 is considered to interact with DNA (*32*). The assembly of Rad51 may be different from that of RecA, despite their overall similarity.

The strong pH dependency of Rad51 self-assembly suggests some conformation change of Rad51 depending on pH. Our CD measurements support this conclusion showing a significant change in the secondary structure of the protein: a helical structure is decreased at lower pH. We can speculate that the interaction of Rad51 with another protein (*4–7*, *13*, *15*) or its phosphorylation (*33*) introduces such a conformation change or facilitates the protonation of residues important for the self-assembly and thus activates Rad51.

Our study also shows how useful FRET measurements are for analyzing the self-assembly of proteins. The method complements the light scattering measurements used to study the self-assembly of RecA (*24*, *29*, *34*). FRET occurs with the formation of dimers, so it mainly

provides information about the first step (dimerization) of self-assembly. In contrast, light scattering detects large oligomers more easily than dimmers, and provides information about the global state of self-assembly rather than the first step. FRET measurements can also provide information about the kinetics of association and dissociation. Rad51 quickly associates to form oligomers, which in turn dissociate rapidly.

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