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Modulation of RAG/DNA complex by HSP70 in V(D)J recombination

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Abstract

V(D)J recombination, a site-specific gene rearrangement process, requires two RAG1 and RAG2 proteins specifically recognizing recombination signal sequences and forming DNA double-strand breaks. The broken DNA ends tightly bound to RAG proteins are joined by repair proteins. Here, we found that heat shock protein 70 was associated with RAG2 following two-step affinity chromatography purification. It was also co-immunoprecipitated with RAG2 in pro-B cells. Purified HSP70 protein disrupted RAG/DNA complexes assembled in vitro and also inhibited the V(D)J cleavage (both nick and hairpin formation) in a dose-dependent manner. This HSP70 action required ATP energy. These data suggest that HSP70 might play a crucial role in disassembling RAG/DNA complexes stably formed during V(D)J recombination.

Keywords: V(D)J recombination; RAG2; HSP70; Protein disassembling

The variable region genes of antigen receptors are assembled by a combinatorial recombination of three different gene segments: V (variable), D (diversity), J (joining). This site-specific V(D)J recombination is a major process responsible for providing the diversity of antigen receptor repertories during the B or T cell development [1].

V(D)J recombination begins with recognition and cleavage of recombination signal sequences (RSS) flanking V, D, or J gene segments by recombination activating gene products (RAG1 and RAG2). A RSS is composed of relatively conserved heptamer (CACAGTG) and nonamer (ACAAAAACC) separated by a less conserved spacer of either 12 or 23 bp sequences, called 12 RSS or 23 RSS, respectively. RAG1/2 complexes only select a pair of 12 RSS and 23 RSS in cells (''12/23 rule'') [2]. In vitro, the RAG complex can bind independently to 12 RSS or 23 RSS and form a nick at the border between coding sequence and heptamer sequence, followed by hairpin formation at the coding sequence end [3]. The 12/23 rule makes more efficient hairpin production after stable synaptic complex formation of RAG1/2, RSSs, and an additional non-histone chromatin protein HMG1 [4,5]. After cleavage, RAG proteins stay on the RSS DNA to prevent DNA degradation by some nucleases or keep the integrity of the broken DNA ends [6]. The broken DNA is further processed by non-homologous DNA repair system. The RSS ends are precisely joined in the head-to-head manner, and the coding hairpin ends are processed before joining. The repair system in V(D)J recombination requires a general DNA double-strand break repair proteins such as DNA-dependent protein kinase (DNA-PK) catalytic subunit, Ku70, Ku80, XRCC4, Artemis, DNA ligase IV, etc [1,7].

The transient conformational change of stable RAG/RSS complexes might be necessary for access of DNA repair proteins to the broken DNA ends during V(D)J recombination. In fact, bacterial Mu transposition, a similar mechanism to V(D)J recombination, requires a host heat shock protein ClpX that can promote overall transposition by disassembling MuA tetramers [8]. Here, we identified heat shock protein 70 associating with RAG2 and analyzed its molecular chaperone activity to disassemble RAG/RSS DNA complexes.

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Materials and methods

Materials. Ni^{2+}-NTA Agarose was from Qiagen. Pre-cast TBE (4–20% gradient) polyacrylamide gels were purchased from Invitrogen. Protein A agarose beads were obtained from PIERCE. Anti-RAG2 and anti-HSP70 antibodies were purchased from BD Pharmingen and Santa Cruz, respectively. RAG proteins and HMG1 protein were purified as described previously [9,10].

Mass spectrometry. A specific protein associating with RAG2 was cut out from the gel and identified by the method described previously [11].

Cloning, overexpression, and purification of human HSP70. Human heat shock protein 70 gene was amplified by PCR using two HSP70-specific primers and human placenta cDNA library (Clontech) and subcloned into a bacterial expression vector pDRK-N(M) [12]. The *hsp70* gene was confirmed by DNA sequencing. The resulting plasmid pHSP70 was transformed into *Escherichia coli* DH5α. Cells were grown in LB medium with shaking at 37 °C until an OD_{600} reaches 0.7–0.8, then induced with 1 mM IPTG at 37 °C for 3 h. Cells were collected by centrifugation at 6000 rpm at 4 °C for 15 min. The cell pellet was suspended in 1× Ni^{2+}-binding buffer (25 mM Tris, pH 7.5, 0.5 M NaCl, 10% glycerol) with 2 mM imidazole, 2 mM β-mercaptoethanol, and 1 mM PMSF. Cells were lysed by sonication for 30 s 9 times on ice, and soluble proteins were separated by a centrifugation at 25,000 rpm at 4 °C for 30 min. The 6× histidine-tagged HSP70 was loaded on 1 mL Ni^{2+}-column, washed with Ni^{2+}-binding buffer containing 30 mM imidazole and eluted with 200 mM imidazole. After pooling fractions, the protein solution was dialyzed in the buffer (25 mM Hepes, pH 7.5, 150 mM K-Glu or NaCl, KCl, 10% glycerol, 2 mM DTT) for 4 h and stored at −70 °C after being frozen in liquid nitrogen.

Co-immunoprecipitation. Mouse pro-B lymphoma cells (NSF-70 C-10) were cultured as described [11]. Cells were washed by cold 1× PBS twice and were scraped in immunoprecipitation buffer (25 mM Tris–Hcl, pH 8.1, 10% glycerol, 0.01% NP-40, 1 mM EDTA, 100 mM KCl, 1 mM DTT, 1 mM PMSF). Then, cells were swirled at 4 °C for 20–30 min. The cells were harvested by centrifugation at 13,000 rpm at 4 °C for 15 min. RAG2/HSP70 complexes were immunoprecipitated using anti-RAG2 antibody as described [13]. Proteins were separated in a 10% SDS–polyacrylamide gel and blotted using anti-HSP70 antibody.

V(D)J cleavage and RAG/DNA binding analyses. Both cleavage and binding assays were modified from the methods described [9,10]. The reaction mix (25 mM Hepes (pH 7.5), 2 mM DTT, 60 mM K-Glu, 1 mM $CaCl_2$, 0.025 pmole ^{32}P-labeled oligonucleotide substrates, 50 ng competitor salmon sperm DNA, 100 ng RAG1, 50 ng RAG2, and 2 mM ATP) was incubated at 30 °C for 30 min to form RAG/DNA complexes and further incubated at 30 °C for 1 h in the presence of HSP70 or BSA (0.2 μM, 2 μM, 10 μM). RAG/DNA complexes were separated on a 4–20% gradient native polyacrylamide gel with 0.5× TBE and detected by autoradiography. For cleavage analysis, RAG/DNA complexes formed in the condition with $CaCl_2$ were further incubated at 30 °C for 1 h in the presence of 2 mM $MnCl_2$ and HSP70 or BSA (0.2 μM, 2 μM, 10 μM). DNA products were separated in a 10% 7 M urea superdenaturing polyacrylamide gel and identified by autoradiography.

Results

HSP70 interacts with RAG2

V(D)J recombinase, a complex of RAG1 and RAG2, is necessary for recognition and cleavage of two RSSs to be recombined. The critical amino acid residues of V(D)J recombinase for catalytic activity are exclusively located at the RAG1 protein [10]. However, RAG2 proteins are absolutely required for formation of an active recombinase in V(D)J recombination.

While RAG1 purified from bacterial cells is fully active, RAG2 is completely inactive when it is obtained from bacterial cells. Therefore, RAG2 is typically purified from mammalian cells and used for the V(D)J recombination assay in vitro. We purified RAG2 proteins from HeLa cells transfected with *rag2-flag*-overexpressing vectors using two-step affinity chromatography: Ni^{2+}-chelating and anti-flag antibody chromatography. Throughout this two-step affinity purification, a specific protein at the 70 kDa range was co-purified with RAG2 (Fig. 1A). All five fractions collected from the second anti-flag antibody chromatography showed the similar amount of RAG2 and a 70 kDa-protein. We applied the 70 kDa-protein to MALDI-TOF mass spectrometry analysis and identified it as human heat shock protein 70 (HSP70). We further verified it by the western blot analysis using anti-HSP70 antibody. We detected a specific HSP70 band in the RAG2 protein purified from HeLa cells but not in RAG1 and RAG2 purified from insect or bacterial cells (Fig. 1B).

If RAG2–HSP70 interaction is a critical event during V(D)J recombination, this interaction can be observed in pro-B cells that endogenously express RAG2. To determine this interaction in pro-B cells, we carried out co-immunoprecipitation using anti-RAG2 antibody in NSF-70 C-10 pro-B lymphoma. HSP70 protein was specifically detected at the same size of bacterial purified HSP70 when anti-RAG2 antibody was added to the reaction (Fig. 2, lane 3), but not in the absence of anti-RAG2 antibody (lane 2). These results suggest that HSP70 is specifically associated with RAG2 during V(D)J recombination.

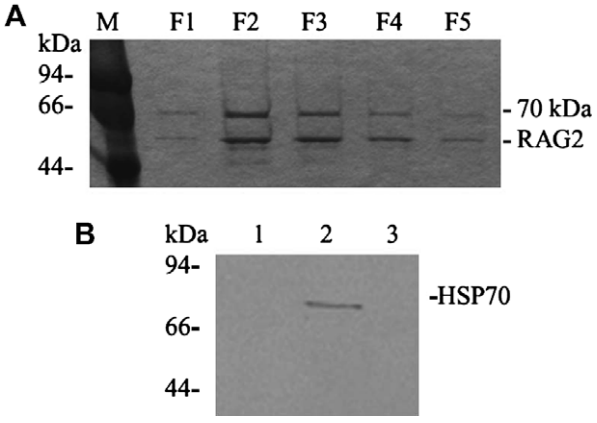

Fig. 1. Co-purification of HSP70 with RAG2. (A) RAG2 purification from the anti-flag antibody affinity chromatography. RAG2 was similarly purified as described previously [9,10]. The fraction pool of the first Ni^{2+}-chelating chromatography was applied to the anti-flag antibody column, and each fraction of RAG2 proteins was collected by addition of flag peptide after washing the column. Proteins in each fraction (indicated by F1–F5) were separated in a 10% SDS–polyacrylamide gel and stained with Coomassie blue. (B) Western blot of purified proteins. Purified proteins: RAG2 from *E. coli* (lane 1), RAG2 from HeLa cells (lane 2), RAG1 from insect cells described [9] (lane 3) were analyzed by the Western blot analysis using anti-HSP70 antibody.

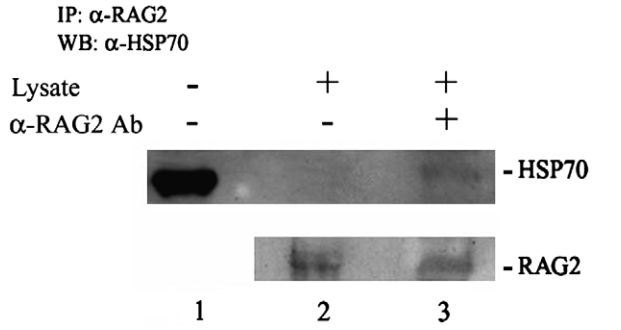

Fig. 2. Interaction of HSP70 and RAG2 in pro-B cells. The co-immunoprecipitation of HSP70 and RAG2 in pro-B cells was described in Materials and methods. First, RAG2/HSP70 complexes were immunoprecipitated using anti-RAG2 antibody, and then HSP70 was detected with Western blot using anti-HSP70 antibody. Lane 1 is HSP70 purified from *E. coli*. Lane 2 and lane 3 are omission and presence of anti-RAG2 antibody, respectively, in the precipitation reaction. The low panel shows RAG2 amount in each reaction.

HSP70 can disrupt RAG/DNA complexes

HSP70 is typically responsible for protein folding or unfolding as molecular chaperone under cellular stress [14]. Also, HSP70 is implicated in many other cellular processes such as protein translocation, activation of steroid hormone complexes, cell death, DNA replication, and gene expression [15,16]. In order to look at how HSP70 is involved in the V(D)J recombination process, we first analyzed whether HSP70 can disrupt RAG/DNA complexes assembled in vitro.

RAG proteins were incubated with 12/23 RSS DNA to form RAG/DNA complexes in the presence of Ca^{2+} ions, and then further incubated in the presence or absence of purified HSP70 or BSA. The RAG/DNA complexes were detected by the gel mobility assay. As expected, HSP70 inhibited the formation of RAG/DNA complexes in a concentration-dependent manner. But, BSA did not affect disassembly of RAG/DNA complexes (Fig. 3A). We further tested how HSP70 influences the V(D)J cleavage following the RAG/DNA complex formation. Addition of Mn^{2+} to the reaction with RAG/DNA complexes inhibited the V(D)J cleavage by RAG proteins in the presence of HSP70 (Fig. 3B). Both hairpin and nick formation were similarly inhibited in the reaction with HSP70. However, BSA did not affect V(D)J cleavage by RAG proteins. In conclusion, HSP70 plays an important role in the transient conformational change of RAG/RSS DNA complexes during V(D)J recombination.

Disassembly of RAG/DNA complexes by HSP70 requires ATP energy

Heat shock proteins usually require ATP hydrolysis for the molecular chaperone activity [14]. If HSP70 is used for deforming RAG/DNA complexes as a molecular chaperone, both RAG binding and V(D)J cleavage activity might

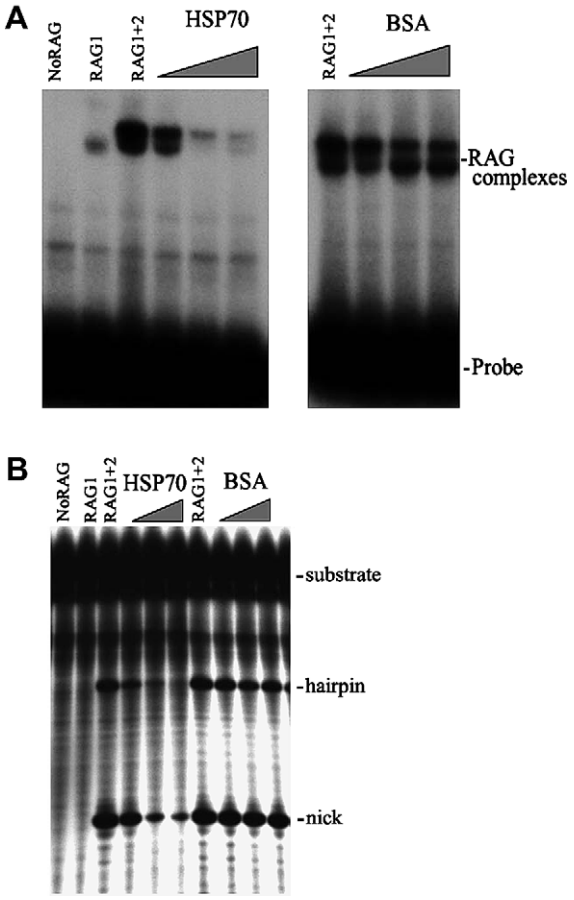

Fig. 3. Effect of HSP70 on V(D)J recombination activity. In vitro binding activity (A) and V(D)J cleavage activity (B) were determined as described in Materials and methods. HSP70 or BSA as a control (0.2, 2, 10 μM) was added to each binding reaction with 2 mM ATP.

be affected by the presence of ATP in the reaction with HSP70. As mentioned above, both RAG/DNA complex formation and V(D)J cleavage were inhibited in the presence of ATP because of disruption of RAG/DNA complexes by HSP70 (Fig. 4). However, they were not affected by HSP70 in the absence of ATP, suggesting that HSP70 disrupts RAG/RSS DNA complexes in an ATP-dependent manner.

Discussion

V(D)J recombination is a site-specific gene rearrangement process that contributes to the diversity of antigen receptor repertoires in immune system. Two lymphoid-specific proteins, RAG1 and RAG2, collaborate to initiate this process by binding two RSSs and making a double-strand break between a RSS and adjacent coding DNA, resulting in production of a hairpined-coding end and a double-strand break at the signal end. Two broken DNA coding ends are joined by a double-stranded break repair system after being reopened. During this process, RAG complexes remain to be tightly bound to the broken DNA ends after cleavage [6]. Therefore, some proteins might be required

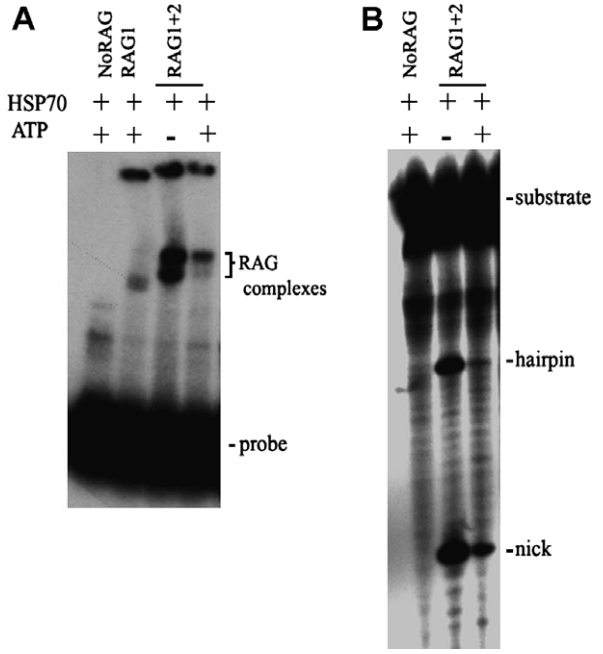

Fig. 4. ATP requirement for HSP70 in RAG/DNA binding and V(D)J cleavage. Both binding (A) and cleavage (B) analyses were carried out as described in Materials and methods in the presence (+) or absence (−) of 2 mM ATP.

for control of transition between cleavage and repair in V(D)J recombination. According to a previous study, it has been suggested that Ku80 might play in disassembly of post-cleavage complex [17]. In addition, Ku80 is required to allow TdT access to RAG post-cleavage complexes, providing support for the hypothesis that Ku proteins are involved in disassembling or remodeling the post-cleavage complex [18]. However, Ku80 still is unlikely as protein disassembler.

Many heat shock proteins have been implicated as molecular chaperones capable of remodeling other proteins. Mu transposition, a similar situation of V(D)J recombination, proceeds through a series of stable synaptic complexes to yield finally the strand transfer complex (STC) [19]. Disassembly of the STC is necessary for completion of the transposition reaction, and it requires the action of the bacterial chaperone protein ClpX, which binds to MuA and facilitates its dissociation from the STC complex [8,20]. Like this, such a chaperone protein may also function in V(D)J recombination to promote disassembly of the RAG/DNA cleavage complex.

In this report, we showed that HSP70 directly interacted with RAG2 and disrupted RAG/RSS DNA complexes assembled in vitro. HSP70 might unfold RAG2 and sequentially induce RAG protein degradation in cells by destabilizing the entire RAG/DNA complexes as shown in Mu transposition. In fact, HSP70 promotes many cellular reactions by enhancing protein degradation. Apoprotein B degradation is largely increased by HSP70 [21]. In addition, HSP70 binds to human apurinic/apyrimidinic endonuclease and promotes base excision repair [22]. Taken together, we here suggest that HSP70 can be involved in V(D)J recombination as a protein dissembler to remodel RAG/DNA complexes.

Acknowledgments

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