

Recombination Activating Gene (RAG)-1 and 2 Encoding Proteins Expressed by the Baculovirus System

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We have been attempting to obtain mouse recombination activating gene-1 (RAG-1) and RAG-2 protein for biochemical analyses. First of all, we obtained truncated products of these genes expressed and purified using the *E. coli* expression system and then established the polyclonal and monoclonal antibodies by means of *E. coli* expressed peptides as antigens. Subsequently, whole RAG-1 and RAG-2 gene products were expressed the baculovirus expression system. Since it has been difficult to achieve the significant gene expression of full-length cDNA, we employed the glutathione S-transferase (GST)-fused gene-expression system which facilitated the massive expression of gene products. This system was also advantageous in that we could detect the expressed protein molecules not only with anti-RAG antibody but also with anti-GST antibody.

Introduction

The diversity of immunoglobulins and T-cell receptors for antigens is created by a site-specific recombination of their genes during lymphocyte development, so-called V (D) J recombination (Tonegawa, 1983). Many researchers have recognized V (D) J recombinase to be a complex and have attempted to identify one of the components of V (D) J recombinase (Aguilera et al., 1987 ; Halligan and Desiderio, 1987 ; Matsunami et al., 1989).

Terminal deoxynucleotidyl transferase (TdT) was shown to be directly involved in V (D) J recombinase, a template-independent DNA polymerase. The function of TdT is the N nucleotide insertion at the recombined sites. The TdT activity, however, is not essential for the recombination step because occurrence of V (D) J recombination without TdT molecules has been detected by the analysis of V (D) J recombination activity-implementing gene transfection experiments and TdT knockout mice (Kallenbach et al., 1992 ; Gilfillan et al., 1993 ; Komori et al., 1993).

Melvin Bosma et al. reported in 1983 that the SCID mouse exhibits an aberration in V (D) J recombination during lymphocyte development and then produces aberrant recombined products (Bosma et al., 1983 ; Schuler et

al., 1986). Recently, the catalytic subunit of DNA dependent protein kinase (DNA-PKcs) was proposed as a candidate for the SCID factor (Blunt et al., 1995 ; Kirchgessher et al., 1995). Further analyses require for understanding the role of the DNA-PK activity to V (D) J recombination.

Recombination activating gene (RAG)-1 and RAG-2 were identified as genes possessing an ability to induce lymphocyte-specific nonhomologous recombination : V (D) J recombination activity into the fibroblast cell line NIH/3T3 (Schatz and Baltimore, 1988 ; Schatz et al., 1989 ; Oettinger et al., 1990). Their vital role in V (D) J recombination in both B and T cells was confirmed by study of RAG-1 and RAG-2 gene knock-out mouse (Mombaerts et al., 1992 ; Shinkai et al., 1992). Recently, the functions of RAG-1 and RAG-2 products are going to be disclosed with truncated RAG-1 and RAG-2 products which exhibits soluble characteristics (McBlane et al., 1995 ; Sadofsky et al., 1995 ; van Gent et al., 1995).

Biochemical analyses of RAG-1 and RAG-2 products seem to be essential for an understanding of the V (D) J recombination machinery. However, the instability and sparsity of RAG-1 and RAG-2 makes their detection in vivo and their purification difficult (Lin and Desiderio, 1993). Furthermore, their expression in other species has also been difficult so far (Gellert, 1992 ; Oettinger, 1992). To overcome these problems, therefore, we attempted to obtain a large amount of RAG products using the baculovirus system. In this study, we succeeded in mass-expression of RAG-1 and RAG-2 products using the baculovirus expression system with GST-fused RAG genes.

Materials and methods

1. Expression of truncated RAG proteins in *E. coli*.

M6-BSK⁺ and MR2-BSK⁺ contain mouse full-length RAG-1 cDNA and mouse full-length RAG-2 cDNA, respectively. Each cDNA fragment is cloned at the *Not* I site of pBluescript SK⁺ (Stratagene, La Jolla, Ca., U. S. A).

Amino acid number of the truncated products were shown in figure 1. M6-BSK₊ *Pvu* II -*Sac* I fragment (216 bp) prepared from M6-BSK₊ and *Pvu* II -*Hinc* II fragment (442 bp) prepared from MR2-BSK₊ were transferred to pGEX-3X (Pharmacia P-L Biochemicals Inc., Milwaukee, Wisconsin, U. S. A.). Following the transfection of these vectors into *E. coli* DH5 α , the cells were grown in LB broth and fusion proteins were purified with anti-GST affinity chromatography to the method reported previously (Smith and Johnson, 1988).

2. Preparation of antibodies

Polyclonal antibody was prepared by immunizing New Zealand white rabbits with affinity-purified truncated RAG proteins and monoclonal antibodies were prepared by immunizing Dahl R rats with the same antigens according to the method reported previously (Yoshida et al., 1988). Goat anti-GST polyclonal antibody was purchased from Pharmacia.

3. Cell and virus

Spodoptera frugiperda insect cells (Sf9) and *Autographa californica* nuclear polyhedrosis virus (AcRP23. LacZ) containing the LacZ gene were used (Possee and Howard, 1987). The insect cells were cultured at 27°C and grown in Grace's medium (Gibco Laboratories, Grand Island, N. Y., U. S. A.) supplemented with 10% fetal bovine serum (FBS) and 0.26% Bacto tryptose broth (Difco Laboratories, Detroit, Mich., U. S. A.).

4. Expression of recombinant RAG-1 and RAG-2 proteins in insect cells

pAcYM1 was used for baculovirus expression vector (Matsuura et al., 1987). pAcRAG-1-12 was constructed by inserting a *Stu*I-*Ssp*I fragment of M6-BSK₊ into the *Sma*I site of pAcYM1. pAcRAG-2-20 was constructed by inserting a *Pst*I (162)-*Eco*RV (1963) fragment of MR2-BSK₊ into the *Sma*I site of pAcYM1. The construction of pAcGST-RAG-1, pAcGST-RAG-2, and pAcGST is shown in Figure 2. Virus vector pAc23. LacZ (10-20 ng) linearized by restriction enzyme *Eco*81 I was mixed with the circular form of recombinant plasmid vectors pAcRAG-1-12, pAcRAG-2-20, pAcGST-RAG-1, pAcGST-RAG-2 or pAcGST (1 μ g). After adding distilled water up to 8 μ l, an equal volume of 50% (v/v) lipofectin (BRL, Gaithersburg, MD, U. S. A.) was added and incubated at room temperature for 15 min. The mixture solution was poured over 1×10^6 of Sf9 cell in a 35-mm dish in which the medium had been replaced by serum-free medium beforehand. After 2 days of culture at 27°C, a plaque assay was carried out with the culture supernatant. The plaque assay procedure is as follows: The cultured supernatant of transfectants was diluted with culture medium and inoculated into 0.8×10^6 cells of Sf9 cell without medium. After 1 h of incubation at 27°C, the supernatant was discarded. Two milliliters of 1% agar and 1 mL of medium were added. Following 48-72h-incubation at 27°C, one milliliter of 0.01% (w/v) neutral red-0.04% (w/v) X-gal in PBS was added. After

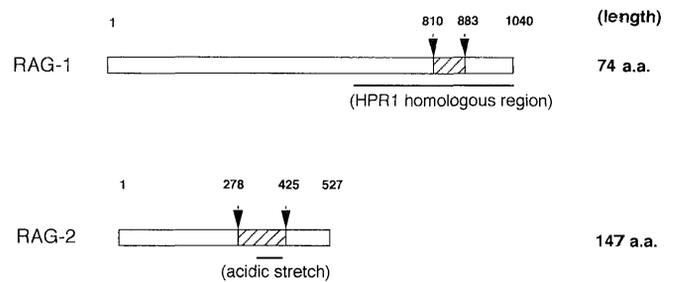


Fig. 1 Expressed regions of RAG-1 and RAG-2 genes using the *E. coli* system. Open rectangles denote the open reading frames. Areas marked with diagonal lines indicate the regions for expression. The numbers above the genes indicate the amino-acid number.

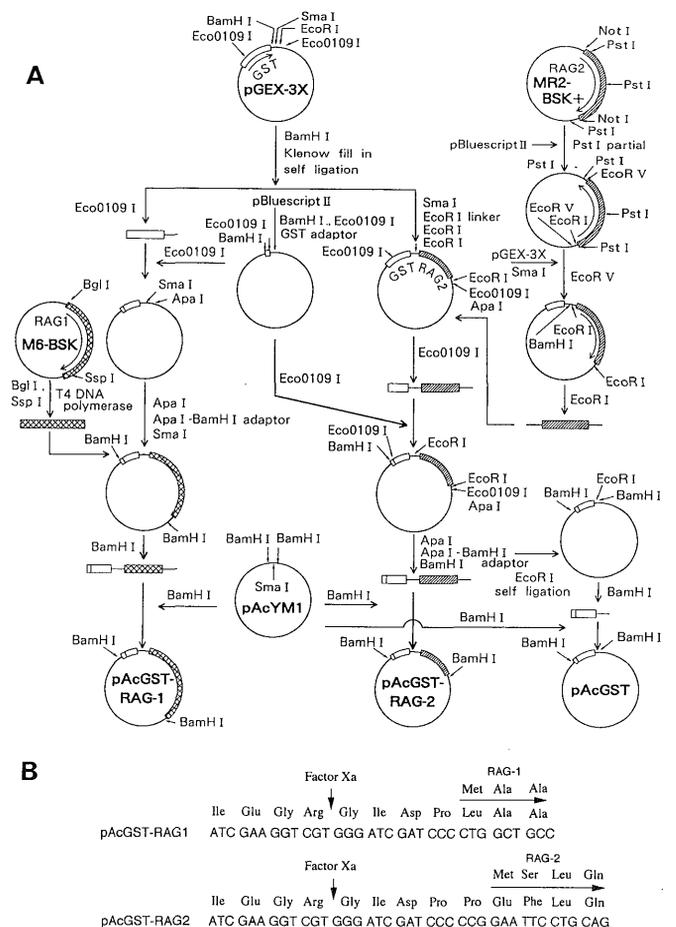


Fig. 2 The construction of the GST-fused RAG-1 and RAG-2 protein-expression vectors for the baculovirus expression system. (A) The RAG-1 gene is denoted by the areas marked with diagonal lines and the RAG-2 gene by the cross-hatched areas. Open boxes denote GST genes. M6-BSK and MR2-BSK contain full-length cDNA segments of RAG-1 and RAG-2, respectively. (B) The amino-acid sequence of the GST-RAG joining regions. The arrows indicate the digestion site by Factor Xa. The amino-acid sequences are shown over the nucleotide sequence.

a 24-h incubation, white plaques were picked up. After three rounds of plaque assay, single plaques were isolated. The method for large-scale gene-expression with recombinant virus is as follows : After infection of recombinant virus at a multiplicity of 5PFU/cell, the cells were cultured for 48 h and then collected.

5. Western blot analysis

Western blot was carried out as described previously (Towbin et al., 1979). For secondary reagents, horseradish peroxidase (HRP) labelled protein-A, protein-G and anti-rat IgM (Zymed, So. San Francisco, CA., U. S. A.) were used. The molecules were visualized with the ECL chemiluminescence system (Amersham, Buckinghamshire, U. K.).

6. Preparation of synthesized recombinant RAG-1 and RAG-2 proteins fraction.

The synthesized protein fraction was prepared as described previously (Cockerill and Garrard, 1986). Briefly, the protocol is as follows : 1×10^6 cells were washed once in phosphate-buffered saline, suspended in two to four volumes of RSB [10 mM NaCl-3 mM MgCl₂-10 mM Tris HCl (pH 7.4)-0.5 mM PMSF] and put on ice for 10 min. Suspended cells were homogenized using the Dounce homogenizer B pestle with 30 strokes. A pellet was washed twice in RSB-0.25 M sucrose. After suspension in RSB-2 M sucrose and centrifugation at $103700 \times g$ at 4°C for 30 min, the pellet was washed three times in RSB-0.25 M sucrose. Nuclei were suspended in 100 μ L of RSB-0.25 M sucrose-1 mM CaCl₂ and digested with DNase I (Takara Co. Ltd., Kyoto, Japan) at a final concentration of 100 μ g/ml at 23°C for 2 h. After centrifugation for 10 min at $750 \times g$ at 4°C, the pellets were suspended in 2 ml of RSB-0.25 M sucrose, and 2 ml of 4 M NaCl-20 mM EDTA-20 mM Tris HCl (pH 7.4) was added and the same solutions were utilized except for the NaCl concentration at the protein elution experiment to determine the optimum condition. After incubation (15 min, on ice) and centrifugation ($1500 \times g$, 15 min), the pellets were extracted twice by suspension in 1 ml of 2 M NaCl-10 mM Tris HCl (pH 7.4)-0.5 mM PMSF-0.25 mg/ml BSA. The pellets collected by centrifugation ($4500 \times g$, 15 min) were washed with RSB-0.25 M sucrose-0.25 mg/ml BSA. Extracted fractions were stored at -20°C after adding an equal volume of 100% glycerol. Digestion of the extracted fractions with Factor Xa was performed according to the manufacturer's recommendations (Denzyme APS, Aarhus C., Denmark).

Results and discussion

The biochemical analyses of the RAG proteins remain to be elucidated because of following three practical reasons ; first, the absence of high quality antibodies against RAG proteins, secondly, the extremely low quantity of RAG proteins in vivo, and thirdly, the difficulty of heterologous

expression of RAG genes.

In this study, we prepared polyclonal antibodies and monoclonal antibodies designated as MR-1-1, MR-1-100 and MR-1-130 against RAG-1, and MR-2-10, MR-2-12 and MR-2-32 against RAG-2, respectively. All of these antibodies exactly detected full-length nonfused RAG proteins expressed in the insect cells (Figure 3). At the initial step of our study, however, another difficulty was encountered in determining conclusively whether the molecules expressed by the baculovirus of *E. coli* expression systems were truly RAG molecules, because the anti-RAG antibodies did not clearly detect the mammalian RAG molecules in vivo (data not shown). This was the greatest obstacle we faced in our experiments.

To overcome this problem, we employed GST-fused RAG proteins in the baculovirus expression system to detect the products only by anti-RAG antibodies but also by anti-GST antibodies. We also expected a change in the molecular weight of the target molecules by the digestion of the fused products with restriction endopeptidase Factor Xa of which recognition sequence exists between GST and RAG peptides.

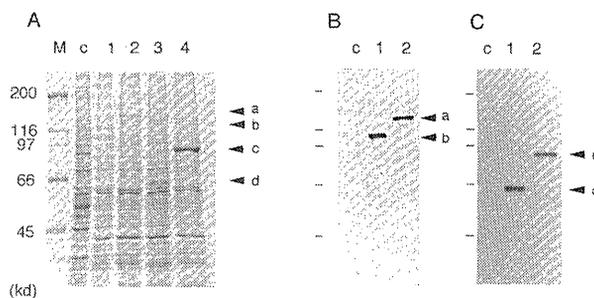


Fig. 3 Expression of the recombinant proteins in insect cells. Sizes are indicated in kilodaltons (kd). (A) Whole-cell extracts of RAG-1-or RAG-2-infected insect cells and GST-fused RAG-1-or RAG-2-infected insect cells were analyzed. Each lane contains 7.5×10^4 cells. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. Non-infected Sf9 (lane c) ; RAG-1-infected Sf9 (lane 1) ; GST-fused RAG-1-infected Sf9 (lane 2) ; RAG-2-infected Sf9 (lane 3) ; GST-fused RAG-2-infected Sf9 (lane 4). (B) The expression of RAG-1 proteins was analyzed by Western blot. Anti-RAG-1 monoclonal antibody MR-1-100 was used for detection. Whole-cell extract prepared from 3.25×10^4 cells was applied on each lane. The loaded samples on each lane are as follows : non-infected Sf9 (lane c) ; RAG-1-infected Sf9 (lane 1) ; GST-fused RAG-1-infected Sf9 (lane 2). (C) The expression of RAG-2 proteins was analyzed by Western blot. Sizes are indicated in kilodaltons (kd). Anti-RAG-2 monoclonal antibody MR-2-10 was used. Whole-cell extract prepared from 3.25×10^4 cells was applied on each lane. The loaded samples on each lane are as follows : non-infected Sf9 (lane c) ; RAG-2-infected Sf9 (lane 1) ; GST-fused RAG-2-infected Sf9 (lane 2). The arrows [a], [b], [c], and [d] indicate GST-fused RAG-1, RAG-1, GST-fused RAG-2 and RAG-2 and RAG-2, respectively.

The nonfused forms of RAG-1 and RAG-2 proteins were detected faintly by Coomassie staining (Figure 3A), while clear bands were demonstratable in Western blot analysis (Figure 3B and C). As shown in Figure 3, RAG-1 molecule

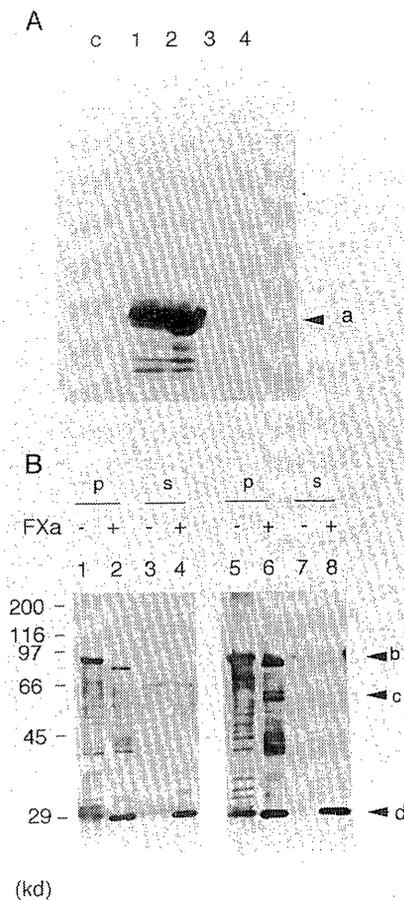


Fig. 4 Confirmation of the recombinant products by Factor Xa digestion. (A) The expression of GST-protein in Sf9 was analyzed with goat anti-GST polyclonal antibody (pharmacia). The loaded samples of lane c were prepared from whole-cell extract of non-infected Sf9. The other loaded samples were prepared from GST-infected Sf9. The preparing condition is as follows ; whole-cell extract (lane 1) ; extract from cytoplasm (lane 2) ; 2.0 M NaCl eluate (lane 3) ; residual pellet-fraction after 2.0 M NaCl eluate (lane 4). Samples of lane c and 1 were prepared from 2.5×10^6 cells and samples of lanes 2, 3 and 4 were prepared from extraction of 2.5×10^6 cells. The arrow [a] indicates GST. (B) The GST-fused RAG-2-product was digested with Factor Xa. Anti-GST polyclonal antibody and anti-RAG-2 polyclonal antibody were used to detect the sample lanes 1-4 and lanes 5-8, respectively. The residual pellet-fractions of 2.0 M NaCl extraction were used for Factor Xa digestion were prepared by centrifugation. Mock experiment samples without Factor Xa digestion were also loaded as controls : pellet-fractions [P] (lane 1 and 5) ; supernatant-fractions [S] (lane 3 and 7). Sizes are indicated in kilodalton (kd). The arrows [b], [c] and [d] indicate GST-fused RAG-2, RAG-2 divided by Factor Xa and GST divided by Factor Xa, respectively.

was detected at approximately 125 kd and RAG-2 molecule at 65 kd. The molecular weight of RAG-1 molecule is equal to that predicted by amino-acid sequence. However, that of RAG-2 was higher than that predicted (56 kd). The increase in molecular weight of RAG-2 was also observed in the GST-fused RAG-2 molecule, as described below. In the case of RAG-1, we cannot definitely conclude that the RAG-1 molecule does not undergo a molecular-weight change because the resolution of SDS-PAGE is not sufficient at the region where the RAG-1 molecule is detected. The molecular weight of GST-fused RAG-1 and GST-fused RAG-2 (155 kd and 94 kd, respectively) was higher than that predicted (148 kd and 85 kd, respectively).

The quantity expressed GST-RAG-1 and GST-RAG-2 was estimated on the basis of SDS-PAGE pattern to be about $130 \mu\text{g}/10^8$ cells $1 \text{mg}/10^8$ cells, respectively.

These fused proteins could be digested into the expected molecular size by restriction endopeptidase Factor Xa. Factor Xa recognizes the amino-acid sequence Ile-Glu-Gly-Arg and digests the C-terminal side of Arg. Both of our GST-fused RAG genes contain the DNA sequence encoding the Factor Xa recognition sequence at the joining region between GST and RAG genes. In order to digest the recombinant protein, we have been attempting to obtain purified products. When cells infected with GST-fused products of RAG-1 and RAG-2 were fractionated with 2M NaCl solution, the products existed in insoluble nuclear fractions. Using these fractions, each half of the molecule divided with Factor Xa was detected by anti-RAG antibodies and anti-GST antibody, respectively (Figure 4B). The molecular weight of divided molecules was appropriate. It is confirmed that the commercially available anti-GST antibody could detect the GST region expressed in insect cells (Figure 4A). These observations show that the molecules detected in our system are definitely GST-RAG fused proteins.

The expression of a large number of genes has been reported to date in studies using the baculovirus expression system (Luckow and Summers, 1988). Unlike the *E. coli* system, this baculovirus system allows proteins to maintain their original function. Although our RAG-1 and RAG-2 products were detected in an insoluble fraction of the nuclei, they will undoubtedly prove to be useful for research on V(D)J recombinase when we succeed in making them soluble.

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