

The Half-Life of RAG-1 Protein in Precursor B Cells Is Increased in the Absence of RAG-2 Expression

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Summary

Site-specific recombination of immunoglobulin and T cell receptor gene segments in B and T lymphocytes is dependent on the expression of two recombination activation genes, *Rag-1* and *Rag-2*. Here, we show that RAG-1 protein turnover in pre-B cells depends on the expression of RAG-2. The apparent half-life of RAG-1 protein is increased when RAG-2 is not expressed in differentiating pre-B cells.

The variable domains of both Ig and TCR molecules are encoded by multiple germline V, D, and J gene segments that are separated in the germline (1). Site-specific recombination of these gene segments occurs during early B and T lymphopoiesis (1). The recombination activating genes *Rag-1* and *Rag-2* are involved in V(D)J recombination (2–4). Targeted mutation of either *Rag-1* or *Rag-2* results in the inability to initiate DNA double-strand breaks at recombination signal sequences (5, 6). Further steps of the V(D)J recombination reaction appear to involve factors that are also essential for DNA double-strand break repair, like the catalytic subunit of the DNA-dependent protein kinase, that may be encoded by the *scid* gene (7, 8) or the Ku 80 protein, encoded by the *XRCC-5* gene (9–11).

Though each of these factors is required to allow a complete V(D)J recombination reaction with unmethylated extrachromosomal rearrangement substrates, assembly of endogenous V, D, and J gene segments in the chromosomes is also regulated by accessibility of Ig or TCR loci for the components of the V(D)J recombinase(s) (12). The expression of sterile transcripts, derived from germline Ig and TCR gene loci before V(D)J recombination (13–15), is taken as one early sign for such accessibility. Transcription is controlled by promoters and enhancers within the Ig and TCR gene loci (16, 17), which function together with developmentally and tissue-specifically expressed transcription factors.

V(D)J recombination is controlled by the expression of the *Rag* genes themselves, which is confined to precursor lymphocytes (5, 6). In addition to the transcriptional control of both *Rag* genes, it has been shown that RAG-2 pro-

tein levels are regulated during the cell cycle and are highest at G₀/G₁ and hardly detectable during S/G₂/M phases of the cell cycle (18). This posttranslational control is apparently mediated by modulation of RAG-2 protein stability resulting from cell cycle-dependent phosphorylation (19).

To analyze whether a lack of RAG-2 expression in cells from *Rag-2*-deficient (T)¹ mice affects the expression of *Rag-1*, *Rag* expression was monitored in *Rag-2* T B cell precursors both on the RNA as well as on the protein level.

Mouse B cell precursors can be isolated from either fetal liver or bone marrow and can be grown in vitro for extended periods of time on stromal cells in the presence of exogenously added IL-7 (20). The proliferating cells remain DJ rearranged on their IgH chain loci and keep their L chain gene loci in germline configuration. Removal of IL-7 from these cultures leads to growth arrest and induces a program of differentiation, which is characterized by the loss of pre-B cell and gain of B cell-specific surface markers, the loss of IL-7 responsiveness, the induction of apoptosis, and the induction of V-to-DJ and V-to-J rearrangements on H and L chain gene loci, respectively (21). Induction of apoptosis during the in vitro differentiation of stromal cell/IL-7-dependent pre-B cells, which hampers an analysis of the differentiating cells, can be prevented in cells derived from mice expressing an *Eμ-bcl-2* transgene (22, 23). Expression of the *bcl-2* transgene has been shown not to interfere with the overall in vitro differentiation process but inhibits apoptosis of IL-7-deprived cells for up to 7 d of culture (24). Undifferentiated pre-B cells transcribe low or even undetectable *Rag* mRNA levels. When differentiation is induced, transcription of both *Rag-1* and *Rag-2* genes can easily be monitored within 2–3 d (24).

Normal numbers of stromal cell/IL-7-dependent B cell precursors are present also in *Rag-1*- or *Rag-2*-deficient mice (15). This has allowed us to analyze how *Rag* expres-

¹Abbreviations used in this paper: tg, transgenic; T, deficient.

sion is regulated in these mutant cells after IL-7 withdrawal. The results suggest that the absence of *Rag-2* expression in RAG-2T-derived pro-B cells increases the half-life of RAG-1 protein so that up to 30–40-fold higher concentrations of RAG-1 protein are found in *Rag-2T* versus wild-type cells.

Materials and Methods

Mice. Homozygous *Rag-2T* mice (5) obtained from Dr. Fred Alt (The Children's Hospital, Howard Hughes Medical Institute, Boston, MA) were bred under special pathogen-free conditions at the Basel Institute for Immunology. *Eμ-bcl-2*-transgenic (tg) mice (23) obtained from Dr. Andreas Strasser (The Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia) were bred onto *Rag-2T* background at the Basel Institute for Immunology. Hemizygous *Rag-2* (-/+)/*Eμ-bcl-2*-tg males were backcrossed on female *Rag-2T* mice and tested by PCR for the presence of the transgene and homozygosity of the neomycin resistance gene insertion into the *Rag-2* locus. BDF₁ (C57Bl/6 × DBA/2)F₁ embryos from time-pregnant C57Bl/6 females, *Eμ-bcl-2*-tg embryos were provided by the breeding facilities at the Basel Institute for Immunology. The appearance of vaginal plugs was counted as day 0 of gestation. *Rag-1T* mice (6) (provided by Dr. S. Tonegawa, Massachusetts Institute of Technology, Cambridge, MA) were obtained as breeding pairs from Dr. Georges Köhler (Max Planck Institute for Immunobiology, Freiburg, Germany).

Cell Lines and Cell Culture. Stromal cell/IL-7-dependent pre-B cell lines and clones were established from day 16 fetal liver or from 4–6-wk-old bone marrow as described (20). They were maintained on a semiconfluent layer of 2,000-rad γ -irradiated ST-2 stromal cells (25) (a kind gift of Dr. S.I. Nishikawa, University of Ohu, Japan) in IMDM (GIBCO BRL, Gaithersburg, MD) containing 5×10^{-5} M 2-ME, $1 \times$ nonessential amino acids (GIBCO BRL), 0.03% primatone (Quest International, Naarden, The Netherlands), 1% FCS, and 100–200 U of recombinant IL-7, which was added as a conditioned supernatant obtained from J558 cells transfected with an IL-7 expression vector (the cell line was a kind gift of Dr. T.H. Winkler, The Basel Institute for Immunology).

ST-2 stromal cells were grown in the same IMDM-based medium with 2% FCS, but without IL-7. For the induction of in vitro differentiation, pre-B cells growing on stromal cells and IL-7 were removed from the feeder cell layer, washed three times with medium containing no IL-7, and were cultured at $1-2 \times 10^6$ cells/ml on γ -irradiated stromal cells in medium without IL-7. All cells were grown in a humidified incubator at 37°C in the presence of 10% CO₂.

Northern Blot Analysis. Total cellular RNA was prepared according to the acid guanidinium-thiocyanate method (26), and Northern blot analysis was performed as described (24). 15 μ g of total RNA per lane was analyzed for the expression of *Rag-1* and *Rag-2* mRNAs. The same blots were hybridized to the indicated probes (β -actin served as a control for RNA loading), which were derived from the following constructs: *Rag-1*, a mixture of 410-, 950-, and 1,300-bp fragments obtained by HindIII digestion of M6-BSK⁺ containing the entire *Rag-1* cDNA (2); *Rag-2*, a 2,100-bp fragment obtained by NotI digestion of MR2-BSK⁺ containing the mouse *Rag-2* cDNA (3); β -actin probe, a 522-bp fragment (from positions 220 to 742 of GenBank sequence MUSACTBR). This probe has been cloned by PCR into

M13mp19, and the insert was verified by sequencing. M13 DNA was isolated as single-stranded DNA, which was labeled by primer extension.

Western Blot Analysis and Antibodies. Cellular lysates were prepared according to the method described by Lin and Desiderio (19). Briefly, after washing in PBS, cells were lysed for 15 min on ice in RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM NaF, and proteinase inhibitors: 1 mM PMSF, 1 μ g/ml pepstatin A, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin [Sigma Chemical Co., St. Louis, MO]). The lysates were centrifuged (14,000 g, 10 min, 4°C), and the supernatant was frozen in liquid nitrogen. Lysates corresponding to 10^6 cells were fractionated per lane. Western blotting was performed for 5 h at 300 mÅ in 25 mM Tris, 192 mM glycine, and 20% methanol. Before incubation with the primary anti-RAG-1 and anti-RAG-2 reagents, the filters were usually cut into an upper half containing proteins >97 kD (including RAG-1) and a lower half containing proteins <97 kD (including RAG-2) and incubated independently with the primary antibodies. Affinity-purified primary antibodies specific for RAG-1 and RAG-2 protein (see below) bound to the filters were detected using horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG antibody (Southern Biotechnology Associates, Birmingham, AL) in conjunction with an ECL kit (Amersham International, Little Chalfont, UK) according to the recommendations supplied by the manufacturer.

For the generation of polyclonal anti-RAG-1 and anti-RAG-2 antibodies, amino acids 56–123 of murine RAG-1 and 70–516 of murine RAG-2 were expressed in bacteria as fusion proteins with a mutant *Pseudomonas* exotoxin (27), and the fusion proteins were used to immunize rabbits. The anti-RAG-1 and anti-RAG-2 antibodies were affinity purified using bacterially expressed RAG polypeptides, expressed as fusion proteins with maltose-binding protein (pMAL; New England Biolabs; Beverly MA). The purity of the purified Ig was confirmed by SDS-PAGE followed by silver staining. The generation and affinity purification of the polyclonal rabbit anti-RAG-1 and anti-RAG-2 antibodies will be described in detail elsewhere (Leu, T.M.R., K.R. McConnell, E. Corbett, S. Bennett, and D.G. Schatz, manuscript in preparation).

Analysis of the Half-Life of RAG-1 Protein. For the analysis of RAG-1 protein half-life, 5×10^8 cells from each *Eμ-bcl-2*-tg and *Rag-2T/Eμ-bcl-2*-tg mice were cultured in the absence of IL-7 for 24 h, and then pulse-labeled with 0.25 mCi/ml [³⁵S]L-methionine for 6 h at 37°C. [³⁵S]L-methionine-labeled proteins were chased by culturing cells in medium containing excess (30 mg/ml) unlabeled methionine, diluting cells to 10^7 cells/ml. Aliquots of 5×10^7 cells were lysed after various time points as described above. Lysates were precleared for 1 h at 4°C with normal rabbit serum and protein A-Sepharose (Pharmacia, Uppsala, Sweden) and were immunoprecipitated with rabbit anti-mouse RAG-1 antiserum 307 (19) (a kind gift of Dr. Stephen Desiderio, Howard Hughes Medical Institute, The Johns Hopkins University, Baltimore, MD) and protein A-Sepharose for another hour at 4°C according to standard protocols (28). Immunoprecipitated RAG-1 was fractionated by 10% SDS-PAGE, and specific radioactivity on dried gels was quantitated by means of a Phosphorimager supplied with Imagequant software (Molecular Dynamics, Sunnyvale, CA). In different experiments, the amounts of initial radioactive label in RAG-1 protein was different by a factor of three. For the presentation of the data shown in Fig. 3, the initial intensity of radioactivity detected by the Phosphorimager at time 0 was taken as 100%, and all subsequent measurements during the chase period related accordingly as relative radioactivity in percentage

of the initial 100%. All intensity measurements were normalized to the radioactivity of a background protein with apparent long half-life and migrating at ~300–400 kD. The cpm values for 100% in Fig. 3 varied between 800 and 2,000 cpm.

Results

Rag-1 and Rag-2 mRNA Expression Is Equally Induced in Wild-type and Rag-2T Cells. Stromal cell/IL-7-dependent pre-B cell lines from normal as well as *Eμ-bcl-2-tg* mice can be induced to upregulate *Rag-1* and *Rag-2* transcription within 24–48 h of in vitro differentiation (24). As shown in Fig. 1, this transcriptional activation of the *Rag* genes was also observed in cell lines from *Rag-2T* mice or *Rag-2T* mice expressing an *Eμ-bcl-2* transgene (Fig. 1, left), regardless of whether they were derived from bone marrow or fetal liver. Due to the insertion of the neomycin resistance gene into the coding region of the *Rag-2* gene, the mutant *Rag-2* transcripts migrated at ~2.4 kb instead of 2.1 kb.

The levels of induction of *Rag-1* mRNA expression on IL-7 withdrawal appear to be similar in cells from *Rag-2T* and wild-type mice capable of expressing RAG-2 protein. This excludes a role for the RAG-2 protein in the control of *Rag-1* or *Rag-2* transcription or a role for RAG-2 protein in stabilizing the *Rag* mRNAs. It further demonstrates that the neomycin resistance gene insertion (5) does not interfere with the function of the *Rag-2* promoter.

Expression of RAG-1 and RAG-2 Protein during In Vitro Differentiation of Pre-B Cells. Analyses of RAG-1 and RAG-2

protein expression at different time points after induction of in vitro differentiation shown in Fig. 2 *A* demonstrates that RAG-2 protein levels in wild-type or *Rag-1T* cells rise with similar kinetics, as do the levels of *Rag-2* mRNA assayed by Northern blotting. As expected, RAG-2 protein was undetectable in lysates prepared from *Rag-2T* mice. However, the RAG-1 protein expression in wild-type cells differed markedly from the expression pattern of its mRNA during in vitro differentiation (compare Fig. 1). Whereas *Rag-1* mRNA appeared to reach, at day 2 of differentiation, a level that was maintained for the next 3–4 d (not shown), RAG-1 protein expression levels increased within the first day but thereafter dropped to hardly detectable levels within the next 2–3 d. Even when analyzed in apoptosis-resistant *Eμ-bcl-2-tg* pre-B cells, RAG-1 protein expression did not recover for up to 5 d of in vitro differentiation (Fig. 2 *B*), with RAG-2 protein levels remaining high at least until day 5 after induction of differentiation (Fig. 2 *B*). On the contrary, RAG-1 protein levels in the absence of RAG-2 were found to accumulate during in vitro differentiation, reaching a maximum after ~2–3 d. A longer term analysis of RAG-1 protein expression for up to 5 d after IL-7 withdrawal revealed no significant drop in RAG-1 protein levels in *Rag-2T/Eμ-bcl-2-tg* cells (Fig. 2 *B*). *Rag-1* and *Rag-2* mRNA levels became low, or even undetectable, after 5 d of in vitro differentiation (data not shown). This indicates that RAG-1 protein in *Rag-2T* cells does not turn over rapidly. Quantitation of the RAG-1 protein levels assayed in serial dilution analysis after 24 and

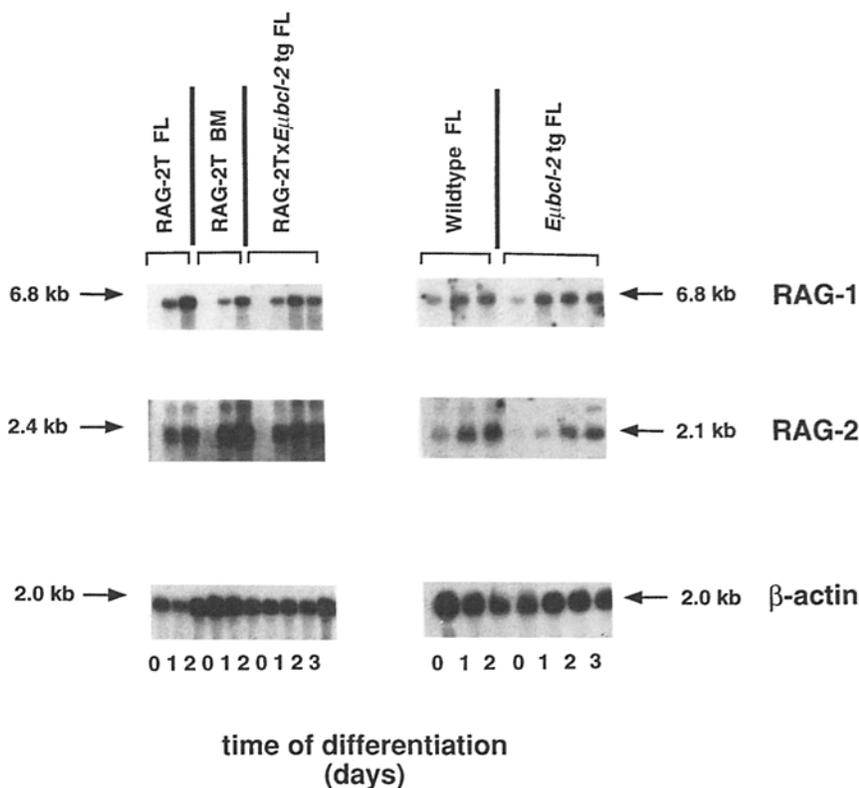


Figure 1. Upregulation of *Rag-1* and *Rag-2* mRNA expression during the in vitro differentiation of stromal cell/IL-7-dependent cell lines from wild-type and *Rag-2T* mice. Total RNA was extracted from cell lines either derived from bone marrow (BM) or fetal liver (FL) of *Rag-2T* and *Rag-2T/Eμ-bcl-2-tg* mice (left) or from fetal liver of normal BDF₁ and *Eμ-bcl-2-tg* mice (right). Expression of *Rag* mRNAs was assayed in undifferentiated cells and after 1, 2, or 3 d of in vitro differentiation as indicated. The same blots were (re)probed with the indicated probes. The Northern blots containing samples of the *Rag-2T* cells and the control samples were blotted on different filters and are therefore separated in the presentation. Repeated experiments of this type have shown that the differences in expression levels of RAG in RNAs between wild-type and RAG-2T cells are minimal, if at all significant.

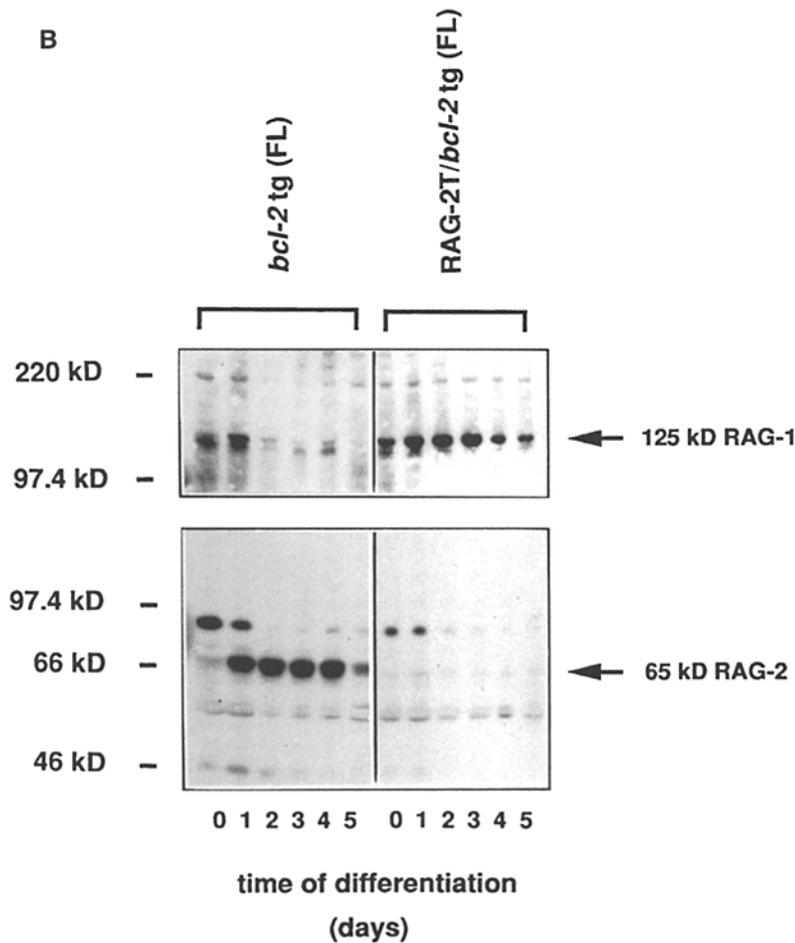
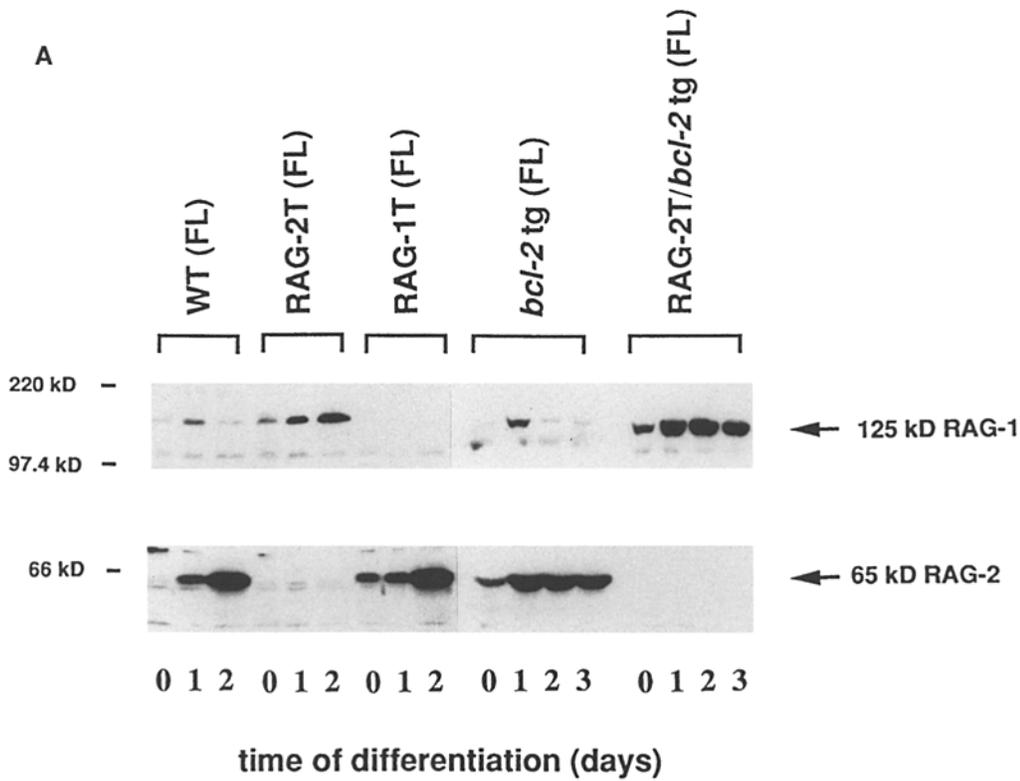


Figure 2. RAG-1 and RAG-2 protein expression in differentiating pre-B cells. (A) Different wild-type (*WT*), *Rag-1T*, and *Rag-2T* cell lines (some of them expressing an *E μ -bcl-2* transgene, as indicated) were analyzed for the expression of RAG-1 and RAG-2 proteins at different time points after IL-7 withdrawal. Cellular lysates corresponding to 10^6 viable cells/lane were analyzed by Western blotting. *FL*, fetal liver. (B) Analysis of RAG-1 and RAG-2 expression during a long-term in vitro differentiation kinetic (5 d) with one *E μ -bcl-2*-tg (*left*) and one *Rag-2T/E μ -bcl-2*-tg cell line (*right*). *FL*, fetal liver.

48 h of induction revealed a 3–4-fold difference at day 1 and a 30–40-fold difference of RAG-1 protein levels at day 2 of differentiation between wild-type and *Rag-2T* cells, respectively (data not shown).

We conclude that the induction of RAG-2 protein expression after 24 h of in vitro differentiation in wild-type cells appears to be responsible for the observed decrease of RAG-1 protein levels. This decrease has been seen in six different wild-type and one SCID/*bcl-2*-tg pre-B cell lines analyzed so far (data not shown).

Western blot analysis for the expression of RAG-1 and RAG-2 protein during the in vitro differentiation of *Rag-1T* cells shows that the lack of RAG-1 protein expression does not influence the accumulation of RAG-2 protein (Fig. 2). This suggests that the turnover of RAG-1 protein is influenced by RAG-2, but not vice versa.

The Half-Life of RAG-1 Is Prolonged in the Absence of RAG-2 Protein. Since steady state *Rag-1* mRNA levels were found to be equally induced during the in vitro differentiation of both wild-type and *Rag-2T* cells (see Fig. 1), we investigated whether a changed half-life of RAG-1 protein could account for the difference in the observed levels of RAG-1 protein. Analysis of the RAG-1 protein half-life was performed after 24 h of induction of differentiation, since RAG-1 protein levels became too low at day 2 of differentiation in wild-type cells to be detected in the assay. Data in Fig. 3 show that the half-life in wild-type, *bcl-2*-tg cells was ~10 min, whereas it was ~30 min in *Rag-2T*, *bcl-2*-tg cells at 24 h after the induction of differentiation. This ~3-fold difference in the half-life of RAG-1 protein could account for the observed 3–4-fold difference in levels of RAG-1 protein between wild-type and *Rag-2T* cells at day 1 in vitro differentiation (see Fig. 2). It appeared that RAG-1 expression levels differed by >30-fold after 2 d of in vitro differentiation. This makes it likely

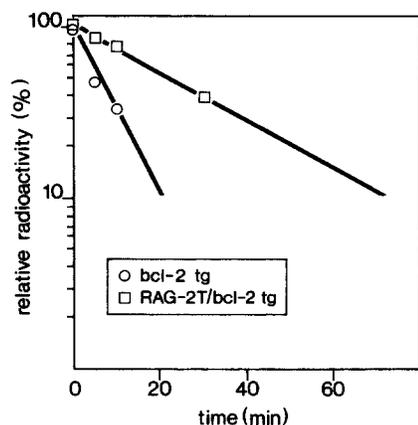


Figure 3. The half-life of RAG-1 protein in differentiated pre-B cells. Analysis of the half-life of RAG-1 protein at day 1 of differentiation in wild-type/*Eμ-bcl-2*-tg (○) and *Rag-2T/Eμ-bcl-2*-tg cells (□). One out of two independent experiments, yielding similar results, is depicted. From these data, the half-time of RAG-1 protein in wild-type cells is estimated to be in the range of 8–10 min, whereas it is in the range of 28–30 min in *RAG-2T* cells. The determination of the percentage of radioactivity is described in detail in Materials and Methods.

that the difference in the apparent half-lives of RAG-1 protein in wild-type and *Rag-2T* cells is even greater in cells differentiated for longer periods of time.

Discussion

The experiments presented here indicate that the expression of normal levels of RAG-2 protein in pre-B cells decreases the half-life of RAG-1 protein. This finding adds a new dimension to the regulation of the V(D)J recombinase activity during lymphocyte differentiation. It is known that RAG-2 protein levels vary during the cell cycle (18), and that the posttranslational control of RAG-2 expression and stability is apparently modulated by phosphorylation (19). Our previous results suggest that RAG-2 protein expression also changes at different stages of lymphocyte development (29). It is high in pre-B-I cells (for the characterization of different B lineage precursors see reference 30), low in large, pre-B receptor-expressing pre-B-II cells, high again in small pre-B-II cells and immature B cells, and, finally, downregulated again in mature B cells.

However, it has so far been difficult to analyze the levels of RAG-1 protein expression during these developmental stages (U. Grawunder, F. Melchers, and T. Winkler, unpublished observations). The stability (half-life) of RAG-1 protein may be low at these stages and hence hard to detect whenever RAG-2 is strongly expressed. In this context, it is worth noting that in a recent study, initiation of proper V(D)J recombination in a cell-free system could only be achieved if recombinant RAG-1 protein was added to cellular extracts obtained from induced temperature-sensitive A-MuLV-transformed pre-B cell lines (31). Addition of recombinant RAG-2 protein did not increase the efficiency of the reaction, although it remains to be established by these investigations whether the added RAG-2 protein, expressed in bacteria, was active at all. The temperature-sensitive A-MuLV-transformed pre-B cell lines (32, 33), in their capacity to differentiate at the nonpermissive temperature, resemble the stromal cell/IL-7-dependent pre-B cells used in our study, which differentiate upon removal of IL-7. It is therefore likely that nuclear extracts derived from the temperature-sensitive pre-B cell lines after induction of differentiation (i.e., growth at 39°C) have a comparably low concentration of RAG-1 protein that can be supplemented by exogenously added recombinant RAG-1 protein in the assay.

The influence of RAG-2 protein expression on RAG-1 protein levels in pre-B cells suggests cross-talk between the two proteins. It has been found that RAG-1 and RAG-2 proteins can be coimmunoprecipitated with specific antibodies, suggesting a direct (or indirect) physical interaction of the two proteins (34). Such direct interactions could well change the accessibility of the RAG-1 protein for post-translational modifications that might control proteolytic degradation or the accessibility to proteases themselves.

It appears that two nuclear pools of RAG-1 protein exist, one that is easily extractable at low salt concentrations

and one that is tightly associated with the nuclear matrix and only extractable at high salt concentrations (34). If we assume that our extraction procedure solubilizes both pools, and that it solubilizes all RAG-1 protein in the cell, one might speculate that the form of RAG-1 associated with the nuclear matrix is protected from rapid degradation. In the presence of high RAG-2 concentrations, more RAG-1 may be recruited into an active V(D)J recombinase complex together with RAG-2, where RAG-1 might be more accessible to degradation. It is also conceivable that the extraction procedure used in the preparation of the cell lysates does not solubilize all forms of RAG-1 protein. If the absence of RAG-2 protein induces or increases the insolubility of RAG-1 protein so that it is removed from the

cell lysate in the first centrifugation step, the apparent change in the half-life would then not be due to degradation, but would indicate a change in conformation, possibly even as a result of a translocation in the cell, of the RAG-1 protein. Since it is likely that the V(D)J recombinase complex contains additional components (35, 36), the modulation of the half-life of RAG-1 protein by posttranslational modifications might be even more complicated. Future experiments will have to assess not only the pool sizes, turnover rates, and intracellular localization of the RAG-1 and RAG-2 proteins and their posttranslational modification status but, in all likelihood, also determine those parameters for the other partners in the V(D)J recombinase complex.

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