Ubiquitin-dependent Degradation*

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Periodic accumulation and degradation of RAG2 (recombination-activating gene 2) protein controls the cell-cycle-dependent V(D)J recombination of lymphocyte antigen receptor genes. Here we show the molecular mechanism of RAG2 degradation. The RAG2 protein is translocated from the nucleus to the cytoplasm and degraded through the ubiquitin/proteasome system. RAG2 translocation is mediated by the Thr-490 phosphorylation of RAG2. Inhibition of this phosphorylation by p27Kip1 stabilizes the RAG2 protein in the nucleus. These results suggest that RAG2 sequestration in the cytoplasm and its subsequent degradation by the ubiquitin/proteasome system upon entering the S phase is an integral part of G0/G1-specific V(D)J recombination.

During B and T cell development, the genes encoding the variable region of immunoglobulin (Ig) and the T cell receptor (TCR) are assembled from germ line-variable (V), diversity (D), and joining (J) gene segments by V(D)J recombination (1–3). V(D)J recombination is initiated by the introduction of site-specific double-strand breaks (DSBs) between two recombining gene segments and their flanking recombination signal sequences (RSSs). The essential components of this reaction are RAG1 and RAG2 (recombination-activating genes 1 and 2) proteins, which are expressed specifically in the developing B and T lineage cells under V(D)J recombination (4, 5). Subsequent reaction steps employ more generally expressed proteins involved in non-homologous end joining (6, 7): the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), the Ku70/80 dimer, XRCC4, and DNA Ligase IV. More proteins may be involved in V(D)J recombination to ensure the strict regulation of this system.

Several lines of evidence suggest that V(D)J recombination is restricted to the G0/G1 stage of the cell cycle and that periodic accumulation and destruction of the RAG2 protein regulate cell-cycle-dependent V(D)J recombination (8). According to Lee and Desiderio, Thr-490 of RAG2 is phosphorylated by cyclin A/CDK2 when the cells are at the G1-S transition of the cell cycle, thereby triggering the rapid degradation of RAG2 (9). However, the precise molecular mechanism of the degradation process remains elusive.

The ubiquitin/proteasome system plays a major role in target-specific protein degradation and in the regulation of protein expression levels (10, 11). The formation of ubiquitin-protein conjugates proceeds via a three-step cascade. First, a ubiquitin-activating enzyme (E1) activates ubiquitin, which is then transferred by a ubiquitin-conjugating enzyme (E2) to a ubiquitin ligase (E3) with which the substrate protein is associated. Finally, E3 catalyzes the conjugation of ubiquitin to the substrate protein. Proteins polyubiquitinated by these enzymes are subjected to degradation by the 26S proteasome. Recent reports have suggested that many proteins, such as p53, IκB, β-catenin, and p27Kip1, are degraded by the ubiquitin/proteasome pathway (10–13).

p27Kip1, a cyclin-dependent kinase inhibitor, increases RAG2 stability by inhibiting the cyclin A/CDK2 activity (9). Interestingly, p27Kip1 and RAG2 have similar sequences in the CDK-phosphorylation sites: QT187PKKGPL in p27Kip1 and QT490PKRNPPL in RAG2. This CDK-phosphorylation site has a critical role in p27Kip1 degradation (13–15). At the G1-S transition of the cell cycle, CDK2 phosphorylates Thr-187 of p27Kip1. The phosphorylated p27Kip1 is translocated from the nucleus to the cytoplasm, associated with the SCF (Skp1/cullin-1/F-box protein) E3 ubiquitin-ligase complex through this CDK-phosphorylation site, then ubiquitinated and degraded by the 26S proteasome, which promotes the cell-cycle transition from G1 to S. We speculated that a similar scenario may occur for RAG2 degradation. Thus, we examined the ubiquitination of the RAG2 protein and the correlation between RAG2 subcellular localization and its stability.

EXPERIMENTAL PROCEDURES

Plasmids—To generate RAG2 expression vectors (Fig. 1A), mouse RAG2 cDNA fragments were amplified by the reverse transcriptase-polymerase chain reaction (RT-PCR) method from the mouse thymus RNA using specific primers. To amplify wild-type RAG2 cDNA fragments, primers 5′-CAACTCGAGATGGTCCCTGCAGATGTAAC-3′ and 5′-GCTCTAGATTAATCAAACAGTCTTCTAAGG-3′ were used. An amplified fragment was introduced into a pCR-Blunt-TOPO vector (Invitrogen). After digestion of this plasmid with XhoI and EcoRV, the RAG2 cDNA fragment (XhoI-EcoRV) was inserted into the SalI-SmaI site of the pCA7-neo vector (16) to generate pT7-RAG2 containing the T7-tag sequence upstream of the RAG2 sequence. To generate pEGFP-RAG2, the same RAG2 cDNA fragment was also introduced into the SalI-SmaI site of the pCAEGFP vector in which the T7-tag sequence of pCA7-neo vector (16) had been replaced with the EGFp sequence of pEGFP-C1 (Clontech). To generate pT7-388 and pEGFP-388, primers 5′-CAACTCGAGATGGTCCCTGCAGATGTAAC-3′ and 5′-GCTCTAGATTAATCAAACAGTCTTCTAAGG-3′ were used. DNA fragments encoding deletion mutant 388 were amplified with these primers with RT-PCR, and the subsequent procedure was same as that of the wild-type RAG2 vectors. To generate pT7-388 and pEGFP-388, primers 5′-CAACTCGAGATGGTCCCTGCAGATGTAAC-3′ and 5′-GCTCTAGATTAATCAAACAGTCTTCTAAGG-3′ were used. DNA fragments encoding deletion mutant 388 were amplified with these primers with RT-PCR, and the subsequent procedure was same as that of the wild-type RAG2 vectors.
RAG2 Clearance System

C'-3' were used for this purpose. Human XRCC4 cDNA fragment was prepared from pMyCR-1 (343) (Myc-tagged full length human XRCC4 expression vector, Ref. 17) by digestion with XhoI and NotI. The 3'-NotI site was converted to a blunt end by Klenow treatment, and this fragment (XhoI-blunt) was introduced into the SalI-Smal site of the pCAT7-neo vector to generate pT7-XRCC4. The histidine-tagged ubiquitin expression plasmid (pMT107) and HA-tagged p27Kip1 expression plasmid (pHA-p27Kip1) were generous gifts from D. Bohmann (18) and J. Kato (15), respectively. Nuclear localization signal (NLS) sequences or nuclear exporting signal (NES) sequences (19) were introduced into the XhoI-blunt site of pT7-RAG2 vector. Second, an annealed DNA fragment of two synthesized oligonucleotides 5'-TCGACTCGTTCGCTCAGCTCAAAAAAGCAGGGCGAGGCTTCTTTTTATTATT-3' and 5'-GGGATTTGATTTTGCTTGATATTCTTTTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTA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RESULTS

Ubiquitination of RAG2 Protein—To show that the RAG2 protein is a substrate for ubiquitination, we adopted a highly sensitive in vivo ubiquitination assay system originally developed by Treier et al. (Fig. 1B) (18). We generated three T7 epitope-tagged RAG2 vectors to express wild-type RAG2 (pT7-RAG2), a point mutant where Thr-490 was replaced by alanine (pT7-T490A) and a deletion mutant that contained the N-terminal 388-amino acid region (pT7-388) (Fig. 1A). The core functional domain of RAG2 for V(D)J recombination has been defined as an N-terminal 1–383-amino acid region (21, 22). We confirmed the recombination activity of the three constructs (data not shown). Each construct, together with the histidine-tagged ubiquitin expression vector (pMT107), was transfected into COS7 cells (18). The T7-tagged XRCC4 expression construct (pT7-XRCC4) was used as a control. Half of the cells were treated with lactacystin, a specific inhibitor of the 26S proteasome (20). Polyubiquitinated proteins were precipitated by Ni2+-charged resin and subjected to Western blot analysis using the anti-T7 antibody (Clontech) or anti-HA tag antibody (12CA5, Boehringer Mannheim). Polyubiquitinated proteins were visualized with the anti-T7 antibody (α-T7, bracket). Equal aliquots of whole-cell lysate were subjected to SDS-PAGE and Western blot analysis using the α-T7 (INPUT), D, subcellular localization of GFP-RAG2 fusion proteins in COS7 cells: EGFP-T490A (lower right panel), EGFP-388 (lower right panel).

As shown in Fig. 1C, RAG2 proteins were clearly polyubiquitin-ated, as evidenced by the blockade of proteasome-mediated degradation with lactacystin (lanes 1 and 2). Ubiquitinated XRCC4 was not detected even after lactacystin treatment (lanes 3 and 4). In crude cell lysates, the expression level of RAG2, but not that of XRCC4, was increased by blocking the proteasome-mediated degradation with lactacystin treatment (Fig. 1C, INPUT, lanes 1–4). These results clearly indicate that RAG2 is ubiquitinated and degraded by the 26S proteasome.
Lee and Desiderio reported that Thr-490 phosphorylation targets the RAG2 protein for rapid degradation, relieving the T490A mutant of this destruction (9). Therefore, we next examined the ubiquitination and proteasome-mediated degradation of mutant RAG2 proteins, T490A and 388 (Fig. 1C, lanes 5–10). As expected, the T490A protein was more abundant compared with the RAG2 protein in the absence of lactacystin (INPUT, lanes 8 and 6). After lactacystin treatment, the expression level of T490A increased and the ubiquitination became visible (lane 7) but was weaker than that of wild-type RAG2 (compare lanes 5 and 7). This result suggests that the phosphorylation of Thr-490 is not essential for but facilitates the ubiquitination and subsequent proteasome-mediated degradation of the RAG2 protein. Unexpectedly, a deletion of the C-terminal 140-amino acid region including the Thr-490 rendered the RAG2 protein more sensitive to ubiquitination and proteasome-mediated degradation (lanes 9 and 10; compare with lanes 5 and 6). These data suggest that the following: 1) Sites for the ubiquitination and interaction with a ubiquitin ligase are located in the N-terminal 1/2/388-amino acid region of RAG2. 2) The C-terminal 140-amino acid region inhibits ubiquitination and proteasome-mediated degradation. 3) The phosphorylation of Thr-490 abrogates such inhibitory function of the C-terminal 140-amino acid region.

**Regulation of Subcellular Localization of RAG2**—As mentioned before, upon phosphorylation, p27Kip1 is translocated from the nucleus to the cytoplasm and degraded through the ubiquitin/proteasome system (13, 15). To investigate whether RAG2 degradation correlates with its subcellular localization similar to p27Kip1, we examined the localization of three GFP fusion proteins, EGFP-RAG2, EGFP-T490A, and EGFP-388, transiently expressed in COS7 cells (Fig. 1D). Although it is believed that RAG2 resides in the nucleus, EGFP-RAG2 is located in both the cytoplasm and the nucleus at various ratios (Fig. 1D, upper two panels, and see Fig. 3B, upper left panel). On the other hand, the T490A mutant is located exclusively in the nucleus (Fig. 1D, lower left panel), whereas the 388 mutant is predominantly located in the cytoplasm (Fig. 1D, lower right panel). RAG2 is physiologically co-expressed with RAG1 in lymphocyte precursors (1–3). Thus, its localization may be affected by the RAG1 protein. However, the co-expression of the RAG1 protein did not alter the localization pattern for the RAG2 protein in our system (data not shown). Thus, it is...
presumably that RAG2 shuttles between the nucleus and the cytoplasm independent of RAG1, that the C-terminal 140-amino acid region is inhibitory to the cytoplasmic localization of RAG2, and that the phosphorylation of the Thr-490 in this region abrogates this inhibition. Together with the result shown in Fig. 1C, the cytoplasmic localization of RAG2 by Thr-490 phosphorylation or the C-terminal deletion accelerates its degradation through the ubiquitin/proteasome system.

Correlation of Subcellular Localization and Stability of RAG2 Protein—To confirm the correlation between the subcellular localization and the stability of the RAG2 protein (Fig. 2), we first examined the stability of the RAG2 protein that is restricted to the nucleus or the cytoplasm by fusing a NES or a NES to the RAG2 protein, respectively. The expression constructs encoding the wild-type RAG2, NES-fused RAG2, T490A mutant, and NES-fused T490A mutant were transfected into COS7 cells, and their subcellular localization was confirmed by immunofluorescent detection using the anti-RAG2 antibody (Fig. 2A). We next examined the amount of RAG2 protein in whole-cell lysates by Western blot analysis. The NES-fused RAG2 protein was expressed in the cells at a higher level than the wild-type RAG2 protein (Fig. 2B, lanes 1 and 2). By contrast, the expression level of the T490A protein exclusively localized in the nucleus was markedly diminished by the fusion to NES (lanes 3 and 4). The expression level of these proteins was equivalent to that of the wild-type RAG2 protein in the cells treated with lactacystin (lanes 5–8, top). Lactacystin treatment did not affect the expression level of co-expressed EGFP (lanes 5–8, bottom). Thus, the cytoplasmic RAG2 is less stable than the nuclear RAG2 due to its proteasome-dependent degradation.

Co-expression of p27Kip1 Increases RAG2 Localization and Stability in the Nucleus—We next examined the localization of RAG2 when it was stabilized by co-expression with p27Kip1. Co-expression of p27Kip1 resulted in the increased expression of the EGFP-RAG2 expression level as reported previously (9) (Fig. 3A) and, strikingly, in the exclusive localization of the EGFP-RAG2 protein in the nucleus (Fig. 3B, upper panels) without affecting the localization of EGFP (lower panels). Thus, p27Kip1 induces the localization of RAG2 in the nucleus, leading to the stabilization of the RAG2 protein. Taken together, we conclude that the nuclear localization of the RAG2 protein increases its stability and that the cytoplasmic sequestration accelerates its degradation through the ubiquitin/proteasome system. RAG2 translocation from the nucleus to the cytoplasm appears to be mediated by Thr-490 phosphorylation. Thus, the p27Kip1-inhibition of this cyclin A/CDK2-mediated Thr-490 phosphorylation stabilizes the RAG2 protein in the nucleus.

DISCUSSION

V(D)J recombination, the process by which lymphocytes acquire immune diversity, is potentially deleterious, because the process involves DNA cleavage. This rapid cytoplasmic sequestration/degradation of RAG2 at the G1-S transition before the onset of genomic DNA replication would ensure that the S phase does not initiate with RAG-mediated DSBs being present in the DNA. Moreover, G0/G1-specific V(D)J recombination is also consistent with the fact that the non-homologous end-joining pathway that is responsible for the repair of DSBs in V(D)J recombination is most active during G0/G1 phase (23). Thus, the cell cycle regulation of V(D)J recombination serves to protect cells against deleterious effects of DNA cleavage.

Here we show the molecular mechanism of the rapid sequestration/degradation of the RAG2 protein. A similar mechanism might be employed in other proteins whose nuclear localization may be harmful.

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RAG2 Clearance System

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