Regulation of V(D)J Recombination Activator Protein RAG-2 by Phosphorylation

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Antigen receptor genes are assembled by site-specific DNA rearrangement. The recombination activator genes *RAG-1* and *RAG-2* are essential for this process, termed V(D)J rearrangement. The activity and stability of the RAG-2 protein have now been shown to be regulated by phosphorylation. In fibroblasts RAG-2 was phosphorylated predominantly at two serine residues, one of which affected RAG-2 activity in vivo. The threonine at residue 490 was phosphorylated by $p34^{cdc2}$ kinase in vitro; phosphorylation at this site in vivo was associated with rapid degradation of RAG-2. Instability was transferred to chimeric proteins by a 90-residue portion of RAG-2. Mutation of the $p34^{cdc2}$ phosphorylation site of the tumor suppressor protein p53 conferred a similar phenotype, suggesting that this association between phosphorylation and degradation is a general mechanism.

Immunoglobulin (Ig) and the T cell receptor are the antigen receptors of B and T lymphocytes and are encoded in multiple germline DNA segments, V, D, and J, that are joined during lymphocyte development (*1*). Assembly of antigen receptor genes, or V(D)J rearrangement, is mediated by conserved DNA sequence elements and is the only known example of site-specific recombination in vertebrates.

Several genes are essential for V(D)J rearrangement. The recombination activator genes *RAG-1* and *RAG-2* were originally identified by their ability to activate rearrangement of an exogenous recombinational substrate in fibroblastoid cells; both genes are required for this activity (*2*). The products of V(D)J joining in fibroblastoid cells transfected with *RAG-1* and *RAG-2* are identical to their counterparts in lymphoid cells. The sequences of *RAG-1* and *RAG-2* predict proteins of 118 and 58 kD, respectively. These genes are required for antigen receptor gene assembly; in homozygous *RAG-1*– or *RAG-2*–deficient mice, functional B and T cells are absent and V(D)J rearrangement is completely blocked (*3*). Both *RAG-1* and *RAG-2* could encode components of the V(D)J recombinase itself or regulatory proteins that potentiate V(D)J recombination indirectly; the available evidence does not distinguish these possibilities.

In mice, homozygous mutation of the *scid* (severe combined immune deficiency) gene results in a paucity of functional B and T cells and impairment of the joining of coding sequences, which is a specific step in V(D)J recombination (*4*). Homozygous mutant *scid* mice also suffer a general defect in the repair of double-stranded DNA breaks (*5*). Mutations in at least two other genes that function in double-strand DNA repair are also associated with impaired V(D)J recombination (*6*). The appropriation of part of the enzymatic machinery for DNA repair by the V(D)J recombinational apparatus suggests that the regulation of these two DNA transactions may be coordinated.

V(D)J recombination is normally restricted to the B and T lineages and is subject to developmental control (*7*). The lineage and developmental stage specificity of V(D)J rearrangement are established, at least in part, by regulation of *RAG-1* and *RAG-2* transcription (*2, 8*). In developing B cells, all of the V(D)J rearrangements required for Ig expression occur over several cell divisions (*9*). The occurrence of Ig gene rearrangement during cell division suggested the need for finer temporal control of V(D)J recombination than could be provided by transcriptional regulation. Such control could be effected by post-translational modification of either RAG-1 or RAG-2, because both proteins are required for V(D)J recombination.

Phosphorylation of RAG-2. To analyze the product of the *RAG-2* gene, we used rabbit antibodies to four synthetic RAG-2 peptides (*10*). Cell lines that stably express RAG-2, singly or in combination with RAG-1, were generated by transfection of NIH 3T3 cells with plasmids carrying *RAG* coding sequences (*11*). Expression of *RAG* RNA in these cell lines was assayed by hybridization. In addition, RAG-2 activity was verified by stable coexpression with RAG-1 in an NIH 3T3 derivative that carries a single integrated copy of a retroviral substrate for recombination, LJHCR (*12*). In cells expressing both RAG-2 and RAG-1, the integrated substrate was shown to undergo V(D)J recombination, as assessed by polymerase chain reaction (PCR) and hybridization to an oligonucleotide probe specific for signal joints; rearrangement was not detectable in cells that had not been transfected with both *RAG* genes (*13*).

The NIH 3T3 cells that expressed RAG-2 were metabolically labeled for 3 hours with [^{35}S]methionine. Cells were lysed and RAG-2 was immunoprecipitated with RAG-2–specific antibodies (Abs). Three of the Abs to RAG-2 (Ab428, Ab432, and Ab435) immunoprecipitated a 65-kD, ^{35}S-labeled species from cells that stably express RAG-2; a fourth Ab (Ab429) immunoprecipitated this species less efficiently because of low affinity (*13*) and was not used further (Fig. 1A). The 65-kD species was not recovered from NIH 3T3 cells nor was it precipitated by control antibody (Fig. 1A). Both Ab432 and Ab435 specifically precipitated a 65-kD phosphoprotein from RAG-2–expressing cells that had been metabolically labeled with [^{32}P]orthophosphate (Fig. 1B). After acid hydrolysis, the major phosphorylated amino acid recovered from RAG-2 was phosphoserine, a minor fraction was recovered as phosphothreonine, and no phosphotyrosine was detected (Fig. 1C).

Two major phosphorylation sites in vivo. The major amino acids of RAG-2 that are phosphorylated in vivo were identified by in vitro mutagenesis of candidate phosphorylation sites and phosphopeptide mapping. The number of potential phosphorylation sites was delimited by computational analysis. First, samples of [^{32}P]RAG-2 that were isolated from metabolically labeled cells by immunoprecipitation and electrophoresis were digested with each of four proteases: trypsin, thermolysin, chymotrypsin, and endoproteinase Glu-C. The two-dimensional phosphopeptide maps generated by each enzyme were then compared to the mobilities of phosphopeptides predicted by the programs PEPTIDESORT and MOBILITY (*14*). The predicted mobilities of all possible phosphopeptides generated by each protease were compared to the four actual phosphopeptide maps. We identified six residues whose phosphorylation could account for the observed patterns: Ser^{356}, Ser^{375}, Thr^{376}, Thr^{391}, Ser^{392}, and Ser^{381}.

To determine which of these amino acid residues represent the major sites of RAG-2 phosphorylation in vivo, we introduced the following single or double alanine substitutions (*15*): S356A; S375A,T376A; T391A,S392A; and S381A. As described below, the RAG-2 protein was unstable in vivo and amino acid substitutions at Thr^{490} prolonged its half-life without affecting its steady-state phosphorylation pattern (*13*). To facilitate analysis of the alanine substi-

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tution mutants described above, we introduced, in each case, an additional T490A mutation. These mutants were transiently transfected into the human embryonal kidney cell line 293 and labeled with [^{32}P]orthophosphate. RAG-2 was recovered by immunoprecipitation with Ab435 and isolated by gel electrophoresis; the recoveries of wild-type and all mutant proteins were similar (*13*). RAG-2 was then digested with thermolysin. The S356A mutation was associated with the selective disappearance of two closely migrating phosphopeptides (Fig. 2, A and B). The appearance of two phosphopeptides is explained by incomplete digestion between Asp^{354} and Leu^{355}: thermolytic cleavage following an acidic residue is inefficient (*16*), and the observed mobilities of these two phosphopeptides agree with the mobilities calculated for completely and incompletely digested thermolytic peptides spanning Ser^{356}. The T391A,S392A mutation resulted in selective disappearance of the remaining major species (Fig. 2, A and C); phosphoamino acid analysis of this major phosphopeptide yielded phosphoserine exclusively (Fig. 2D). The S375A,T376A and S381A mutations had no effect (*13*). We conclude that, at steady state in vivo, RAG-2 is phosphorylated principally at two sites: S356 and S392.

Ser^{356} effects on Ig gene rearrangement. To address whether phosphorylation at either of these sites might affect RAG-2 activity, we assessed the ability of wild-type RAG-2 and a series of RAG-2 mutants to activate rearrangement of an extrachromosomal substrate, pJH200 (*17*), when cotransfected with the *RAG-1* gene into nonlymphoid cells. An alanine substitution at Ser^{356} significantly reduced V(D)J recombination in both the 293 cells (Fig. 3, A and B) and NIH 3T3 cells (Fig. 3C); impairment of rearrangement required mutation at Ser^{356}; mutations at Ser^{375}, Thr^{376}, Ser^{381}, Thr^{391}, or Ser^{392} had no effect (Fig. 3A). RAG-2 is found predominantly in the nucleus (*13*). The reduction in activity observed in this experiment was not the result of decreased expression or altered localization of RAG-2, as total and nuclear RAG-2 protein amounts, as assayed by immunoblotting with Ab435, were not affected by these mutations (*13*).

The major RAG-2 phosphopeptides obtained after steady-state labeling with ^{32}P in vivo were a subset of those obtained after in vivo phosphorylation of bacterially expressed RAG-2 by casein kinase 2 (CK2) (*13*). The Ser^{356} and Ser^{392} residues lie within amino acid sequence motifs favored by acidotropic protein kinases such as the casein kinases (*18*).

Phosphorylation of RAG-2 by p34^{cdc2} in vitro. Bacterially expressed RAG-2 is also selectively phosphorylated by the protein kinase p34^{cdc2} in vitro (*19*). The kinase preparation used in these experiments, the E2 fraction of RNA polymerase II carboxyl-terminal domain (CTD) kinase, contains p34^{cdc2} in complex with a 58-kD subunit (*20*). Phosphorylation of RAG-2 by p34^{cdc2} in vitro occurred only at threonine, and immunoprecipitation of RAG-2 proteolytic fragments showed that the sites of in vitro phosphorylation by p34^{cdc2} lie within 25 kD of the RAG-2 COOH-terminus (*13*). Within that segment of RAG-2, the only threonine residues that conform to the p34^{cdc2} consensus recognition sequence (*21*) are Thr^{490} and Thr^{515}. Synthetic peptides spanning amino acid residues 487 to 493 and 508 to 527 were both phosphorylated by p34^{cdc2} in vitro (Fig. 4), and in each case phosphoamino acid analysis revealed

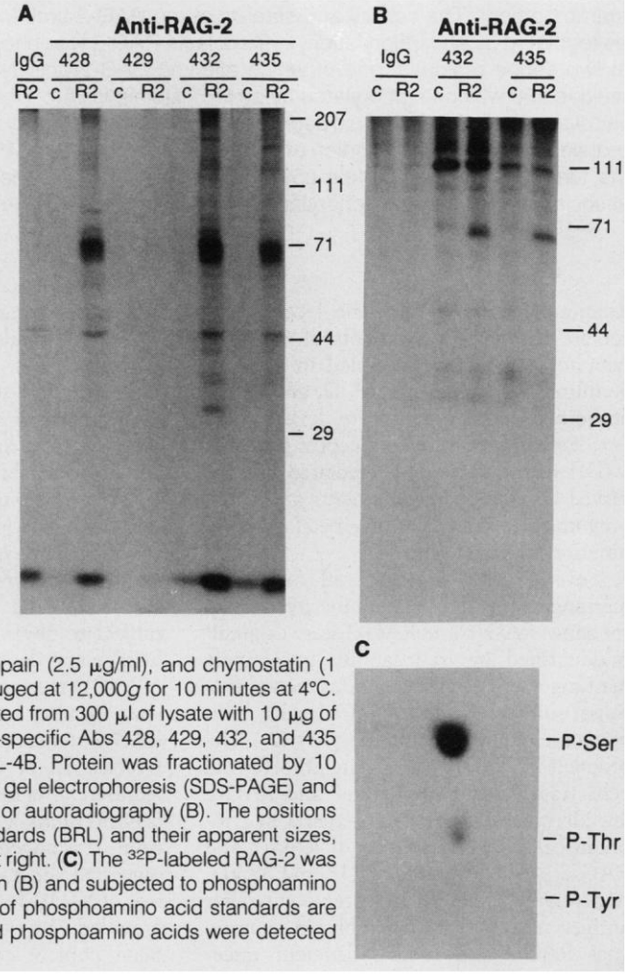

Fig. 1. Immunoprecipitation and phosphoamino acid analysis of metabolically labeled RAG-2. (**A** and **B**) NIH 3T3 cells, stably transfected with pBCMGNeoRAG-2 (R2) or with vector alone (c) were grown to 50 percent confluence on 10-cm plates. Cells were starved for 30 minutes in methionine-free or phosphate-free Dulbecco's modified Eagle's medium, then labeled for 3 hours with [^{35}S]methionine at 0.3 mCi/ml (A) or with carrier-free [^{32}P]orthophosphate at 2.5 mCi/ml (B). Cells were lysed in RIPA buffer [50 mM tris (pH 8.0), 150 mM NaCl, 0.5 percent deoxycholate, 1 percent NP-40, 0.1 percent SDS], supplemented with 50 mM NaF, 1 mM Na_3VO_4, aprotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin (1 μg/ml), 0.25 mM phenylmethanesulfonyl fluoride, antipain (2.5 μg/ml), and chymostatin (1 μg/ml); the lysate was centrifuged at 12,000*g* for 10 minutes at 4°C. Protein was immunoprecipitated from 300 μl of lysate with 10 μg of normal rabbit IgG or RAG-2–specific Abs 428, 429, 432, and 435 and protein A–Sepharose CL-4B. Protein was fractionated by 10 percent SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and detected by fluorography (A) or autoradiography (B). The positions of prestained molecular standards (BRL) and their apparent sizes, in kilodaltons, are indicated at right. (**C**) The ^{32}P-labeled RAG-2 was eluted from lane 4 of the gel in (B) and subjected to phosphoamino acid analysis (*31*). Positions of phosphoamino acid standards are indicated at right; ^{32}P-labeled phosphoamino acids were detected by autoradiography.

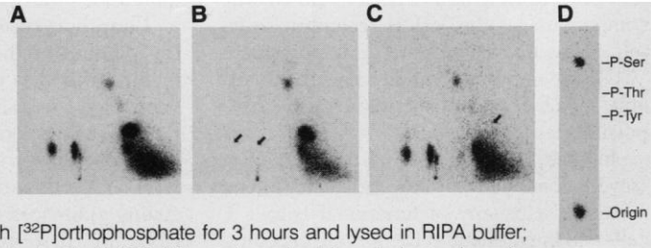

Fig. 2. Localization of the major RAG-2 phosphorylation sites in vivo. Plasmids pcRAG-2T490A (**A**), pcRAG-2T490A,S356A (**B**), and pcRAG-2T490A,T391A,S392A (**C**) were cotransfected with pRSV-T into 293 cells (*11*). After 2 days, cells were labeled with [^{32}P]orthophosphate for 3 hours and lysed in RIPA buffer; protein was immunoprecipitated with Ab435. After polyacrylamide gel electrophoresis, protein was transferred to polyvinylpyrrolidone, digested with thermolysin, and oxidized in performic acid (*32*). Peptides were resolved in two dimensions by electrophoresis and ascending chromatography (*33*). The ^{32}P-labeled peptides were detected with a PhosphorImager (Molecular Dynamics). Arrows indicate the major phosphopeptides that are eliminated by mutation. (**D**) The phosphopeptide that was eliminated by the T391A,S392A mutation was subjected to phosphoamino acid analysis; the positions of phosphoamino acid markers are indicated.

phosphorylation at threonine exclusively (*13*); thus Thr^{490} and Thr^{515} are the targets of phosphorylation by $p34^{cdc2}$ in these two peptides.

When native, bacterially expressed RAG-2 was phosphorylated by $p34^{cdc2}$, tryptic digestion released a single predominant phosphopeptide (Fig. 4A, spot d); when RAG-2 was denatured before phosphorylation, tryptic digestion released three phosphopeptides (Fig. 4B, spots a, b, and d). Tryptic digestion of peptide 508 to 527 after phosphorylation by $p34^{cdc2}$ yielded two phosphorylated products (Fig. 4C) that comigrated with RAG-2 phosphopeptides a and b (Fig. 4D). The two phosphopeptides result from inefficient tryptic digestion at repeated lysine residues 518 and 519 (Fig. 4C) (*14*). Tryptic digestion of peptide 487 to 493 after phosphorylation by $p34^{cdc2}$ yielded a phosphopeptide (Fig. 4E) that comigrates with RAG-2 phosphopeptide d (Fig. 4F). Thus, in vitro, $p34^{cdc2}$ can phosphorylate RAG-2 at two sites, Thr^{490} and Thr^{515}; Thr^{490} is the predominant target in the native protein.

Mutation of the major $p34^{cdc2}$ phosphorylation site. When wild-type or mutant *RAG-2* plasmids were transfected into 293 cells, expression of the RAG-2(T490A) mutant consistently increased; mutations at seven other serine or threonine sites did not affect RAG-2 expression (*13*). Using transient transfection, we further examined this increase in RAG-2 expression; we avoided stable transfection because this might select for cells that process the RAG-2 protein aberrantly, or for cells in which the RAG-2 gene had undergone mutation. Wild-type and T490A mutant *RAG-2* plasmids were combined in various proportions so that the total amount of DNA was constant, and the mixtures were transfected into 293 cells; the cells were labeled and RAG-2 was immunoprecipitated with Ab435. The yield of ^{35}S-labeled RAG-2 protein increased as the ratio of mutant to wild-type *RAG-2* cDNA (Fig. 5A). The amount of RAG-2 protein was not directly related to steady-state amounts of *RAG-2* RNA, which were also assayed for each transfectant at the time of harvest (Fig. 5B, upper panel). (The species migrating faster represents mature *RAG-2* RNA; several slower-migrating species may represent incompletely processed transcripts.) Similar amounts of total RNA were present in each lane, as assessed by hybridization to a mouse β-tubulin probe (Fig. 5B, lower panel). The RAG-2 protein in 293 cells transfected with wild-type or mutant *RAG-2* genes was also assayed by immunoblotting with antibody to RAG-2, with similar results (Fig. 5C). The effect of mutation at Thr^{490} was not specific for substitution with alanine; substitution with aspartic acid resulted in a similar increase in RAG-2 expression (*13*).

Expression of wild-type and T490A mutant RAG-2 proteins might have differential physiologic effects on the transiently transfected cell population, leading to nonspecific differences in protein expression. Accordingly, the wild-type *RAG-1* gene was cotransfected with wild-type or T490A mutant *RAG-2* genes into 293 cells; RAG-1 and RAG-2 expression were assayed by immunoblotting with RAG-1 or RAG-2 antibodies. Although the steady-state expression of RAG-2 in T490A transfectants was elevated relative to wild type, the amount of RAG-1 was identical in cells expressing wild-type or mutant RAG-2 (Fig. 5C).

Because the T490A mutation appeared to exert its effect after transcription, we determined the effect of this mutation on the rates of synthesis and degradation of RAG-2 protein. Although the synthetic rates of wild-type and T490A RAG-2 proteins were similar (Fig. 6, inset), their degradation rates were not. The wild-type protein had a half-life of about 12 minutes; in contrast, the T490A mutant had a half-life of about 205 minutes (Fig. 6). The relative increase in half-life (about 17 times longer) is sufficient to account for the increase (20 times) in the steady-state amount of mutant RAG-2 protein, relative to that of wild-type protein, as determined by immunoblotting and densitometry (*13*). The T490A mutation did not impair RAG-2 activity as measured by the extrachromosomal assay, suggesting that phosphorylation at Thr^{490} regulates the amount of RAG-2 but not its activity. In addition, the T490A mutation did not affect nuclear localization of RAG-2 and coexpression of RAG-1 did not affect the relative amounts of wild-type RAG-2 and RAG-2(T490A) proteins [Fig. 5C and (*13*)].

Regulation of chimeric protein stability by the COOH-terminal 90 amino acid residues of RAG-2. The difference in steady-state expression of wild-type RAG-2 and RAG-2(T490A) could be conferred on chimeric proteins by transfer of a RAG-2 polypeptide sequence spanning Thr^{490}. Constructs (*11*) encoding chimeric proteins were made (Fig. 7C); each chimera contained the NH_2-terminal 410 residues of

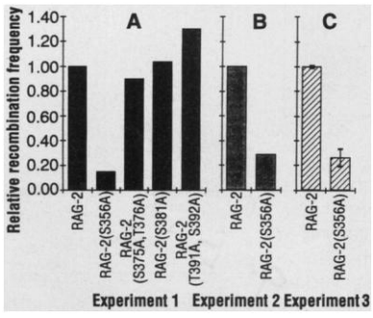

Fig. 3. Impairment of RAG-2 activity by mutation of S356. Plasmids encoding wild-type or mutant RAG-2 proteins were cotransfected with pcRAG-1 and the recombination substrate pJH200 into 293 cells (experiments 1 and 2) or NIH 3T3 cells (experiment 3); V(D)J rearrangement within pJH200 was assayed as described (*7*). Recombination frequencies were normalized to the frequency observed with wild-type RAG-2. In experiment 3, transfections were performed in duplicate and for each RAG-2 construct the mean and standard error are indicated. In separate experiments, the recombination frequencies obtained with wild-type RAG-2 varied from 1 to 4 percent.

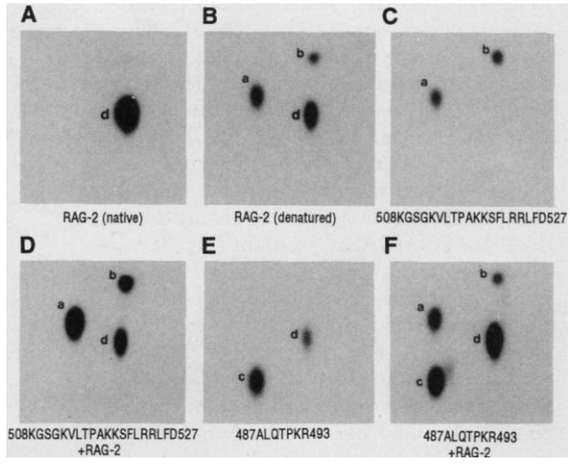

Fig. 4. RAG-2 amino acid residues phosphorylated in vitro by $p34^{cdc2}$. (**A**) *Escherichia coli*–expressing RAG-2 were lysed under nondenaturing conditions; the lysate was radiolabeled with CTD kinase E2 and [γ-^{32}P]ATP (*19*). RAG-2 was isolated and digested with trypsin; phosphopeptides were resolved in two dimensions and detected by autoradiography. (**B**) RAG-2 was immunoprecipitated from SDS-solubilized bacterial lysates and incubated with CTD kinase E2 in the presence of [γ-^{32}P]ATP (*19*). RAG-2 was isolated and subjected to tryptic phosphopeptide mapping. (**C**) Tryptic phosphopeptide map of a synthetic peptide (KGSGKVLTPAKKSFLRRLFD) corresponding to residues 508 to 527 of RAG-2, which had been phosphorylated by CTD kinase E2. (**D**) Equal radioactive amounts of denatured RAG-2 and peptide 508 to 527, labeled with ^{32}P by CTD kinase E2, were mixed and the tryptic phosphopeptides were mapped. (**E**) Tryptic phosphopeptide map of a synthetic peptide (ALQTPKR), corresponding to residues 487 through 493 of RAG-2, which had been phosphorylated by CTD kinase E2. Spot (c) represents undigested phosphopeptide. (**F**) Tryptic phosphopeptide map of a mixture of denatured, ^{32}P-labeled RAG-2 and peptide 487 to 493, as in (D).

RAG-1 and residues 437 to 527 of either wild-type RAG-2 (R1-R2) or the RAG-2(T490A) mutant (R1-R2T490A). After transfection into 293 cells, steady-state amounts of chimeric proteins were determined by immunoblotting with an antibody to the COOH-terminus of RAG-2 (Ab432; Fig. 7A) and an antibody to the NH_2-terminus of RAG-1 (Ab516; Fig. 7B). Expression of R1-R2T490A was greater than that of R1-R2 (Fig. 7, A and B). Similar results were obtained for chimeric proteins in which the COOH-terminal 90 residues of RAG-2 were fused to the NH_2-terminal 649 residues of *Escherichia coli* β-galactosidase (*13*). Thus the essential determinants of the T490A phenotype lie within 90 amino acid residues of the COOH-terminus of RAG-2. These results indicate that increased expression of RAG-2(T490A), relative to wild-type protein, did not result from differential effects of the wild-type and mutant RAG-2 proteins themselves, because similar differences in protein expression were conferred by small portions of wild-type or mutant RAG-2.

Phosphorylation of RAG-2 in vivo at Thr^{490}. The observation that RAG-2 was stabilized in vivo by mutations at Thr^{490} indicated that phosphorylation of RAG-2 by a cyclin-dependent kinase may target it for degradation. In the steady state, phosphorylation of RAG-2 at Thr^{490} in vivo was not detected. A stabilizing mutation of RAG-2 at a site different from Thr^{490} might result in accumulation of the Thr^{490} phosphorylated form. Deletion of the COOH-terminal 29 amino acid residues of RAG-2 (RAG-2ΔC; Fig. 8B) spares the Thr^{490} site and resulted in increased RAG-2 expression, presumably by increasing the half-life of the protein (Fig. 8A). The amounts of RAG-2(T490A,ΔC) and RAG-2(T490A) were similar, suggesting that the COOH-terminal deletion partially suppresses transit through the degradation pathway affected by the T490A mutation. When the phosphopeptide maps of RAG-2(ΔC) and RAG-2(T490A,ΔC) were compared, a phosphopeptide was present in the RAG-2(ΔC) map (Fig. 8C) that was absent from the RAG-2(T490A,ΔC) mutant (Fig. 8D). This peptide comigrated with the RAG-2 tryptic peptide that was phosphorylated at Thr^{490} in vitro (*13*). Thus Thr^{490} was phosphorylated in vivo; the identity of the responsible kinase potentially could be one with a specificity similar to a cyclin-dependent kinase (cdk) like p34^{cdc2}. Removal of the terminal 29 amino acid residues of RAG-2 could, in principle, create a cdk phosphorylation site that is not used in the wild-type protein in vivo; this seems unlikely for two reasons. First, as shown above, the intact protein is readily phosphorylated by p34^{cdc2} in vitro; second, the RAG-

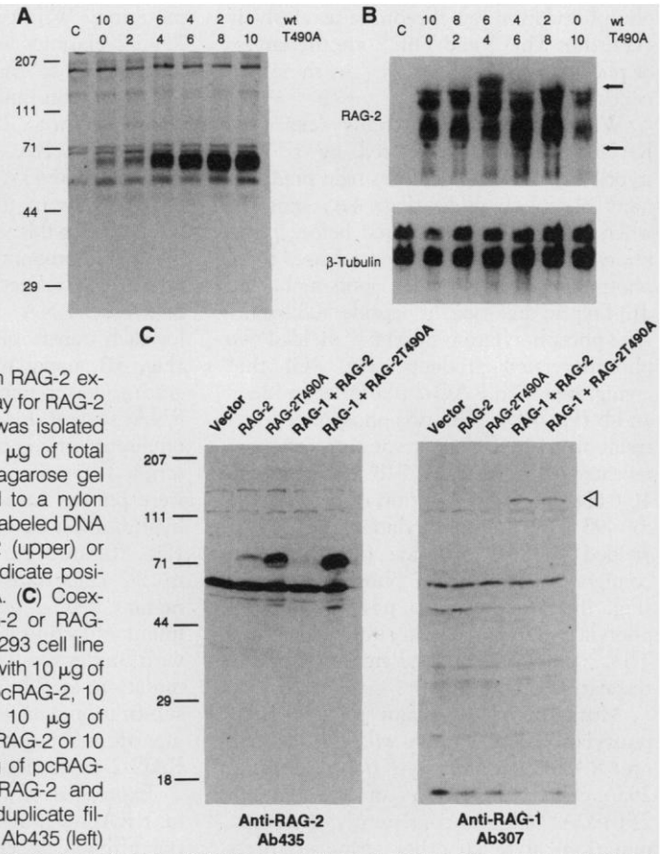

Fig. 5. (**A**) Increased expression of RAG-2 upon mutation of Thr^{490}. The 293 cell line was transiently transfected with 10 μg of pcDNA (lane c) or with mixtures of pcRAG-2 (wt) and pcRAG-2T490A (T490A) as indicated in micrograms. RAG-2 was metabolically labeled with [^{35}S]methionine, immunoprecipitated with Ab435, and analyzed by SDS-PAGE as in Fig. 1. (**B**) The effect of T490A mutation on RAG-2 expression. At the time of assay for RAG-2 protein, total cellular RNA was isolated from transfected cells; 20 μg of total RNA was fractionated by agarose gel electrophoresis, transferred to a nylon filter, and hybridized to ^{32}P-labeled DNA probes specific for RAG-2 (upper) or β-tubulin (lower). Arrows indicate positions of 28S and 18S RNA. (**C**) Coexpression of wild-type RAG-2 or RAG-2(T490A) with RAG-1. The 293 cell line was transiently transfected with 10 μg of pcDNA (vector), 10 μg of pcRAG-2, 10 μg of pcRAG-2(T490A), 10 μg of pcRAG-1, and 10 μg of pcRAG-2 or 10 μg of pcRAG-1 and 10 μg of pcRAG-2(T490A). After 2 days, RAG-2 and RAG-1 were detected on duplicate filters by immunoblotting with Ab435 (left) and Ab307 (right), respectively. Transfected plasmids are indicated at top. The open arrowhead indicates RAG-1.

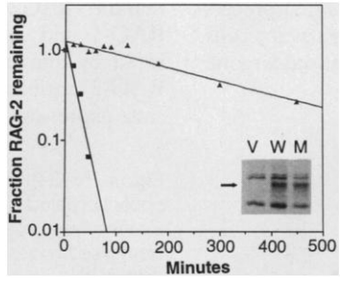

Fig. 6 (**Left**). The prolonging effect of the T490A mutation on the half-life of RAG-2 protein. Transfection of 293 cells with equimolar amounts of vector (pcDNA; lane V), pcRAG-2 (lane W), or pcRAG-2(T490A) (lane M). After 2 days, cells were labeled with [^{35}S]methionine (0.3 mCi/ml) for 15 minutes; RAG-2 was isolated by immunoprecipitation and electrophoresis. Arrow at left indicates ^{35}S-labeled RAG-2 (inset). Alternatively, label was stopped by the addition of medium containing a 30,000-fold excess of unlabeled methionine and cyclohexamide (10 μg/ml). At various times, ^{32}P-labeled RAG-2 was assayed as above. [^{35}S]RAG-2 was quantitated with a PhosphorImager. Data were normalized to [^{35}S]RAG-2 or [^{35}S]RAG-2(T490A), measured when the labeling was stopped, and this value (fraction RAG-2 remaining) was plotted logarithmically as a function of time. ■, Wild-type RAG-2; ▲, RAG-2(T490A).

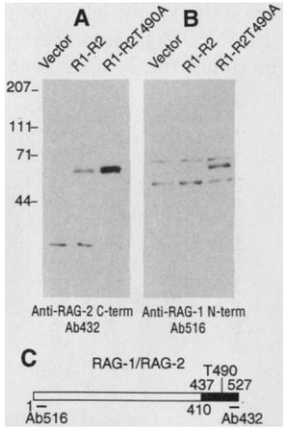

Fig. 7 (**Right**). Comparison of expression of chimeric proteins generated from RAG-1 and RAG-2. Chimeric proteins containing the NH_2-terminal 410 residues of RAG-1 and residues 437 to 527 of wild-type RAG-2 (R1-R2) or RAG-2(T490A) (R1-R2T490A) were expressed in 293 cells. At 2 days after transfection, an equal amount of protein from each transfectant was fractionated by 10 percent SDS-PAGE and transferred to nitrocellulose. Ponceau-S staining verified that equivalent protein was present in each lane. Duplicate filters were incubated with Ab432 (**A**) or Ab516 (**B**) (*10*); bound antibody was detected with horseradish peroxidase–conjugated goat antibody to rabbit IgG and an enhanced chemiluminescence assay (Amersham). Transfected plasmids are indicated at top. The expected size of the chimeric protein is 57 kD. (**C**) Diagram of the chimeric protein. Regions derived from RAG-1 and RAG-2 are indicated by open and shaded boxes, respectively. Residues included in these regions are indicated above (RAG-2) and below (RAG-1) the boxes. Positions of peptides used to generate antibodies 516 and 432 are indicated.

2(ΔC) retains activity in vivo (*13*), suggesting that its structure is not greatly altered by this mutation.

Mutation of the p34^{cdc2} phosphorylation site of tumor suppressor protein p53. The prolongation of RAG-2 half-life by mutation of Thr^{490} was not restricted to experiments performed in the 293 cell line; half-life was also extended in NIH 3T3 cells (*13*). Thus, the regulation of protein half-life by phosphorylation may be a general mechanism. A search of the SwissProt database with the COOH-terminal 90 residues of RAG-2 revealed that the tumor suppressor protein p53 had limited similarity in a region surrounding the p34^{cdc2} phosphorylation site at Ser^{315} (*22*). We tested whether mutation of Ser^{315} in p53 would result similarly in enhanced protein expression.

Human wild-type p53 or a p53(S315D) mutant plasmid was transfected into NIH 3T3 cells. Immunoblotting with an antibody to human p53 showed that the S315D mutation conferred a higher steady-state protein level than wild type (Fig. 9A). It seemed unlikely that this relative increase in expression resulted from differences in the ability of wild-type p53 and p53(S315D) to inhibit cell growth, because these proteins have been found to have similar effects on the growth of yeast (*23*). Nonetheless, to address the possibility that the difference in expression of wild-type p53 and p53(S315D) was the indirect result of differential physiologic effects of these proteins, chimeras containing the NH_2-terminal 627 amino acids of RAG-1 and the COOH-terminal 234 amino acids of p53 or of the p53(S315D) mutant (*11*) were constructed (Fig. 9D), expressed in NIH 3T3 cells, and detected by immunoblotting with Abs to RAG-1 (Fig. 9, B and C). The portion of p53 retained in the chimera lacked the transactivator domain, which is essential for p53 function (*24*). The chimeric protein carrying the S315D mutation was expressed in greater amounts at the steady state than its wild-type counterpart (Fig. 9, B and C). Thus, as observed for RAG-2, mutation of the major site of p53 phosphorylation by p34^{cdc2} was associated with increased expression.

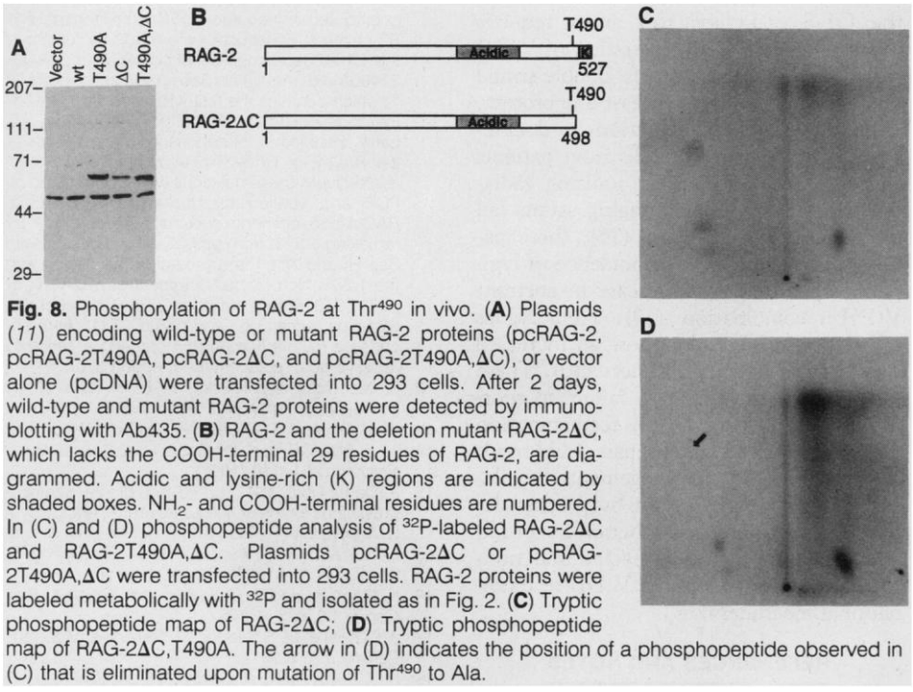

Fig. 8. Phosphorylation of RAG-2 at Thr^{490} in vivo. (**A**) Plasmids (*11*) encoding wild-type or mutant RAG-2 proteins (pcRAG-2, pcRAG-2T490A, pcRAG-2ΔC, and pcRAG-2T490A,ΔC), or vector alone (pcDNA) were transfected into 293 cells. After 2 days, wild-type and mutant RAG-2 proteins were detected by immunoblotting with Ab435. (**B**) RAG-2 and the deletion mutant RAG-2ΔC, which lacks the COOH-terminal 29 residues of RAG-2, are diagrammed. Acidic and lysine-rich (K) regions are indicated by shaded boxes. NH_2- and COOH-terminal residues are numbered. In (C) and (D) phosphopeptide analysis of ^{32}P-labeled RAG-2ΔC and RAG-2T490A,ΔC. Plasmids pcRAG-2ΔC or pcRAG-2T490A,ΔC were transfected into 293 cells. RAG-2 proteins were labeled metabolically with ^{32}P and isolated as in Fig. 2. (**C**) Tryptic phosphopeptide map of RAG-2ΔC; (**D**) Tryptic phosphopeptide map of RAG-2ΔC,T490A. The arrow in (D) indicates the position of a phosphopeptide observed in (C) that is eliminated upon mutation of Thr^{490} to Ala.

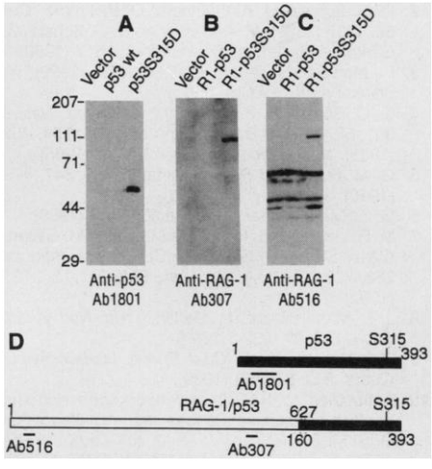

Fig. 9. Mutation of the p34^{cdc2} phosphorylation site is associated with increased steady-state expression of p53. Chimeric proteins RAG-1–p53 and RAG-1–p53(S315D) contain residues 1 to 627 of RAG-1 fused to residues 160 to 393 of wild-type human p53 (RAG-1–p53) or a p53(S315D) mutant (RAG-1–p53S315D), respectively. NIH 3T3 cells were transfected with plasmids encoding wild-type human p53, p53(S315D), RAG-1–p53, or RAG-1–p53(S315D). At 2 days after transfection, proteins were detected by immunoblotting with Ab1801 (**A**), which is specific for human p53 (Santa Cruz Biotechnology), or with antibodies 307 (**B**) and 516 (**C**), which are specific for RAG-1 (*10*). The expected size of the RAG-1–p53 chimeras is 103 kD. For (A) to (C), transfected plasmids are indicated at top. (**D**) Diagrams of p53 (upper) and RAG-1–p53 (lower). Regions corresponding to RAG-1 and p53 are indicated by open and shaded boxes, respectively. Amino acid residues present in these regions are numbered. Antibody recognition sites are indicated by heavy lines. Ser^{315}, the p34^{cdc2} phosphorylation site of p53, is indicated (S315).

Phosphorylation and regulation of V(D)J rearrangement. In that V(D)J recombination requires both RAG-1 and RAG-2, rearrangement could be regulated by controlling the activity of either of these proteins. Our data indicate that the activity and stability of RAG-2 may be regulated by phosphorylation. The RAG-2 protein used in our studies was expressed in the NIH 3T3 or 293 cell lines by transfection, yielding sufficient protein for phosphopeptide analysis and facilitating the use of mutagenesis to determine phosphorylation sites. There are several reasons why this approach is valid. (i) With respect to its dependence on recombinational signal sequences and the structures of the recombination products, V(D)J rearrangement was faithfully mimicked in nonlymphoid cells expressing RAG-1 and RAG-2 (*2*); the functionality of RAG-2 was confirmed for the cell lines used in our study. (ii) The phosphorylation patterns of RAG-2 in NIH 3T3 and 293 cells were identical. (iii) Mutations at two of the three sites of phosphorylation that we mapped by this approach, Ser^{356} and Thr^{490}, had significant effects on RAG-2 function or expression.

Ser^{356} and Ser^{392}, the principal phosphorylation sites of RAG-2 at steady state in vivo, lie within an acidic region (*2*) of RAG-2 (25 out of 63 residues being either Asp or Glu). The S356A mutation impairs the ability of RAG-2 to activate recombination without affecting its expression or its nuclear localization, suggesting that phosphorylation of Ser^{356} increases RAG-2 activity. Although mutation of Ser^{392} did not affect rearrangement of an extrachromosomal substrate, the effect of this mutation on rearrangement of endogenous antigen receptor gene segments has yet to be tested.

Protein destabilization targeted by phosphorylation. The hypothesis that phosphorylation at Thr^{490} targets RAG-2 for rapid degradation is supported by several observations: (i) Mutation at Thr^{490} pro-

longed RAG-2 half-life; (ii) phosphorylation of RAG-2 at Thr^{490} was not detected in the wild-type protein at steady state; (iii) when degradation of RAG-2 was suppressed by a COOH-terminal deletion, accumulation of a Thr^{490} phosphorylated form of the protein was observed in vivo; (iv) a similar difference in steady-state expression was conferred on chimeric proteins by residues 437 to 527 of wild-type (Thr^{490}) or mutant (Ala^{490}) RAG-2, implying that the phenotype does not require the remainder of the protein.

Several lines of evidence suggest that the enzyme responsible for phosphorylation of RAG-2 at Thr^{490} could be a cdk. (i) A purified $p34^{cdc2}$-containing kinase (*20*) preferentially phosphorylates RAG-2 at Thr^{490} in vitro. (ii) The sequence of RAG-2 near T490 (T*-P-K-R) conforms to the consensus recognition sequence for $p34^{cdc2}$-containing kinases (*21*). (iii) Although the MAP-2 kinase can phosphorylate a subset of $p34^{cdc2}$ sites, these targets conform to the consensus P-X-T/S*-P (*25*), which differs from the sequence surrounding RAG-2 Thr^{490} (L-Q-T*-P). The $p34^{cdc2}$ protein has multiple forms, distinguished by their associated cyclins; in addition, several homologs of $p34^{cdc2}$ have been identified (*26*). Thus, although it seems likely that a cdk with specificity similar to that of $p34^{cdc2}$ phosphorylates RAG-2 at Thr^{490} in vivo, we cannot at present ascribe this function to a particular kinase. The short half-life of wild-type RAG-2 relative to the Thr^{490} mutants and the apparent association between phosphorylation and degradation in this system suggest that RAG-2 is phosphorylated during most of the cell cycle; transient dephosphorylation of Thr^{490} would result in increased RAG-2 protein. RAG-2 expression could therefore be controlled by regulating the activity of a phosphatase, a kinase, or both.

Fibroblastoid cells transfected with the *RAG-2* T490A mutant did not exhibit increased V(D)J joining activity relative to wild-type transfectants (*13*), implying that under conditions of transient transfection RAG-2 protein is not limiting for rearrangement of the extrachromosomal substrate. In lymphoid progenitor cells, however, *RAG-2* RNA is less abundant than *RAG-1* RNA (*2*), and the amounts of both RNA species, as well as the amounts of RAG-1 and RAG-2 protein, are lower than in transiently transfected fibroblastoid cells (*13*). Under these conditions, therefore, changes in the amount of RAG-2 protein could potentially regulate joining of endogenous antigen receptor gene segments.

Human p53 is phosphorylated by p60-$p34^{cdc2}$ and cyclinB-$p34^{cdc2}$ at S315; phosphorylation at this site is cell cycle–dependent, and the abundance of p53 oscillates during cell cycle (*22*). We observed that mutation of Ser^{315} of p53 was associated with increased steady-state p53 protein expression; this phenotype could be conferred on a chimeric protein by the COOH-terminal portion of p53. Thus, the apparent association between phosphorylation and protein degradation may represent a general mechanism. Consistent with this idea, cyclin proteolysis is accelerated in a cell-free extract upon addition of $p34^{cdc2}$ (*27*).

Antigen receptor gene rearrangement occurs in dividing cells; regulation of RAG-2 expression by phosphorylation provides a means by which V(D)J recombinase activity could be restricted to a particular interval of the cell cycle. Agents that cause double-strand breaks in DNA, such as ionizing radiation, induce cell cycle arrest at the G1-S boundary; this arrest requires induction of p53 (*28*). Initiation of V(D)J recombination also creates double-strand breaks and depends on at least one protein, the *scid* product, that functions in double-strand break repair. In cells from patients with ataxia-telangiectasia, ionizing radiation and other DNA damaging agents fail to induce p53 expression (*28*); these patients show an increased incidence of lymphoid tumors and an increase in aberrant V(D)J recombination (*29*). Homozygous p53-deficient mice also show an increased frequency of lymphoid tumors (*30*). These observations suggest that a failure to coordinate initiation of V(D)J recombination and double-strand break repair could result in aberrant V(D)J rearrangement. Regulation of RAG-2 expression by posttranslational modification may function in coordination of the lymphoid-specific and nonspecific components of the V(D)J recombinational machinery.

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10. Antibodies to RAG-2 and their corresponding peptide antigen sequences are: Ab428 to FG-GRSYMPSTQRTTEK^{171}; Ab429 to GQKGWPKRS-CPTGVFHFDIK^{51}; Ab435 to FDGDDEFDTYNEDD-EDDES^{411}K (the COOH-terminal lysine residue of this peptide was added to assist coupling to carrier protein); and Ab432 to KGSGKVLTPAKKS-FLRRLFD^{527}. Antibodies to RAG-1 and their corresponding peptides are: Ab307 EKVLLPGYH-PFEWQPPLK^{524}; and Ab516 to FKLFRVRS-FEKAPEEAQKEK^{49}. Rabbits were immunized and the antibodies were affinity-purified (*34*). Antisera were screened by immunoblot of bacterially expressed RAG proteins and specificity was confirmed by peptide competition.
11. RAG-1 and RAG-2 coding sequences were amplified from BALB/c genomic DNA by PCR. The PCR products were subcloned and verified by DNA sequencing. The RAG-2 sequence was identical to that described (*2*) except for GC in place of CG at nucleotides 1528 and 1529. This difference was observed in products of several independent PCR reactions, and the corresponding amino acid (Cys for Ser at residue 458) conforms to the sequence of chicken RAG-2 (*35*). We conclude that the murine RAG-2 sequence used in our studies is correct. For stable expression, RAG-1 and RAG-2 coding sequences were inserted into pBCMGNeo (*36*) or pBCMGHyg (*37*). Expression plasmids pcRAG-1 and pcRAG-2, used in transient transfection experiments, were constructed by ligating RAG-1 or RAG-2 coding sequences into pcDNA (Invitrogen). R1-R2 chimeric constructs were made by replacing the 1.4-kb Sac I fragment of pcRAG-2 or pcRAG-2T490A with the 1.3-kb Sac I fragment of pcRAG-1, which contains the first 410 codons of RAG-1. Plasmids pcRAG-2ΔC and pcRAG-2T490A, ΔC carry deletions of 29 codons from the 3′ ends of the RAG-2 or RAG-2T490A coding sequences, respectively; these deletions were constructed by PCR and verified by nucleotide sequencing. RAG-1-p53 chimeric constructs were made by removing 1.3 kb from pcRAG-1 by digestion with Bsp HI and Xba I and replacing this region with the 1.2-kb Nco I–Xba I fragment of $pBSKSN_3$ or pSELECT315(Asp). Plasmid $pBSKSN_3$ encodes wild-type human p53 and pSELECT315(Asp) encodes a mutant human p53 carrying a serine to aspartic acid replacement at codon 315.
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19. Murine RAG-2 cDNA was introduced into pET11c (Novagen); after transfection into *E. coli* BL21(DE3), expression of RAG-2 was induced by IPTG (*38*). Cells were lysed by incubation with lysozyme (100 μg/ml) for 15 minutes at 30°C in 50 mM tris (pH 8.0) and 2 mM EDTA. Insoluble protein was removed by centrifugation at 12,000*g* for 2 minutes. The supernatant was incubated with 100 ng of CTD kinase E2 complex (*20*) at 30°C for 30 minutes in a buffer containing 60 mM KCl, 50 mM tris (pH 7.8), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 200 μM adenosine triphosphate (ATP), and 10 μCi of [γ-^{32}P]ATP (specific activity 6000 Ci/mmol). RAG-2 was immunoprecipitated with Ab435 and isolated by SDS-PAGE. Alternatively, the bacterial sediments were solubilized by boiling in SDS sample buffer and diluted in RIPA buffer; RAG-2 was immunoprecipitated with Ab435 and then phosphorylated by the CTD kinase.
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