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Requirement for ubiquitin conjugation and 26S proteasome activity at an early stage in V(D)J recombination

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Abstract

V(D)J recombination, the process that rearranges gene segments to assemble mature antigen receptor genes, relies on a recombinase comprising the RAG1 and RAG2 proteins. RAG1 is a multi-functional enzyme including DNA binding and cleavage as well as ubiquitin ligase activities, all of which appear to contribute to its role in recombination. Here we demonstrate that components of the ubiquitin conjugation machinery and the 26S proteasome are required for an early step in V(D)J recombination. Inhibitors of the 26S proteasome and ubiquitin activating enzyme (E1) blocked both chromosomal and extra-chromosomal recombination when added one hour following transfection/induction, but they had no effect when added 16 hours later. There was no effect on expression of RAG1, and recombination did not require transit through the cell cycle, confirming that inhibition was not due to an indirect effect on cell cycle arrest or protein expression. Experiments in which RAG1 translation was blocked with cyclohexamide after 16 hours of expression indicated that many active recombination complexes were formed within this window, although recombination products continued to accumulate for 48 hours. These data suggest that ubiquitin-dependent degradation is an early step in complex assembly or activation, and are consistent with our previous hypothesis that degradation of a negative regulator is required to trigger recombination.

Keywords

V(D)J recombination; RAG1; 26S proteasome; ubiquitin activating enzyme; E1

1. Introduction

Recombination of variable, joining and sometimes diversity gene segments (V(D)J recombination) is absolutely required for the maturation of B and T lymphocytes and the generation of a diverse antigen receptor repertoire (Gellert, 2002; Schatz and Spanopoulou, 2005; Tonegawa, 1983). This process is initiated by a lymphoid specific recombinase comprising the RAG1 and RAG2 proteins (Oettinger et al., 1990; Schatz et al., 1989), which together recognize recombination signals sequences (RSSs) flanking a pair of gene segments and introduce a double-stranded DNA break adjacent to each (McBlane et al., 1995; van Gent et al., 1996). General non-homologous end joining DNA repair proteins then join the RSSs to

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form “signal” joints (SJ) and the gene segments are ligated to form “coding” joints (CJ) (Taccioli et al., 1993). Recombinase activity is tightly regulated to restrict recombination to the early stages of B and T cell development and to the correct loci. This is mediated both by regulation of *rag1* and *rag2* gene transcription and targeted degradation of the RAG2 protein (Lin and Desiderio, 1994), which is only present during the G1 phase of the cell cycle. The recombination loci are sequestered in heterochromatin at the nuclear periphery until recombination is activated (Kosak et al., 2002), at which point they become nucleocentric and undergo chromatin modifications specifically recognized by RAG2 (Liu et al., 2007; Matthews et al., 2007; Ramon-Maiques et al., 2007). The RAG1 protein, in addition to its DNA binding and cleavage activities, includes a “RING finger” ubiquitin ligase (E3) domain which is also hypothesized to contribute to regulation (Jones and Gellert, 2003; Yurchenko et al., 2003).

Ubiquitin conjugation is involved in regulation of nearly every biochemical system in eukaryotic cells (Pickart, 2001). Ubiquitin can be covalently attached to a wide range of target proteins through a three-enzyme cascade, including the ubiquitin activating (E1) enzyme, one of a family of ubiquitin conjugating (E2) enzymes, and an E3 (Hershko et al., 1983). The E3 confers specificity on the cascade by selecting the target and delivering it to the ubiquitin-charged E2. Through multiple iterations of this process, the lysine residues on target proteins become modified with poly-ubiquitin chains. In most cases, poly-ubiquitylation leads to degradation of the target by the 26S proteasome (Hershko et al., 1984). Mono- and poly-ubiquitylation can also modify target-protein activity or serve as a scaffold for the assembly of additional factors. The presence of intrinsic ubiquitin ligase activity within the V(D)J recombinase suggests that it may employ auto-regulatory mechanisms.

RAG1 ubiquitin ligase activity is necessary to promote robust V(D)J recombination (Simkus et al., 2007; Simkus et al., 2009a). A mutation that disrupts the RAG1 RING finger in humans results in Omenn syndrome, a B-cell negative primary immune deficiency, indicating that an intact RAG1 RING finger domain is required for normal B cell development (Villa et al., 2001). Amino acid substitutions at the interface between the RAG1 RING and ubiquitin conjugating (E2) enzymes that abrogate E3 activity lead to reductions in recombination activity in cells (Simkus et al., 2009a). RAG1 promotes its own ubiquitylation at several highly conserved lysine residues upstream of the RING finger domain (Jones and Gellert, 2003; Simkus et al., 2009a). Auto-ubiquitylation does not appear to promote degradation of bulk RAG1 in the cell, as abrogation of E3 activity does not stabilize the protein and addition of 26S proteasome inhibitor does not lead to accumulation of ubiquitylated RAG1 (Jones and Gellert, 2003; Simkus et al., 2007). The RAG1 RING finger can promote formation of poly-ubiquitin chains linked in an atypical manner through ubiquitin lysine 11 (K11) in addition to the much more typical K48 (Simkus et al., 2009a). It is not known whether K11-linked ubiquitin chains are recognized by the 26S proteasome or whether they play some other role.

We set about to determine whether the 26S proteasome and E1 enzyme were required for V(D)J recombination. We found that on extra-chromosomal recombination substrates both were required at an early stage, but were dispensable once recombinase complexes had been assembled. For chromosomal recombination at the kappa locus, E1 was required throughout recombination, but the 26S proteasome was again only required for an early step. These data suggest that a ubiquitylation and degradation event must occur for the assembly of recombination complexes, and that an additional ubiquitylation event is required later, perhaps for disassembly or remodeling of the complex after it has cleaved the DNA. This second step may be less critical for recombination of extra-chromosomal substrates.

2. Materials and methods

2.1 Cell lines and culture techniques

RAG1^{-/-}/λ^{-/-} pro-B cells (gift of Dr. Chris Roman, State University of New York, Brooklyn, NY), which are devoid of functional RAG1 (Guloglu et al., 2005), were maintained at 37°C in RPMI-1640 (Mediatech) supplemented with 10% FBS (Gibco), Penicillin/Streptomycin (Mediatech), L-glutamine (Mediatech) and β-mercapto-ethanol. Abelson murine leukemia virus transformed 103/Bcl-2/4 Pre-B cells (gift of Dr. Garnett Kelsoe, Duke University Medical Center, Durham, NC) carry a temperature-sensitive variant of the abl protein (Chen et al., 1994). These cells were maintained at the permissive temperature of 34°C in RPMI-1640 with same supplements as RAG1^{-/-} cells. E36ts20 CHO cells, which carry an E1 temperature sensitive allele (Handley-Gearhart et al., 1994), were grown in MEMα (Invitrogen) supplemented with 10% FBS (Gibco), Penicillin/Streptomycin (Mediatech), L-glutamine (Mediatech) and β-mercapto-ethanol at the permissive temperature of 30°C.

2.2 Trypan blue exclusion cell viability assay

In all cases cells were split 24 hours prior to treatment. At time zero, cells were treated with various inhibitors as described below; after 48 hours cell viability was assessed by trypan blue permeability. Cell suspension was mixed with trypan blue (0.4% solution in PBS, MP Biomedical) at a 1:1 ratio, and the number of total cells and dead cells were counted using in a hemocytometer.

2.3 Inhibitors

The following inhibitors were used: nocodazole (100 ng/ml), aphidicolin (1.6 μg/ml), E1 inhibitor Pyr-41 (40 μM), cycloheximide (40 μg/ml), and *clasto*-lactacystin β-lactone (lactacystin, 5 μM). In order to maintain lactacystin at an effective concentration, the inhibitor was boosted every 12 hours from the time of first treatment.

2.4 Proteasome activity assay

Cells were collected at time zero and every 12 hours just prior to re-treatment to assess proteasome activity. Cell pellets were resuspended in lysis buffer (5mM Hepes pH 7.5, 1mM DTT) and disrupted by shearing through a 25 gauge needle. The protein concentration in each lysate was determined using BioRad Protein Assay dye reagent according to the manufacturer's instructions. Lysates (equalized for protein content) were incubated at with proteasome substrate succinyl-LLVY-AMC (Affiniti Biosystems, London, U.K.) in 20mM Tris (pH 7.4) for 1 hour (37°C), transferred to opaque 96 well plate and read on a PerkinElmer Victor³ 1420 Multilabel Counter at using a 348 nm excitation filter and a 440 nm emission filter.

2.5 Plasmids and recombination assays

pJH548 and pJH549 encode full-length murine RAG1 and RAG2 (Sadofsky et al., 1993); pJH200 and pJH299 are extra-chromosomal recombination substrates in which SJ or CJ, respectively, are left in the plasmid backbone after recombination (Hesse et al., 1987). pJMJ071 encodes full-length murine RAG1 with C-terminal myc and 6-histidine tags (Simkus et al., 2007).

Extrachromosomal V(D)J recombination assays in RAG1^{-/-}/λ^{-/-} cells (5×10^6) were performed with 10 μg of recombination substrate plasmid and 10 μg of RAG1 expression plasmid transfected into the cells by electroporation. All transfection experiments included a condition in which the RAG1 expression plasmid was replaced with pEGFP-N1 (Clontech) thus acting as a negative control for recombination and also a measure of transfection efficiency. Transfected cells were harvested after 48 hours and total DNA was isolated using

Qiagen DNeasy according to manufacturer's instructions. Recombination was analyzed by PCR. Reactions (25 μ l) included recovered DNA (1 μ g), 1 U of KlenTaq LA (Sigma Aldrich), 0.1mM of each dNTP, and 0.1 μ g of each primer together in buffer supplied by the manufacturer. Amplification of SJs on pJH200 were carried out using primers RA14 and RA-CR2 and cycling parameters as described previously (Roman et al., 1997). CJs on pJH299 were amplified using primers RA-CR3 (Roman et al., 1997) and JH290F as described previously (Simkus et al., 2007).

Extrachromosomal V(D)J recombination assays in E36ts20 CHO cells were performed by transfecting both RAG1 and RAG2 expression plasmids along with the substrate plasmid using the Profection Mammalian Transfection System – Calcium Phosphate (Promega). Transfected cells were harvested after 48 hours incubation at permissive (30°C) or non-permissive (42°C) temperatures followed by DNA isolation and PCR analysis as described above.

2.6 Chromosomal recombination at the immunoglobulin kappa locus

V $\rightarrow$ J κ 1 recombination was studied in 103/Bcl-2/4 cells by inducing V(D)J rearrangement at 39.6°C for 48 hours. All experiments included a negative control for recombination that was incubated at 34°C for 48 hours. At the end of 48 hours cells were harvested and isolated DNA was amplified by PCR using primers DAR-14 and DAR-24 (Ramsden and Gellert, 1995) to detect the presence of V $\rightarrow$ J κ 1 CJs. PCR reactions (25 μ l) included recovered DNA (1 μ g), 1 U KlenTaq LA (Sigma-Aldrich), 0.1 mM of each dNTP, and 0.1 μ g of each primer in buffer supplied by the manufacturer. Amplification was preceded by incubation at 94°C for 5 minutes followed by 35 cycles each consisting of 30 seconds denaturation at 94°C, 15 seconds annealing at 62°C and primer extension for 30 seconds at 72°C. A control genomic amplification was carried out for the same samples using primers FabPi-200F (5'-TGGACAGGACTGGACCTCTGCTTTCCTAGA) and FabPi-200R (5'-TAGAGCTTTGCCACATCACAGGTCA TTCAG) and the same cycling parameters. SJ were generated by transfer to 39.6°C for 48 hours followed by return to 34°C for 24 hours. SJs were detected by PCR as described previously (Ramsden and Gellert, 1995).

2.7 Protein expression

RAG1^{-/-}/ λ ^{-/-} cells (2×10^7) were transfected with 20 μ g pJMJ071 and treated with sodium butyrate (5 mM) and forskolin (10 μ M) followed by incubation at 37°C for 24 hours. Full-length RAG1 protein was detected by Western blot of 6M urea-solubilized whole cell lysates using anti-RAG1 antibody K20 (1:2000; Santa Cruz Biotechnology). Anti- β -actin antibody (1:2000, Cell Signaling Technologies) was used as a loading control. RAG1 protein expression in 103/Bcl-2/4 cells (2×10^7) was induced at 39.6°C for 24 hours, whole cell lysates prepared in 6M urea buffer (pH 7.4), and RAG1 protein detected by Western blot as above.

2.8 Statistical analysis

Statistical analysis was performed where indicated by paired student's t-test using publicly available tools provided at <http://www.physics.csbsju.edu/>.

3. Results

3.1 The 26S proteasome is required for an early step in recombination

We used chemical inhibitors to the 26S proteasome to examine its requirements in V(D)J recombination. Our standard recombination assay lasts 48 hours, much longer than the cellular half-life of the proteasome inhibitor lactacystin (data not shown), so conditions were optimized to ensure that the proteasome would remain inhibited for the entire period (see Materials and Methods). To test proteasome activity, cells were harvested every 12 hours, and 26S

proteasome activity in cell lysates was measured using the succinyl-LLVY-AMC substrate (Fig. 1A). We also confirmed that a substantial percentage of cells remained viable after extended proteasome inhibition (Fig. 1B).

The general scheme for all recombination experiments is shown in Fig. 1C. Initially, recombination was examined on extra-chromosomal substrates that allowed for detection of either SJ or CJ. These were transfected into RAG1-negative murine pro-B cells along with a plasmid expressing full-length murine RAG1. When proteasome inhibitor was added one hour following transfection and maintained throughout the 48 hour incubation both SJ and CJ formation were severely reduced relative to carrier-treated control (Fig. 1D and E). We confirmed that lactacystin-treated cells produced RAG1 protein (Fig. 1F) and that lactacystin did not reduce transfection efficiency as measured by expression of GFP from a reporter plasmid (data not shown). This indicates that the reduction in recombination was not due to an indirect effect on protein expression or transfection. V(D)J recombination with full length, wildtype RAG1 and RAG2 proteins is confined to the G1 phase of the cell cycle (Desiderio et al., 1996), with CJ accumulating throughout G1 and SJ appearing at G1/S (Ramsden and Gellert, 1995). The 26S proteasome is required for cell cycle progression (King et al., 1996), so we considered the possibility that transit through the cell cycle after transfection may be required prior to initiation of recombination, and that treatment with lactacystin may prevent an adequate number of the cells from entering G1 and initiating recombination. To test with this, we treated the cells with nocodazole, a inhibitor of microtubulin dynamics that arrests cells in G2 (Jordan et al., 1992). FACS analysis indicated that this level of nocodazole was adequate to fully arrest the cells in G2 (Fig. 1G). Nocodazole had no significant effect on recombination (Fig. 1D and E, gray bars). This indicated that extra-chromosomal substrates could undergo recombination immediately following transfection, and did not have to undergo replication first. Thus inhibition of recombination by lactacystin was not due to an indirect effect on cell cycle progression.

To examine whether the proteasome was required throughout recombination, lactacystin was added at various time following transfection, then maintained for the remainder of the incubation. When lactacystin was added 16 or 24 hours following transfection, no significant inhibition of recombination was detected (Fig. 1D and E). This was not the result of robust recombination prior to treatment. Cells treated at 16 hours then allowed to incubate an additional 32 hours showed significantly higher levels of recombination than those harvested at 16 hours (Fig. 1D and E, cf. black and white bars at each time point). Thus the 26S proteasome was required for an early step in recombination but was dispensable after that.

We made similar observations when measuring recombination of the immunoglobulin kappa locus in 103/Bcl-2/4 cells. These cells are infected with a temperature sensitive Abelson leukemia virus, and carry out high levels of CJ formation at the kappa locus when incubated at non-permissive temperature (39.5°C) (Chen et al., 1994). They also arrest in G1 at this temperature, so SJ formation does not occur until transfer back to permissive conditions (34°C). Addition of lactacystin 1 hour following transition to 39.5° substantially inhibited CJ formation on the kappa locus, but it had a much less severe effect when added 15 hours after induction of recombination (Fig. 2A). The same was true for SJ detected after return to 34°C (data not shown). As with the transiently transfected RAG1, there was no effect of RAG1 protein levels in the 103/Bcl cells (Fig. 2B). To determine the window in which proteasome activity is required, a more detailed time course was performed in which lactacystin was added at 5 hour intervals (Fig. 2A). This revealed a critical window of 10–15 hours, after which the proteasome was no longer required. When inhibitor was added at 15 hours, the amount of product was statistically indistinguishable from the product formed in an uninhibited reaction (Fig. 2A(3)).

3.2 Active recombination complexes are formed in the first 16 hours following transfection

RAG1 has a half-life of about 10–15 min (Grawunder et al., 1996; Sadofsky et al., 1993), so cessation of protein synthesis should rapidly deplete RAG1 protein levels. We used cyclohexamide, an inhibitor of protein synthesis (Obrig et al., 1971), to manipulate RAG1 expression patterns. When cyclohexamide was added one hour after transfection of RAG1-negative pro-B cells, both RAG1 expression and recombination were inhibited (Fig. 3 A–C), confirming that an adequate concentration of cyclohexamide was used to inhibit production of RAG1. When cyclohexamide was added 16 hours following transfection, synthesis and accumulation of RAG1 ceased but recombination was restored. SJ formation under this condition was significantly higher than recombination levels seen in samples that were harvested at 16 hours (Fig. 3A). Cyclohexamide is nonspecific, so all proteins required for recombination must be present in adequate supply by 16 hours post-transfection such that ongoing protein synthesis is not required. This indicates that RAG1 protein expressed in the first 16 hours was able to form an active complex that did not require additional protein synthesis to complete recombination. Thus many active complexes that have not yet completed recombination have assembled within the first 16 hours of the assay, the period during which 26S proteasome activity is required.

3.3 Ubiquitin conjugating activity is required at multiple steps in recombination

We used several approaches to examine the requirement for the E1 enzyme during V(D)J recombination. First, the effects of the chemical E1 inhibitor Pyr-41 on extra-chromosomal and kappa locus recombination was determined in a manner similar to the 26S proteasome inhibition studies. Second, recombination was performed in a CHO cell line with a temperature sensitive E1 allele (Handley-Gearhart et al., 1994). In RAG1-negative pro-B cells, when E1 inhibitor was added one hour following transfection, there was a significant reduction in SJ and CJ formation, but Pyr-41 added at 16 hours was far less inhibitory (Fig. 4A and B). Once again there was no effect on RAG1 protein expression or transfection (Fig. 4D and data not shown). To examine recombination in CHO E1ts cells, a plasmid expressing RAG2 was also included, as these cells do not express RAG2. Recombination carried out under permissive conditions, during which E1 is active, was higher than recombination in cells transferred to non-permissive temperature one hour following transfection (Fig. 4E), in agreement with the Pyr-41 data. Semi-quantitative PCR indicated that recombination under non-permissive conditions was about 7-fold lower than permissive (Fig. 4F). Residual recombination may be due to a leaky phenotype, as we were unable to confirm complete inactivation of E1 (data not shown). This indicated that there was a requirement for ubiquitin conjugation at an early step in recombination on extra-chromosomal substrates, but that this activity was less critical at subsequent stages, just as was seen with the requirement for the 26S proteasome. In contrast to this, Pyr-41 was inhibitory to recombination on the kappa locus and signal joint formation in 103Bcl cells regardless of when it was added (Fig. 4C). This indicated that unlike 26S proteasome activity, ubiquitin conjugation was required for chromosomal recombination at multiple stages.

3.4 DNA replication is required for recombination on extra-chromosomal substrates only

The discrepancy in the requirement for E1 activity at multiple stages for chromosomal recombination, but only for an early stage on extra-chromosomal substrates suggested that resolution of recombination intermediates on the chromosome may differ from those on plasmids. On the chromosome, CJ accumulate throughout G1, but SJ can not be detected until G1/S (Ramsden and Gellert, 1995), raising the possibility that replication is required for SJ but probably not CJ formation. On extra-chromosomal substrates recombination is restricted to substrates that undergo replication (Lieber et al., 1988), but it is unclear whether replication is simply a marker for plasmids that have entered cells or whether it plays a direct role in

recombination or detection of recombined substrates. For example, replication may be required for the plasmid to become accessible to the recombinase, in which case it must precede assembly of the RAG1/2 complex, or it may be required to disrupt the post-cleavage complex or amplify the recombined plasmids to a level that can be detected after harvest. Our observation that nocodazole does not inhibit recombination rules out a pre-assembly role. If plasmids had to undergo replication prior to recombination, they would also have to pass through G2 prior to assembly of the recombinase in G1. Thus nocodazole would be expected to be inhibitory.

We used aphidicolin, an inhibitor of the DNA polymerase alpha (Spadari et al., 1985), to examine the role of DNA replication in recombination of extra-chromosomal substrates and on the kappa locus. Aphidicolin inhibited the formation of CJ and SJ on extra-chromosomal substrates regardless of when it was added (Fig. 5A and B). The level of recombination seen when aphidicolin was added 16 hours following transfection was indistinguishable to the level of recombination in a sample harvested at 16 hours; the same was true for samples treated at 24 hours post-transfection. These data along with the nocodazole data indicate that DNA polymerase activity is either required for recombination or for detection of recombination on plasmid substrates, but it is not required to prepare the plasmid prior to assembly of the recombinase.

In contrast to extra-chromosomal recombination, aphidicolin and nocodazole added one hour post-induction had only a mild effect on recombination on the kappa locus and SJ formation in 103Bc12/4 cells (Fig. 5C). This is particularly significant in the case of SJ, which only form when cells are returned to permissive temperature and re-enter the cell cycle. These data demonstrate that DNA polymerase alpha is not required for recombination on the chromosome or SJ formation associated with chromosomal recombination.

4. Discussion

V(D)J recombination is subject to multiple layers of regulation. We have previously proposed that RAG1's integral ubiquitin ligase activity may promote degradation of a negative regulator (Simkus et al., 2007; Simkus et al., 2009a; Simkus et al., 2009b), thus promoting formation of an active recombination complex. Our data indicate that both ubiquitylation and the 26S proteasome are required at an early phase in recombination. The proteasome is required during a critical 10–15 hour window in which active recombination complexes are formed but is dispensable afterwards. This requirement is not due to indirect effects on transfection, protein expression or cell cycle progression. If the reduction in recombination caused by 26S proteasome inactivity was due to inhibition of some other aspect of cellular metabolism, we would expect that it would occur regardless of when the inhibitor was added. Instead, the complexes go on to carry out recombination during the remainder of the assay even when additional RAG1 synthesis is inhibited by the addition of cyclohexamide. These data are all consistent with a model in which a negative regulator of recombination must be degraded in order for an active complex to be assembled.

The requirement for the E1 in recombination was more complicated. On extra-chromosomal substrates, E1 activity was required only during the initial phase of the reaction, presumably to promote ubiquitylation of the substrate to be degraded. For recombination on the kappa locus, E1 activity was required throughout the reaction. This indicates that ubiquitylation is required at a step subsequent to recombinase assembly when recombination is occurring on the chromosome, but not on extra-chromosomal substrates. After the RAG1/2 complex cleaves the DNA, it remains tightly bound to the cleaved RSS ends, and more loosely bound to the coding ends. The coding ends are rapidly and specifically passed to the NHEJ repair proteins (Lu et al., 2008; Ramsden and Gellert, 1995), while the RSS ends remain sequestered from the

repair machinery (Jones and Gellert, 2001). Hypothetically, remodeling of the post-cleavage complex is required to mediate disassembly and the hand-off to the DNA repair complex. Auto-ubiquitylation of RAG1 has been suggested to play a role in this, and the requirement for E1 activity throughout is consistent with this model. RAG1 promotes ubiquitin chains linked both through ubiquitin K48 and K11 (Simkus et al., 2009a). The physiological function of K11 linked chains is unknown. Like the K63-linked chains seen in DNA repair complexes (Hoegge et al., 2002), they may serve as a platform for assembly of additional factors or serve to attract proteins to remodel the complex rather than as a signal for degradation. Alternatively RAG1-independent ubiquitylation may be required.

If ubiquitylation is required for a late step in recombination such as post-cleavage complex remodeling, why is it not required for recombination of extra-chromosomal substrates? One possibility is that the complex of RAG1/2 with cleaved DNA ends is less stable on extra-chromosomal substrates. This is not likely as these complexes can be isolated from cells without chemical cross-linking, and are active for subsequent biochemical reactions such as RAG1/2 mediated DNA transposition (Jiang et al., 2004). Another possibility is that RAG1/2 post-cleavage complexes can be remodeled by an alternate pathway that does not normally occur on the chromosome. We found that on extra-chromosomal substrates DNA replication is required for a step subsequent to complex assembly. Passage of a replisome or altered replication-dependent supercoiling may be required to disrupt the post cleavage complex on the relatively small plasmid substrate, but play no role is remodeling of the complex on the chromosome.

Additional work will be required to identify the putative negative regulator that must be degraded for assembly of the active recombination complex, and the identity of the target of ubiquitylation later in recombination. We have shown previously that RAG1 promotes both auto-ubiquitylation and ubiquitylation of the KPNA1 nuclear import protein (Jones and Gellert, 2003; Simkus et al., 2009b), and suggested that KPNA1 acts as a negative regulator of recombination. Examination of the requirements for recombination in cells lacking KPNA1 and in the KPNA1^{-/-} mouse will be an important next step in determination of its potential role in regulation of RAG1 activity (Shmidt et al., 2007).

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Fig. 1.

Requirement for 26S proteasome for extra-chromosomal recombination. (A) Cells were treated with lactacystin as described in Materials and Methods, and 26S proteasome activity was determined at the times indicated. (B) Cell viability was assessed by trypan blue exclusion after 48 hours lactacystin treatment. (C) General scheme for all recombination experiments. Cells were transfected or induced to undergo recombination at time 0. For treated samples (filled bars), treatment was added at the time indicated and DNA was harvested 48 hrs. after transfection/induction. For control samples (white bars), DNA was harvested at the time indicated. Recombination assays were carried out on pJH200 (D) or pJH299 (E) as described in Materials and Methods in the absence of inhibitor (white bars) or in the presence of

lactacycstin (black bars) or nocodazole (hatched bars). (B–E) depict average and standard deviation of at least three independent trials. (F) RAG1 and β -actin expression were analyzed by Western blot. Results are representative of multiple trials. (G) cells were treated with nocodazole as indicated and DNA content was determined by FACS analysis. (1) $t = -9.99$ [2], $p = 0.01$. (2) $t = -16.6$ [2], $p = 0.004$. (3) $t = -5.08$ [2], $p = 0.037$.

Fig. 2.

Requirement for 26S proteasome for chromosomal recombination. (A) Recombination on the kappa locus in 103Bcl2-4 cells was analyzed as described in Materials and methods. White bars, cells were harvested after incubation at 39°C for the time indicated. Black bars, cells were incubated at 39°C for the time indicated then treated with 26S proteasome inhibitor then incubated at 39°C for a total of 48 hours. Average and standard deviation of three independent trials are shown. (B) RAG1 and β -actin expression were analyzed by Western blot in induced (39°C) and un-induced (34°C) samples. Duplicate trials are shown. PI, 26S proteasome inhibitor; EtOH, ethanol carrier control. (1) $t = -4.32$ [2], $p = 0.05$. (2) $t = -10.6$ [2], $p = 0.009$. (3) $t = 1.67$ [2], $p = 0.237$.

Fig. 3. Affect of protein synthesis inhibition on recombination. Recombination of pJH200 (A) or pJH299 (B) was assessed as described in Materials and Methods. White bars, untreated cells were harvested at the time indicated post-transfection. Grey bars, cells were treated with cyclohexamide at the time indicated post-transfection, and harvested 48 hours post-transfection. Average and standard deviation of three independent trials are shown. (C) RAG1 (R1) and β -actin expression were analyzed by Western blot. Results of triplicate trials are shown. (1) $t = -4.99$ [2], $p = 0.038$.

Fig. 4. Requirement for Ubiquitin Activating Enzyme (E1) for recombination. Recombination assays were carried out on pJH200 (A) or pJH299 (B) or on the kappa locus (C) as described in Materials and Methods in the absence of inhibitor (white bars) or in the presence of E1 inhibitor PYR-41 (grey bars). Average and standard deviation of three independent trials are shown. (D) RAG1 (R1) and β -actin expression in the presence of PYR-41 or DMSO carrier control were analyzed by Western blot. Results of duplicate trials are shown. (E) Recombination of pJH200 in E36ts20 CHO was assessed at permissive (30°C) and non-permissive (42°C) temperature. SJ-specific PCR product is indicated. (F) Semi-quantitative PCR of recombination products shown in (E).

Fig. 5.

DNA replication is required for recombination on extra-chromosomal substrates.

Recombination assays were carried out on pJH200 (A) or pJH299 (B) as described in Materials and methods in the absence of inhibitor (white bars) or in the presence of aphidicolin (hatched bars). (C) Recombination in 103-Bcl 2/4 cells is shown. Inhibitor was added as indicated 1 hour after shift to 39°C. For CJ (light bars) on the kappa locus, recombination was measured 48 hrs after shift to 39°C. For SJ (dark bars) recombination was measured after 48 hrs at 39°C followed by 24 hrs at 34°C. Recombination is shown as a percent of carrier control, ethanol for aphidicolin and DMSO for nocodazole.