Molecular Immunology 46 (2009) 1319–1325

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Karyopherin alpha 1 is a putative substrate of the RAG1 ubiquitin ligase

Carrie Simkus[a], Michelle Makiya[b], Jessica M. Jones[a,*]

[a] Department of Biochemistry and Molecular and Cellular Sciences, Georgetown University, Basic Science Building Room 329, 3900 Reservoir Rd. NW, Washington, DC 20057, USA
[b] National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Building 50, Room 6533, Bethesda, MD 20892-8009, USA

ARTICLE INFO

Article history:
Received 27 October 2008
Accepted 23 November 2008
Available online 31 December 2008

Keywords:
V(D)J recombination
Ubiquitin ligase
RAG1
KPNA1
Karyopherin alpha 1
SRP1

ABSTRACT

The RAG1 recombinase, which participates in DNA manipulation during rearrangement of antigen receptor genes in developing immune cells, possesses ubiquitin ligase activity. The nuclear transport protein karyopherin alpha 1 (KPNA1) binds to RAG1 upstream of its ubiquitin ligase domain, but this interaction is not required for nuclear localization of RAG1. We found that the isolated ubiquitin ligase domain of RAG1 (amino acids 218–389) promoted ubiquitylation of purified KPNA1. While RAG1 auto-ubiquitylation is dependent on the ubiquitin conjugating enzyme CDC34, ubiquitylation of KPNA1 was best supported by UbcH2/Rad6 and UbcH5a. Ubiquitylation of KPNA1 required the lysine/arginine-rich region spanning RAG1 amino acids 218–263 upstream of the RAG1 ubiquitin ligase domain, but RAG1 was still able to undergo auto-ubiquitylation in this region even in the presence of KPNA1. This is the first putative substrate identified for the RAG1 ubiquitin ligase, and to our knowledge it is the first reported case of ubiquitylation of KPNA1.

1. Introduction

The production of intact antigen receptor genes in B and T cells requires rearrangement of the immunoglobulin and T cell receptor loci on the chromosomes by the process of V(D)J recombination (Tonegawa, 1983). Recombination is initiated by a lymphocyte-specific apparatus comprising the RAG1 and RAG2 proteins (Oettinger et al., 1990; Schatz et al., 1989), which together bind DNA sequences flanking the antigen receptor gene segments and introduce double stranded breaks in the DNA (van Gent et al., 1996). These breaks are subsequently joined by the general non-homologous end joining DNA repair machinery (Taccioli et al., 1993) to form diverse coding joints (CJ), which encode the antigen receptors, and signal joints (SJ), which are frequently lost from the cell. Such a process is inherently dangerous, and many chromosomal translocations associated with lymphoid malignancy arise from inappropriate activity of the RAG1/2 recombinase (Showe and Croce, 1987; Tsujimoto et al., 1985). V(D)J recombination is subject to regulation on many levels, including cell-type and developmentally stage specific expression of the recombinase genes at the transcriptional level (Geier and Schlissel, 2006; Llorian et al., 2007; Verkoczy et al., 2007). In addition, the RAG2 protein is phosphorylated, ubiquitylated and degraded at the G1/S transition (Lee and Desiderio, 1999; Lin and Desiderio, 1993; Mizuta et al., 2002), restricting recombinase activity to the G1 phase of the cell cycle. The zinc-binding RING domain of RAG1 displays ubiquitin ligase (E3) activity (Jones and Gellert, 2003; Yurchenko et al., 2003), but RAG1 is not required for ubiquitylation of RAG2 (Jiang et al., 2005; Lee and Desiderio, 1999; Mizuta et al., 2002). Instead it is hypothesized that RAG1 E3 activity participates in some other aspect of regulation of V(D)J recombination or lymphocyte development.

Ubiquitin conjugation is a multi-step process that participates in regulation of virtually every biochemical system in eukaryotic cells (Ciechanover et al., 1980; Hershko et al., 1980; Pickart, 2004). The small ubiquitin protein is first captured by the ubiquitin activating enzyme (E1) then passed to one of several ubiquitin conjugating (E2) enzymes (Hershko et al., 1983). The E3 assists in the final step of the process, transfer of the ubiquitin moiety to the epsilon amino group of lysine residues on the protein substrate (Hershko et al., 1983), thus it is the E3 that is primarily responsible for specificity. Repeated iterations of the cascade can lead to the formation of poly-ubiquitin chains that most often target the substrate for degradation by the 26S proteasome (Baumeister et al., 1998). In other cases, mono- or poly-ubiquitylation serves as a regulatory signal in a manner similar to phosphorylation or methylation (Pickart, 2000). A purified RAG1 fragment (amino acids 218–389) including the E3 domain when combined with ubiquitin, E1 and the E2 CDC34 promotes its own ubiquitylation primarily at two conserved lysine residues in the basic region just upstream of the RING ((Jones and Gellert, 2003) Simkus C. and Jones J.M. manuscript in preparation). Full length RAG1 undergoes ubiquitylation in intact cells (Jones and

* Corresponding author at: Department of Biochemistry and Molecular and Cellular Sciences, Georgetown University, Basic Science Building Room 323, 3900 Reservoir Rd. NW, Washington DC 20057, USA. Tel.: +1 202 687 2624; fax: +1 202 687 2624.
E-mail address: jonesj5@georgetown.edu (J.M. Jones).

0161-5890/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2008.11.009

Gellert, 2003), but it is not known whether this is auto-catalyzed. To date, no other substrates of RAG1 E3 activity have been identified, but known RAG1-interacting proteins are likely candidates.

RAG1 is a 1040 amino acid protein with multiple domains and the potential to interact with a variety of partners. The above-mentioned basic region and RING domain as well as several cysteine-rich segments comprise much of the amino terminal third of the protein (Bellon et al., 1997; Rodgers et al., 1996), while the multi-domain "core", amino acids 383–1008, covers much of the remainder (Sadofsky et al., 1993; Silver et al., 1993). The core includes both DNA binding and cleavage activities in complex with RAG2 (van Gent et al., 1996). RAG1 has been shown to bind to two cellular proteins in addition to RAG2, karyopherin alpha 1 (KPNA1, a.k.a. importin alpha 5, SRP1 and RAG cohort protein 2; (Cortes et al., 1994)) and karyopherin alpha 2 (KPNA2, a.k.a. RAG cohort protein 1; (Cuomo et al., 1994)), both of which are believed to be involved in nuclear trafficking (Fried and Kutay, 2003). KPNA2 binds to a nuclear localization sequence within the core, and is necessary and sufficient for nuclear import of RAG1 (Spanopoulou et al., 1995). KPNA1 binds to the basic region upstream of the RING, and neither this sequence nor interaction with KPNA1 is required for import (Spanopoulou et al., 1995). The significance of this interaction is unknown.

Import into the nucleus begins with recognition of the target protein by one of the karyopherin alpha family members (Goldfarb et al., 2004). A ternary complex is formed including the target, karyopherin alpha and a karyopherin/importin beta family member. The complex passes through the nuclear pore (Goldfarb et al., 2004), at which point it disassociates in a manner dependent on RAN-GTP (Gilchrist et al., 2002; Gilchrist and Rexach, 2003), releasing the cargo into the nucleus. There is increasing evidence that both karyopherin alpha and beta proteins are involved regulation of nuclear processes outside of their roles in import (Gorjanacz et al., 2002; Kussel and Frasch, 1995; Loeb et al., 1995; Tabb et al., 2000). Since import of RAG1 into the nucleus requires only interaction with KPNA2 (Spanopoulou et al., 1995), it is possible that interaction with KPNA1 may play another regulatory function.

We found that a fragment of RAG1 (amino acids 218–389) comprising the RING and basic region promoted ubiquitylation of KPNA1 in a purified system including ubiquitin, E1 and E2. We have found previously that RAG1 E3 activity is required for efficient recombination by the full length protein when RAG1 is present at relatively low levels (Simkus et al., 2007), and suggested that ubiquitylation of negative regulator may be required to enable recombination. The previously established interaction between RAG1 and KPNA1 *in vivo* along with RAG1's ability to ubiquitylate KPNA1 suggests that it may be that regulator.

2. Experimental

2.1. Materials

2.1.1. Cloning and expression of KPNA1

A clone including the cDNA of KPNA1 (GenBank ID AW245333, AW245690, BC002374) was obtained from the American type Culture Collection (Manassas, VA, USA), and the entire coding region was sub-cloned into the bacterial expression vector pMAL-2cx (New England Biolabs, Boston, MA, USA) downstream and in frame with the maltose binding protein (MBP) protein coding region. Correct reading frame was confirmed by sequencing (Retrogen, SanDiego, CA, USA). This plasmid, pMAL-Kα, was transformed into Rosetta DE3 bacterial cells (Novagen, USA), which were grown in LB medium (0.5 l) supplemented with 100 μg/ml ampicillin to mid-log phase (OD = 0.5). Expression was induced by the addition of IPTG to 1 mM, and allowed to proceed for 4 h. Cells were collected by centrifugation, and resuspended in 10 ml Buffer A (20 mM Tris [pH 7.4], 0.2 M NaCl, 1 mM EDTA, 1 mM DTT) supplemented with 50 μg/ml lysozyme and 1 mM PMSF. After 10 min incubation on ice, the suspension was subjected to a single round of freeze–thaw, and subjected to 5 bursts of sonication (15 s each). Cell debris was collected by high speed centrifugation, and the supernatant (FrI) was loaded onto a 4.6 ml amylose resin column (New England Biolabs, Boston, MA, USA) equilibrated in Buffer A. After extensive washing with Buffer A, bound proteins were eluted with Buffer A plus 10 mM maltose. Fractions containing maltose binding protein-KPNA1 (MBP-KPNA1) were pooled, and a portion (0.5 ml) was loaded onto a Sephadex 200 10/300 GL column (GE Healthcare, USA) equilibrated in Buffer B (20 mM Tris [pH7.4], 0.2 M NaCl, 1 mM DTT). Fractions containing MBP-KPNA1 were pooled and flash frozen in small aliquots.

2.1.2. Additional proteins

RAG1[218–389], RAG1[264–389], and protein-kinase tagged ubiquitin (PK-Ubi) were expressed and purified as previously described (Jones and Gellert, 2003; Simkus et al., 2007). Recombinant human UbcH5a was sub-cloned, expressed and purified as previously described for CDC34 (Jones and Gellert, 2003; Simkus et al., 2007). Leporine E1 and all remaining E2 enzymes were purchased from Boston Biochem (Boston, MA, USA).

2.1.3. Reagents

Chemical reagents were purchased from Fisher Scientific unless otherwise indicated. Anti-MBP antibody was purchased from New England Biolabs. Anti-Xpress epitope antibody was purchased from Invitrogen (USA). Anti-mouse horse radish peroxidase conjugate and SuperSignal West Femto detection reagent were purchased from Thermo Scientific (Rockford, IL, USA).

2.2. Methods

2.2.1. Ubiquitylation

For ubiquitylation of KPNA1, purified MBP-KPNA1 (1 μM as monomer), E1 (45 nM), the E2 indicated (0.3 μM), PK-Ubi (500 μM), and RAG1 (4 μM as dimer) were incubated in reaction buffer (50 mM Tris [pH 7.4], 0.001% Brij, 2 mM Mg-ATP, 50 nM NaCl, 0.4 mM DTT, and 0.02 mM $ZnCl_2$) for up to 16 h at room temperature. Products were separated on 4–12% NuPAGE gels (Invitrogen, USA), transferred to nitrocellulose and subjected to Western blot with anti-MBP antibody (1:10,000), followed by anti-mouse-HRP conjugate (1:10,000), and SuperSignal West Femto detection reagent. ECL was detected and quantified with a Kodak ImageStation 3000 (Kodak, Cambridge, MA, USA).

Ubiquitylation of RAG1[218–389] was performed essentially as described above, except that KPNA1 concentration was increased (8 μM). Products were detected with anti-Xpress epitope antibody as previously described.

2.2.2. Gel Filtration

Equimolar concentrations (80 μM) of KPNA1, RAG1[218–389], or RAG1[264–389] as indicated were combined in 0.2 ml and loaded onto a Sephadex 200 10/300 GL column equilibrated in buffer C (20 mM Tris [pH 7.4], 0.5 M NaCl, 1 mM DTT, 10% glycerol). Protein elution was monitored by absorbance at 280 nm and Western blot. The column was calibrated with standards from Bio-Rad (Hercules, CA, USA).

3. Results

3.1. Ubiquitylation of KPNA1 in a reconstituted system

RAG1[218–389] promoted ubiquitylation of purified maltose binding protein (MBP) tagged KPNA1 (Fig. 1). MBP-KPNA1 was