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Cytohesin-associated scaffolding protein (CASP) is a substrate for granzyme B and ubiquitination

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ABSTRACT

Natural killer (NK) cells are a sub-population of cytotoxic lymphocytes that can kill tumor or infected cells without prior exposure, by secreting the contents of preformed cytotoxic vesicles, containing perforin and granzymes, at the immune synapse. Cytohesin-associated scaffolding protein (CASP) is an adaptor molecule uniquely expressed in lymphocytes that forms complexes with both vesicle-initiating and sorting proteins, and has roles in NK cell migration, cytotoxicity, and cytokine secretion. In this study, we show that CASP contains a conserved granzyme B cleavage site that could modify its intracellular localization and interaction with sorting nexin 27. We also provide evidence that CASP is modified by ubiquitination. Both of these post-translational modifications could rapidly modify CASP function and highlight the dynamic regulatory mechanisms that direct its role in NK cell functions.

1. Introduction

Cytohesin-associated scaffolding protein (CASP, a.k.a. CYBR, CYTIP, PSCDBP, B3-1) is a lymphocyte-specific protein that was first identified and cloned in our laboratory from human NK cells using subtractive hybridization with a helper T cell line [1]. The CASP gene encodes an expected 40 kDa (359 aa) final protein product.

CASP is an adaptor protein, lacking any catalytic domains but containing several protein–protein interaction domains/motifs. CASP has been found to have direct binding interactions with three proteins. Most of what is known about CASPs intracellular function is attributed to its first identified binding partner cytohesin-1. We were also the first to clone and identify this protein (B2-1, a.k.a. cytohesin-1) from the same natural killer cell-subtracted library [2]. CASP interacts with cytohesin-1 through the coiled-coil motifs of both proteins [3]. CASP is also able to bind, through its coiled-coil motif, to other members of the cytohesin family (ARNO/cytohesin-2 and ARNO3/GRP1/cytohesin-3) [3]. Cytohesin also contains a Sec7 domain that was previously discovered in yeast [4]. This domain was later shown to bind to ADP-ribosylation factors (ARFs) allowing the ARF GDP to be exchanged for GTP causing the ARF to bind to membranes at sites that are rich in phosphoinositols [5]. In addition, CASP contains a PDZ binding motif (PDZbm) at its carboxyterminus [6]. We showed that this PDZbm of CASP interacts with sorting nexin 27 (SNX27) and is polarized at the immune synapse [6,7]. SNX27 is also involved in recycling from the early endosome to the plasma membrane [8]. The CASP domain structure and its known interactions support a role in the assembly of larger protein complexes which are suspected to be involved in intracellular trafficking and signaling [9]. We have recently shown that CASP is also involved in NK migration, cytotoxicity, and cytokine secretion [10].

CASP is rapidly degraded in cells infected by herpes simplex virus type 1 causing major alterations in cell adhesion and migration [11]. It was also shown that CASP is ubiquitinated in dendritic cells, a report that was published while we were researching CASP ubiquitination in NK cells [12]. It was determined that the suppressor of cytokine signaling 1 (SOCS-1) is also interacts with CASP, by bringing CASP to the proteasome for degradation. In that study, yeast-2-hybrid and co-immunoprecipitation with CASP are used to show that SOCS-1 is present, however delineation of the specific domains mediating the interaction are not described [12].

Granzyme B (GrB) is one of the major components of secretory granules found in cytotoxic T cells (CTL) and NK cells [13,14]. This serine protease cleaves after aspartic acid residues and activates several caspases involved in apoptosis [14]. There appears to be several pathways to GrB-induced killing, either involving direct activation of caspases or the initiation of mitochondrial permeabilization [14,15]. The exact mechanism of GrB entry into target cells has also been controversial in recent years. It was shown that the

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release of perforin during killing activates target cell endocytosis [16]. GrB along with perforin become internalized in large endosomal vesicles [17]. GrB then escapes through the perforin pores in these vesicles thus triggering apoptosis [17].

Here we present findings that CASP is specifically cleaved by GrB. CASP is also shown to be ubiquitinated and targeted to the proteasome for degradation in NK cells through two potential lysine residues. CASP may also be monoubiquitinated suggesting a role in endocytosis. These post-translational modifications likely contribute to the function of CASP in cytolytic NK cells.

2. Methods

2.1. Lysates

Cell lines were grown (~1×10^7 cells/mL) and then lysed with lysis buffer which contained 50 mM Tris (pH 7.5), 5 mM EDTA, 300 mM NaCl, 1% Triton X-100, and a cocktail of protease inhibitors (Roche). Cells were sheared using a 23 guage syringe and were then sonicated. The sample was then centrifuged at 10,000 RPM for 10 min and the supernatant was recovered and protein concentration determined.

2.2. Granzyme B inhibition

1×10^7 cells/ml of NK92 were suspended in a 6 well plate (BD) in 1 ml of Il-2 media supplemented with 2 μM GrB inhibitor II (Z-AAD-CMK, in DMSO) or (6-Aminohexanoic acid, Antipain, Aprotinin, Benzamidine HCl, Bestatin, Chymostatin, E-64, EDTA, N-ethylmaleimide). The cells were incubated at 37 °C, 5% CO_2 for 3 h. After the incubation, the cells were then washed in serum free media and harvested as per lysate protocol found above. For *in vitro* protease activity, 50 μg of cleared (killer cells: YT or NK-92, and other non-cytotoxic cells: Jurkat, Raji, or K562) total lysate, or 1 unit of purified GrB (Calbiochem) in PBS-TX was incubated with 0.5–2 μg of recombinant GST–CASP [6] bound to glutathione beads for 1 h. Beads were washed with PBS-TX five times. Remaining bound protein was electrophoresed and stained.

GrB target sites were predicted with GraBCas online software.

2.3. Western blot

NK92 protein lysates (30 μg) were prepared from the cell lines and probed with antibodies as previously described [10]. The anti-CASP primary antibodies (1:2000 anti-PSCDBP Epitomics or 1:5000 PDZ2F9 rat anti-sera) were then added to the blot and shaken for 2 h.

2.4. Co-Immunoprecipitation (C0-IP)

Cells were lysed with the co-immunoprecipitation (CO-IP) lysis buffer, which contained 50 mM/L Tris (pH 7.5), 5 mM/L EDTA, 300 mM/L NaCl, 1% Triton X-100, 1 mM/L PMSF, 10 μg/mL aprotinin, 1 μg/mL leupeptin, Tyr phosphatase cocktail I, and Ser/Thr phosphatase cocktail II. Cells were sheared by brief sonication, and cellular debris was removed by centrifugation at 10,000*g* for 10 min. Aliquots (1 mg/ml) of lysates were incubated with anti-PDZ 2F9 overnight at 4 °C, and then for 2 h at 4 °C with 20 μL Protein A/G PLUS-Agarose. Immune complexes were collected by centrifugation and washed three times with ice-cold phosphate-buffered saline (PBS). Pellets were resuspended in SDS lysis buffer, and then boiled in 4× SDS loading dye for 5 min. Protein was separated by SDS–PAGE and transferred on PVDF membranes. Membranes were then probed with anti-Ub (P4D1) (1:2000, Santa Cruz).

3. Results

In our recent work documenting CASP protein knockdown in NK92 cells [10], we conducted confirmatory experiments toward determining the specificity of available CASP antibodies. After testing every commercially available antibody (mono and polyclonal) and our own rabbit polyclonal anti-CASP antibodies in a variety of immune cell types known to express the CASP transcript, we consistently detected signals at multiple protein sizes, rather than a single band near the predicted size of ~40 kDa. Although the majority of results showed a prominent 40 kDa band, many also had bands of higher and lower molecular weights, with the most consistent additional bands at approximately 95, 70, 29 and 10 kDa. Trials with monoclonal (PDZ-2F9) or polyclonal antibodies targeting the N-terminus of the protein (PSCDBP), we consistently detected multiple bands in NK92 lysates (Fig. 1). Despite inherent limitations, current convention when reporting western blot results in the literature is to crop blot images and display only bands at the desired size, and as a result, these additional potential CASP-specific bands have gone unreported. However, the consistency of the multiple signals seen with antibodies targeting different epitopes on the CASP protein suggest that these additional bands may indeed be specific and support possible post-translational modifications of CASP, with bands >40 kDa representing additions such as ubiquitination for example, and smaller sized bands potentially due to proteolytic cleavage events.

3.1. CASP is cleaved by granzyme B

Following incubation of recombinant GST–CASP protein with several cell lysates we noticed a consistent 10 kDa truncation product from the C-terminus of CASP. We then employed a protease inhibitor panel to examine the specificity of this cleavage. As shown in Fig. 2A, only antipain and aprotinin inhibit the cleavage of recombinant GST–CASP. Both antipain and aprotinin are serine protease inhibitors. To determine if the cleavage may be due to acidic lysosomal proteases we tested its pH optimum. As shown in Fig. 2B, the cleavage was only evident at pH 7–9 and not below pH 6. We ruled out that this cleavage was due to lysosomal proteases which are acid hydrolases (pH 5 optimum). This is indicative of the putative protease activity on CASP taking place within a physiological pH range. Additionally, 1% SDS treatment inhibits cleavage (not shown).

We suspected that the cleavage of recombinant GST–CASP may be mediated by granzyme B (GrB) since the activity could only be detected in cytotoxic cells. As shown in Fig. 2C, we only detected specific cleavage in YT lysates but not upon incubation with non-cytotoxic cells (eg. K562). We also detected cleavage of recombinant GST–CASP using lysates from other cytotoxic cells (NK92,

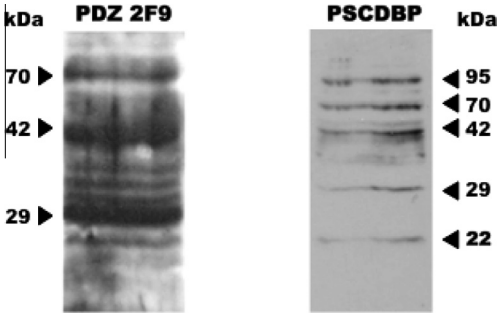

Fig. 1. Characteristic western blot banding pattern results when probing for CASP in NK92 cell line lysates. Monoclonal rat antisera (PDZ 2F9) was used in western blotting experiments to detect CASP in NK92 cell lysates. PSCDBP is a commercially available rabbit polyclonal antibody. In each blot, consistent bands are evident at 70, 42, and 29 kDa. Representative blots are shown.

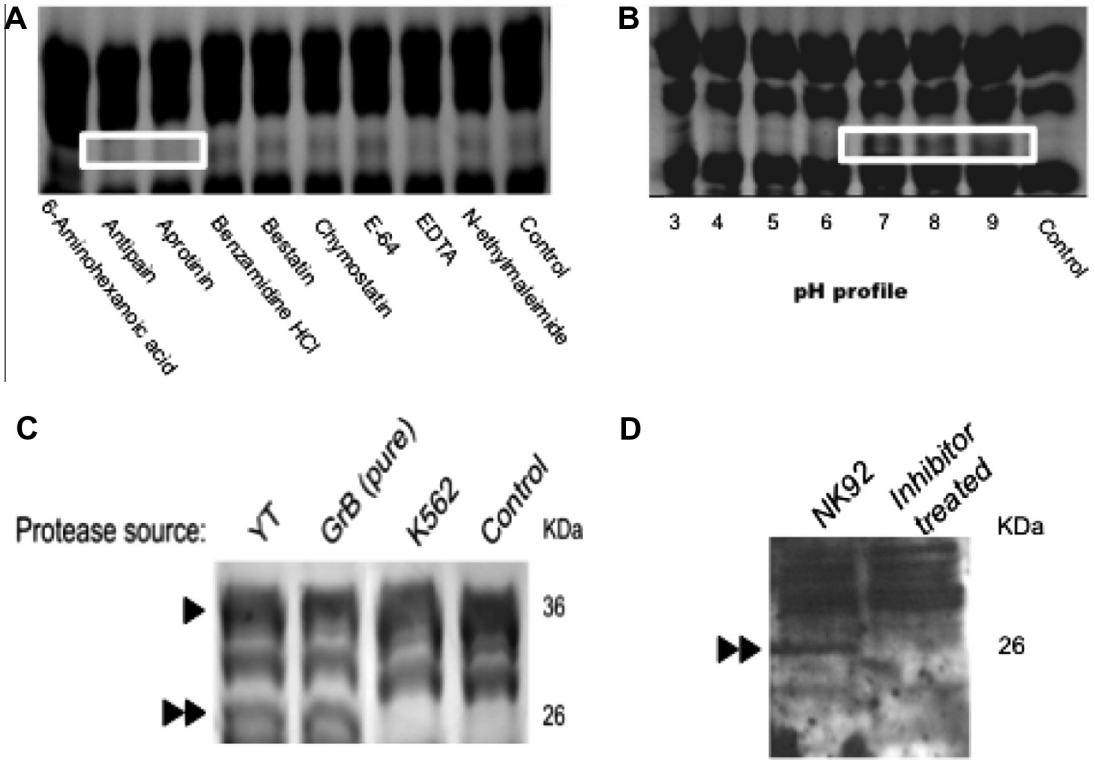

Fig. 2. CASP can be proteolytically cleaved by granzyme B. (A) Protease inhibitor panel. Serine protease inhibitors antipain and aprotinin inhibit GrB cleavage of recombinant GST–CASP when incubated with YT cell lysates. Control is GST–CASP without any inhibitor. (B) Cleavage of GST–CASP is dependent on pH. Lysates of varying pH levels show that CASP cleavage by GrB in within the range of pH 7–9 (indicated with box). Control is GST–CASP protein with YT lysate at pH 7.8. (C) Recombinant CASP is cleaved by granzyme B *in vitro*. GST–CASP protein bound to glutathione beads has approximately 10 kDa removed from the carboxy terminus when incubated with granzyme containing lysates (YT cells) and with purified GrB protein. No protease activity is observed in control cell types (e.g. K562). Single arrow indicates full length recombinant CASP. Double arrows indicate recombinant CASP with the carboxy-terminus removed. Bands between the two arrows are truncated recombinant CASP from the original purification, not products of the *in vitro* reaction. Additional bands are truncation products due to bacterial recombinant production, not due to cleavage. (D) Western blot depicting the proteolytic cleavage of endogenous CASP by granzyme B. The blot was probed with the polyclonal PSCDBP antibody (Epitomics) targeting the N-terminal region of mature CASP protein. In the granzyme B inhibitor treated sample, the 29 kDa product is not present, indicating that endogenous CASP is cleaved by granzyme B.

YTS), but not in other non-cytotoxic lines (Jurkat, Raji, CCRF–CEM) (results not shown). Furthermore, purified recombinant GrB also cleaves GST–CASP (Fig. 2C lane B) giving the identical size product as with cytotoxic lysates indicating that GrB is responsible for this cleavage. Finally, a specific GrB inhibitor II (Z-AAD-CMK) inhibits the cleavage of CASP in NK92 cell lysates (Fig. 2D). This smaller fragment corresponds to the remaining 30 kDa that appears in the western blot when probed with the PSCDBP polyclonal antibody that targets the N-terminus of CASP (Fig. 2D). These results strongly suggest that both native and recombinant CASP is cleaved by the protease GrB.

This cleavage removes approximately 10 kDa from the carboxy terminus of CASP. Using the bioinformatics tool GraBCas [18], the predicted and only GrB cleavage site for CASP is CVSED | SS, consistent with the location of the cleavage product originally detected (Fig. 3A). The GrB cleavage site is conserved amongst primate species, however varies amongst other vertebrates (Fig. 3B). Interestingly, the CASP-related (by gene duplication [19]) protein GRASP does not have a GrB proteolytic cleavage site when analyzed with GraBCas (Fig. 3C). Unlike CASP, GRASP is a neuronal specific protein [20].

3.2. CASP is ubiquitinated and targeted to the proteasome for degradation

To examine the possibility that CASP is ubiquitinated, NK92 cell lysates were incubated with the polyclonal rat anti-PDZ 2F9 antisera and immunoprecipitated with protein A/G coated agarose beads. The eluate was examined by western blotting and probed for ubiquitin to reveal a band at 48 kDa (Fig. 4), indicating ubiquitination of CASP by potentially 2 ubiquitin subunits. The 29 kDa band could be due to cross reactivity of the secondary antibody with the PDZ 2F9 rat antisera, because the ubiquitin antibody is from mouse. The blot was then stripped and re-probed for CASP. Bands at 68 kDa, and two or three additional minor bands between 95 kDa and 50 kDa (Fig. 4) were apparent. This suggests that CASP is found with 1 (near 44 kDa), 2 (48 kDa), 4 (68 kDa) or more ubiquitin subunits attached.

Degradation of CASP is also inhibited when treated with the proteasome inhibitor Bortezomib (Fig 4). The boron atom of Bortezomib binds with high specificity to the catalytic site of the 26S proteasome, preventing targeting of proteins that have been ubiquitinated. The large smear seen in the anti-ubiquitin lane of the bortezomib treated cell population indicates the accumulation of multi-ubiquitinated CASP, which is not degraded due to the fact that the proteasome cannot cleave and recycle the protein due to inhibition (Fig. 4). This result is consistent with the recently reported result by Grabher et al. [12]. Bioinformatic analysis for ubiquitination motifs on mature CASP protein sequences show two potential sites, at lysine residues 247 and 288 (Fig. 3B–C). These sites flank the predicted cleavage site by GrB. Upon comparison of CASP variations amongst vertebrates, both the ubiquitination sites appear to be conserved to a high degree (Fig. 3B).

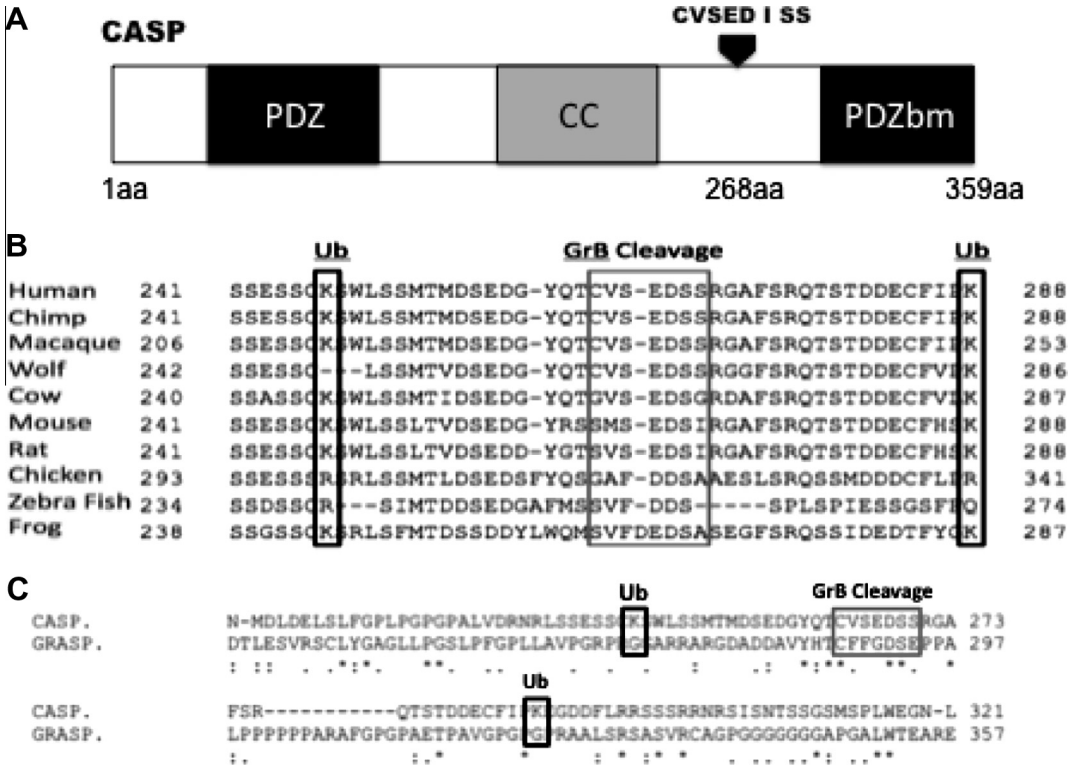

Fig. 3. Identification and conservation of granzyme B cleavage and ubiquitination sites within the CASP sequence. (A) Predicted CASP proteolytic cleavage site by granzyme B (GrB). Bioinformatics tool GraBCas predicts a significant ($p < 0.05$) site for granzyme B cleavage of CASP at amino acid 268, CVSED | SS. This is consistent with experimental findings using recombinant CASP as a target for pure granzyme B–containing lysates or purified GrB. (B) Alignment of vertebrate CASP protein sequences showing the predicted ubiquitination and GrB cleavage region. The GrB cleavage region is highly conserved amongst vertebrate species and those containing sites are highlighted. The lysine residues targeted for ubiquitination are conserved in most species, indicating that this site has retained a common functional binding affinity throughout vertebrate species. (C) CASP and GRASP protein sequence alignments depicting that the GrB and ubiquitination sites are not conserved.

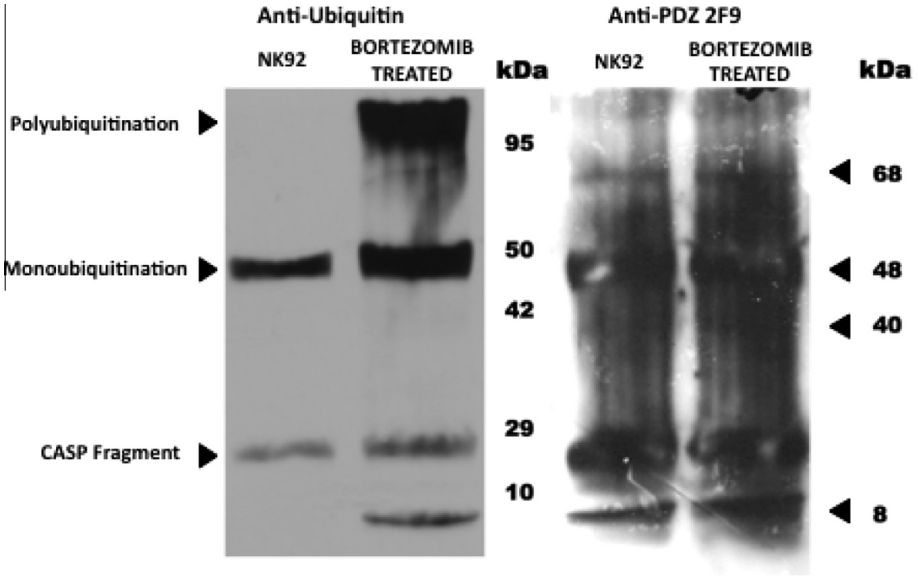

Fig. 4. CASP is ubiquitinated in NK92 cells. Co-immunoprecipitation of CASP from NK92 lysates in untreated conditions and with proteasome inhibition by bortezomib. Anti-ubiquitin probing shows high molecular weight signal when treated with bortezomib, indicating the accumulation of CASP marked for degradation. The 10 kDa band is indicative of a single ubiquitin monomer (about 8.5 kDa). The 50 kDa band seen in the untreated NK92 lysate corresponds to a potential multiubiquitination of GrB-cleaved CASP (potentially 2 subunits added to the 30 kDa cleavage product resulting in a ~48 kDa). PDZ 2F9 antisera shows multiple banding typical of the 68, 48, 40 and 29 kDa forms of CASP in both treated and untreated NK lysates.

4. Discussion

When NK cell line lysates are probed with anti-CASP antisera by western blotting, multiple CASP bands are evident indicating several post translational modifications. The larger CASP modifications are likely due to the ubiquitination of the protein, whereas the smaller CASP protein (below 40 kDa) are due to GrB cleavage. Recombinant or native CASP is cleaved by lysates from

cytotoxic lymphocytes (NK92, YT, YTS) but not by those of non-cytotoxic cell lysates. Serine protease inhibitors and a specific GrB inhibitor also prevent this cleavage of CASP. GrB cleavage consistently removed a 10 kDa fragment from the C-terminus of native or recombinant CASP. In this region GraBCas predicts a GrB proteolytic cleavage site. This proteolytic processing of CASP would eliminate its interaction with SNX27 by removal the PDZbm. The effect of this cleavage may also eliminate the recruitment of CASP to endosomes. Removing the CASP PDZbm and transfecting this construct into cells changes CASPs intercellular localization from vesicular/endosomal to diffusely cytoplasmic (unpublished, not shown). Our laboratory has reported on the active polarization of endosomal SNX27 during lymphocyte migration and tumor cell engagement, indicating that this CASP binding partner plays a role during several polarized events in lymphocytes [7]. The highly limited and regulated expression of CASP in immune cells, coupled with its role in reducing cell adhesion [11] and interaction with the polarizing protein SNX27, suggests an even more diverse role of CASP-related endocytic pathways associated with lymphocytes. An emerging subject of interest in molecular immunology is deciphering the role played by proteins of the endocytic pathway in polarized events such as active maintenance of the immunological synapse in cytotoxic lymphocytes, as well as other polarized events such as migration. Recycling of receptors involved in both activation and adhesion are central to maintaining polarity both as lymphocytes navigate their microenvironment and in communication with antigen-presenting cells.

This disruption of CASP function may be important in apoptosis of hematopoietic cells and/or the destruction of NK cell lymphomas. Studies have shown GrB leakage into the cytosol [21]. In nasal-type NK/T cell lymphomas, GrB leakage into the cytoplasm is a crucial mechanism for apoptosis [22]. GrB cleavage of CASP may act as a fail-safe mechanism to prevent cancerous NK cells from proliferating. If GrB is released into the cytoplasm, it would cleave CASP and may prevent the release, recycling, or maturation of cytotoxic granules, due to the fact that the cell has become compromised, thus helping to limit undesired off-target killing. In addition to GrB being involved in T cell apoptosis, continuous stimulation of cytotoxic lymphocytes cause stress to the cells resulting in GrB release into the cytosol [23]. It was speculated that this GrB release may limit the lifespan of cytotoxic lymphocytes under continuous stimulation. It is also well known that chronic inflammation and repair causes GrB to be found in the extracellular fluids [24–26]. The incidental uptake of extracellular GrB by cytotoxic lymphocytes may result in CASP cleavage and also the cleavage of other cytosolic proteins (e.g. Tubulin [27]), inhibiting their cellular functions.

In addition to targeting proteins to the proteasome for degradation by polyubiquitination, monoubiquitination has been shown to have a role in the endocytosis of membrane bound proteins [28]. Ubiquitin comprises a three dimensional internalization signal that can be appended to proteins destined for downregulation. This is interesting, because the CASP binding partner SNX27 also has a role in the recycling of receptors from endosome to the plasma membrane [8]. The ubiquitin internalization signal located on a membrane bound protein may recruit an adaptor protein such as CASP, that could promote endocytosis. CASP could have a role in the localization of endocytic cargo (such as recycled effector molecules like perforin and granzymes) into subdomains of the plasma membrane competent for vesicle budding or might be part of the vesicle budding machinery through its interactions with cytohesins and SNX27. If CASP is indeed monoubiquitinated, the ubiquitin internalization signal could target endocytic cargo and CASP would be involved in assembling a complex that is necessary for the budding of primary endocytic vesicles.

The two modifications (GrB and ubiquitin) may be regulating each other as well. When CASP is cleaved by GrB, it also loses a potential site of ubiquitination (Fig. 3B and C). This could facilitate the regulation of CASP by being targeted to the proteasome for degradation. CASP would lose ability to bind to SNX27, but would still be able to interact with cytohesin through its coiled coil motif. Conversely, ubiquitination of CASP at the tail-end ubiquitination site could inhibit CASP cleavage by granzyme B.

The conserved sites for ubiquitination and GrB cleavage amongst vertebrates also suggests a value to these functions of modifying CASP. Fish CASP peptide sequence is the most divergent of the vertebrates, however, there has been a novel granzyme identified in fish that has a completely different target site than that of GrB [29].

GrB cleavage of CASP and its ubiquitination highlight the intricate intracellular regulation of CASP, and suggest important roles for CASP in specialized immune cell functions. The post-translational modifications we document here, provide further evidence toward the dynamic role of CASP in the mechanics of natural killer cell function.

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