The Immune System GTPase GIMAP6 Interacts with the Atg8 Homologue GABARAPL2 and Is Recruited to Autophagosomes

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Chemical cross-linking studies performed endogenously, indicated that the two protein normal conditions. The GIMAP6-GABARAPL2 (chromosome 7q36.1 in humans). The mammalian GIMAP family can be divided into members either with (GIMAP1, 2, 3 and 5) or without (GIMAP4, 6, 7, 8 and 9) predicted transmembrane domains near to their carboxy-termini: none of the members expresses known sequence motifs permitting post-translational lipid modifications, such as prenylation or

palmitoylation, that might mediate dynamic membrane associations.

Genetic association studies have implicated *GIMAP* genes in autoimmune diseases *viz*. systemic lupus erythematosus [6,7], Behçet's disease [8] and type 1 diabetes [9]. Consistent with an involvement in autoimmunity, previous work has implicated the GIMAPs in the control of lymphocyte survival. A spontaneous mutation of *GIMAP5* in rats [10-13], as well as both a mutation and a targeted deletion of *GIMAP5* in mice [14,15], produce severe peripheral lymphopenia in the T lymphocyte lineage. Similarly, a conditional lymphocyte-specific deletion of *GIMAP1* results in severe T lymphopenia; however, in contrast to *GIMAP5* mutants in which B cell numbers are relatively normal in young mice (although they reduce in older animals), these GIMAP1 conditional knockout mice show a profound B lymphopenia even in young animals [16]. The pro-survival activity of these proteins is in contrast to the pro-death activity reported for GIMAP4 in mice [17] and rats [18].

Little is known about the molecular mechanisms by which the GIMAPs influence lymphocyte survival. Findings, indicating that some GIMAP proteins can interact with members of the Bcl-2 protein family [1] and that GIMAP5 may exercise its pro-survival properties by stabilising Mcl-1 [19], suggest that the GIMAPs may provide an extra level of apoptosis regulation special to lymphocytes.

In order to extend knowledge of the molecular interactions mediating GIMAP function, we have taken a biochemical approach to identifying *in vivo* binding partners for the GIMAPs. Here we present data that (i) identify GABARAPL2 (also known as GATE-16), a mammalian homologue of the yeast autophagy-related gene Atg8, as a major binding partner of GIMAP6 and (ii) demonstrate the relocation of GIMAP6 to autophagosomes in response to cell starvation or mTOR inhibition.

Materials and Methods

Materials

BirA expressing cell lines were identified by Western blotting (see below). A single myc-BirA-Jurkat cell line was selected for subsequent use.

b) Biot-GIMAP6-His myc-BirA Jurkat cell line. The myc-BirA-Jurkat cell line was maintained in complete medium containing 500µg/ml G418. Approximately 10^7 cells were transfected by electroporation with 20 µg of either *Pvu*I-linearized plasmid biot-GIMAP6-His-pCAG-iPuro or the corresponding vector. Cells were allowed to recover overnight in complete medium containing 500 µg/ml G418. The following day, the cells were spun down and resuspended in 20 ml of complete medium containing 500 µg/ml G418 and plated at 100 µl/well into 96-well plates. The next day an equal volume of complete medium containing 500 µg/ml G418 and 6 µg/ml puromycin was added to each well. Every three days thereafter, half of the medium was replaced with fresh complete medium containing 500 µg/ml G418 and 3 µg/ml puromycin. Cells carrying the parental pCAG-iPuro vector were isolated in parallel, as controls. Biot-GIMAP6-His expressing clones were identified by Western blotting of cell lysates with a horseradish peroxidase-conjugated streptavidin probe. A single vector clone and a single clone carrying Biot-GIMAP6-His [illegible]

20,000 g for 30 min at 4°C. The pellet was washed with 1ml acetone and re-centrifuged as before. The supernatant was aspirated and the pellet dried briefly (30 s) in a 100°C hot block. The pellet was then dissolved in 50 µl 2 x CSB, left at room temperature for 2h and then heated to 100°C for 3 min. Samples were then separated on a 12.5% SDS-PAGE gel and the proteins revealed by staining with Imperial protein stain (Thermo Scientific). Bands of interest were excised and one half of each was reduced, carbamidomethylated and digested overnight with trypsin (Promega sequencing grade, 10 ng/µl in 25 mM ammonium bicarbonate). Approximately 10% of the resulting tryptic digest was analysed by LC-MS/MS. LC separation was achieved on a reversed-phase column (Reprosil C18AQ, 0.075 x 50 mm, 3µm particle size), with an acetonitrile gradient (2 - 35% over 10 min, containing 0.1% formic acid, at a flow rate of 500nl/min). The column was coupled via a nanospray ion source (Proxeon) to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) operated in data-dependent acquisition mode. The acquisition cycle consisted of a high resolution precursor ion spectrum over the m/z range 350 - 1500, followed by up to 5 CID spectra. Mass spectrometric data were processed using Proteome Discoverer (Thermo) and searched against the human entries in Uniprot 15.14 using Mascot software (Matrix Science). m

Figure 1. GIMAP6 interacts with GABARAPL2 in mammalian cells and *in vitro*. A) Jurkat T-cells engineered to over-express myc-tagged BirA and GIMAP6 carrying a biotinylation target sequence were incubated with or without 1% (v/v) formaldehyde at room temperature for the indicated times. Cell lysates were analysed by Western blotting with a streptavidin-HRP conjugate to reveal biotinylated proteins. The mobilities of biotinylated GIMAP6 and the cross-linked species (X-linked species) are shown. The electrophoretic mobility of molecular weight protein standards run in parallel is indicated. Note that the gap between the 15 and 60 min samples indicates that intermediate tracks have been removed. B) Silver-stained SDS-PAGE gel of purified biotinylated GIMAP6 and co-purifying proteins from myc-birA Jurkat cells stably transfected with either plasmid biot-GIMAP6-His-pCAG-iPuro (Lane 2) or the corresponding vector (Lane 1). The electrophoretic mobilities of biotinylated human GIMAP6 and GABARAPL2 are shown, as are those of molecular weight protein standards run in parallel. The asterisks (*) indicate the location of streptavidin released from the streptavidin-agarose beads. C) The sequence of GABARAPL2. Sequence coverage detected in tryptic peptides by the mass spectrometry analysis are shown in bold and underlined. D) HEK293T cells (3×10^6) were transfected with a plasmid (10 µg) encoding myc-tagged human GIMAP6 together with a plasmid (10 µg) encoding either an HA-tagged GABARAPL2 or the corresponding vector. Post-nuclear supernatants were then either directly separated by SDS-PAGE (lysate) or immunoprecipitated with anti-HA mouse mAb 12CA5 and protein-A Sepharose prior to SDS-PAGE (anti-HA IP). Separated proteins were analysed by Western blotting using either anti-HA mouse mAb 12CA5 or anti-myc mAb 9E10 followed by HRP-conjugated goat anti-mouse IgG. E) Jurkat T cells (approximately 9×10^7) were incubated in PBS with or without 1% (w/v) formaldehyde for 1 h at room temperature. The reaction was terminated by adding $1/10^{th}$ volume of 1.25 M glycine, and cells solubilised into 200 µl TX100 lysis buffer containing mammalian protease inhibitors (Sigma). After centrifugation (20000 g, 5 min, 4°C) an equal volume of 2 x CSB was added and the samples either heated at 100°C (boiled) for 30 min to reverse the formaldehyde-induced cross-links or left untreated. Aliquots of the boiled and unboiled (untreated or cross-linked) lysates were then separated on SDS-PAGE gels and then Western blotted, using rat mAb MAC445 to human GIMAP6 - (left panel) or rat mAb MAC446 to GABARAPL2 (right panel) followed by horse-radish peroxidase (HRP) conjugated goat $F(ab')_2$ fragment anti-rat IgG. Blots were then developed using Immobilon ECL western blotting substrate. Cross-linked$ vroq fragmentÈmen אl פ men .

Figure 2. Specificity of GIMAP6-GABARAPL2 interactions. A) HEK293T cells were transfected with 10 µg GABARAPL2 in pcDNA3Biot1His6iresBirA and 10 µg myc-tagged human GIMAP-encoding plasmids as indicated. 48 h later, cell lysates were prepared and biotinylated GABARAPL2 and associated proteins recovered on streptavidin-agarose beads. Aliquots of the lysates

Although these results showed a requirement for the C-terminal region of the protein, when we introduced mutations into the GTPase domain of GIMAP6, we could show that this region too was important. Introduction of mutations into the

Figure 4. GIMAP6 over-expression leads to the induction of GABARAPL2. A) Cells lysates were prepared from three myc-GIMAP6 HEK293 cell lines (lanes 3-5) or two cell lines carrying the corresponding vector (lanes 1-2) and expression of GIMAP6, GABARAPL2, and β-actin analysed by Western blotting as indicated. B) Three T-Rex™-HeLa cell lines carrying plasmid pcDNA4.TO (lanes 1-3) or three myc-GIMAP6 T-Rex HeLa cell lines (lanes 4-6) were grown in the presence or absence of 2 μg/ml tetracycline for four days. Cells lysates were prepared and analysed for myc-GIMAP6, GABARAPL2 or β-actin expression, as indicated, by Western blotting. C) A myc-GIMAP6 T-Rex Hela cell line was grown for various times in the presence or absence of tetracycline. After 4 days, some dishes of cells that had been grown in the presence of tetracycline were extensively washed and maintained in the absence of tetracycline for further time intervals. At each time-point, cell lysates were prepared and analysed for GIMAP6, GABARAPL2 or β-actin expression (with primary antibodies: rat anti-GIMAP6 monoclonal antibody MAC 445, rat anti-GABARAPL2 MAC446, and mouse anti-β-actin monoclonal antibody AC-15 respectively, followed by the corresponding HRP-conjugated second antibodies) by Western blotting. The experiment was performed on both clones 5 and 6 from panel B with similar results – that from clone 6 is shown. D) A myc-GIMAP6 T-Rex HeLa cell line was incubated in the presence (plus) or absence (minus) of tetracycline for four days. Lysates were prepared, separated by SDS-PAGE, and Western blotted for the expression of GIMAP6 (using rat monoclonal antibody, MAC445), GABARAPL2 (rat monoclonal antibody, MAC446), or other Atg8 members and SQSTM1, using antibodies as detailed in the Materials and Methods section. The results shown are representative of two independent experiments. E) Total RNA was isolated from a myc-GIMAP6 T-Rex HeLa cell line which had been grown in the presence or absence of tetracycline for four days. qPCR was performed as described in the Materials and Methods section. Expression levels were normalised between samples to GAPDH and then the levels of individual RNA species represented as a fold-stimulation of the plus-tetracycline samples relative to those from cells maintained in the absence of tetracycline. Data are presented as mean ± range of two independent experiments. F) myc-GIMAP6 HEK293 cells (right-hand panels) or the corresponding vector cells (left-hand panels) were treated with 10 μM emetine for the indicated times. Cell lysates were prepared ≠MM=

short half-life, CYCLIN D1 [29]. As expected, this protein was undetectable after only 3h of emetine treatment (Figure 4F). These results indicate that, even in the absence of GIMAP6,

Figure 5.

Figure 6. Endogenously expressed GIMAP6 is relocated to punctate structures in response to starvation A) Jurkat-T cells were grown on poly-L-lysine-treated coverslips and were either left untreated or maintained in starvation medium for 90 min. Cells were then processed for immunocytochemistry using rat anti-human GIMAP6 monoclonal antibody MAC445 or rabbit anti-MAP1LC3B (Sigma product number L7543) followed by an Alexafluor 488-conjugated anti-rat IgG or an Alexafluor 568-

when the effect of GIMAP6 over-expression in myc-GIMAP6 HEK293

Figure 7. The C-terminal 10 amino acids of GIMAP6 are required for its recruitment to autophagosomes.

functions in which it participates. Finally, to draw an analogy with the recent findings on the interaction of the Rab33-GAP, OATL1, with Atg8 family members [42], it is possible that GABARAPL2 could serve as a scaffold from which GIMAP6 may perform (as yet unknown) functions within the autophagic pathway.

It should be noted that the studies we have reported here suggest that there is no simple role for GIMAP6 in direct

consequences for lymphocyte survival. Activation of core apoptotic mechanisms is, of course, a likely consequence of autophagic failure or defects, and regulatory links between the two processes have been established. It is interesting to note here that the lymphopenic T cell phenotypes of GIMAP5-deficient rats and mice share features with those of mice in which core autophagy genes (e.g. *Atg*5, *Atg*7) have been ablated conditionally in the T cell lineage. In both cases, intrathymic T cell development is relatively normal while the peripheral T cell population is severely depleted and the remaining T cells [47,48] are unusual in phenotype, with marked absence of resting, naïve T cells [49]. In the ATG5/7-deficient models this developmental hiatus at the thymus-to-periphery transition has been ascribed to a failure of mitophagy. It has been shown that thymocytes normally exhibit a much higher mitochondrial load than peripheral T cells and it is proposed that a programmed and autophagy gene-dependent reduction in this load is necessary to protect T cells from death via oxidative stress in the more oxygen-rich environment of the peripheral compartments. Indeed, measurements of mitochondrial load in thymocytes, recent thymic emigrants and peripheral T cells from GIMAP5-mutant rats have revealed deficient reduction of mitochondrial load in this model (L. Webb, G.W.B., data not shown). Deficiencies in GIMAP5 and Atg7 also impose similar effects deleterious to quiescent haematopoietic stem cells and impair their repopulating capacity [19,50]. Considering other members of the GIMAP family, the phenotype of GIMAP1-deficient T and B

16. Saunders A, Webb LM, Janas ML, Hutchings A, Pascall J et al. (2010) Putative GTPase GIMAP1 is critical for the development of mature B and T lymphocytes. Blood 115: 3249-3257. doi:10.1182/blood-2009-08-237586. PubMed: 20194894.
17. Schnell S, Démollière C, van den Berk P, Jacobs H (2006) Gimap4 accelerates T-cell death. Blood 108: 591-599. doi:10.1182/blood-2005-11-4616. PubMed: 16569770.
18. Carter C, Dion C, Schnell S, Coadwell WJ, Graham M et al. (2007) A Natural Hypomorphic Variant of the Apoptosis Regulator Gimap4/IAN1. J Immunol 179: 1784-1795. PubMed: 17641045.
19. Chen Y, Yu M, Dai X, Zogg M, Wen R et al. (2011) Critical role for Gimap5 in the survival of mouse hematopoietic stem and progenitor cells. J Exp Med 208: 923-935. doi:10.1084/jem.20101192. PubMed: 21502331.
20. Drakas R, Prisco M, Baserga R (2005) A modified tandem affinity purification tag technique for the purification of protein complexes in mammalian cells. Proteomics 5: 132-137. doi:10.1002/pmic.200400919. PubMed: 15602769.
21. Saunders A, Lamb T, Pascall J, Hutchings A, Dion C et al. (2009) Expression of GIMAP1, a GTPase of the immunity-associated protein family, is not up-regulated in malaria. Malar J 8: 53. doi:10.1186/1475-2875-8-53. PubMed: 19338674.
22. van de Wetering M, Oving I, Muncan V, Pon Fong MT, Brantjes H et al. (2003) Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 4: 609-615. PubMed: 12776180.
23. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D et al. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A 92: 7297-7301. doi:10.1073/pnas.92.16.7297. PubMed: 7638184.
24. Wong VW, Saunders AE, Hutchings A, Pascall JC, Carter C et al. (2010) The autoimmunity-related GIMAP5 GTPase is a lysosome-associated protein. Self Nonself 1: 259-268. doi:10.4161/self.1.3.12819. PubMed: 21487483.