Gimap3 & Gimap5 in apoptosis

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Preface

This study was carried out in Biomedicum Helsinki, Research Program of Molecular Neurology, University of Helsinki during the summer and fall 2009.

I am grateful for the opportunity to work in Dr. Brendan Battersby's research group, thank you for your time giving me encouraging supervision and guidance and for providing me an excellent atmosphere in the laboratory. I want to thank all the lab members and Anu Wartiovaara's group members for their assistance, company and the hilarious moments inside and outside the laboratory. In addition to a pleasant start for my career, the time gave me a wonderful experience of life.

Abstract

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Abstract:
Apoptosis is a natural mechanism of an organism to get rid of the cells which are not wanted. Apoptosis is a phenomenon vital for the correct function of the organism. Mitochondria has an important role in apoptosis; cytochrome c release from mitochondrial intermembrane space is a central event in apoptosis which is regulated by several proteins inside the cell.

Gimap protein family members are known to function during apoptosis but the mechanisms and effects are not that well studied. In this study we concentrate on Gimap3 and Gimap5 which are highly homologous and share the same characteristics. Both regulate positive T-cell selection. B-cell Lymphoma (Bcl-2) protein family members are the most important group controlling the OM permeability and cytochrome c release. The localization of Gimap3 and Gimap5 is thought to be similar with Bcl-2 proteins and they both can interact with several Bcl-2 family members which would suggest that they can regulate apoptosis by altering Bcl-2 protein function.

In this study we started to go deeper into the mechanisms of Gimap3 and Gimap5 under apoptotic stimuli. We found out that Gimap3 gets processed, most likely degraded, during the induction of apoptosis by staurosporine (STS) and tumor necrosis factor alpha (TNF-α) while Gimap5 does not show any effect. We tested the effect on several different lengths of Gimap3 constructs and came to the conclusion that the essential part of Gimap3 protein signaled for processing has to be in the transmembrane (TM) part where the most of the sequence difference between Gimap3 and Gimap5 resides.
Gimap3 got processed despite caspase inhibition which suggest that it's caspase independent and probably an upstream process. This would fit the suggestion that it functions in apoptosis through BCL-2 proteins. In the cell death assay we saw neither protective or any clear pro-apoptotic effect by Gimap3 or Gimap5 proteins against apoptotic stimuli by TNF-α and saw no difference in Gimap3 expression level during different phases of T-cell proliferation. In our experiments Gimap3 is degraded during apoptosis but it is still unclear what is the function since there is no effect on survival or alterations in the expression in different phases of T-cell proliferation.

Keywords: Gimap, IAN, apoptosis

Tiivistelmä

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Tiivistelmä:
Ohjelmoitunut solukuolema eli apoptoosi on organismin luonnollinen ja elintärkeä mekanismi karsia ei-halutut solut. Apoptoosin säätelyssä mitokondrioilla on tärkeä osa; mitokondriossa soluhengityksessä toimivan sytokromi c:n vapautuminen mitokondriosta solulimaan on apoptoosin keskeinen tapahtuma. Kyseinen ilmiö on useiden eri proteiinien säätelemä.

Gimap proteiiniperheen tiedetään omaavan roolin apoptoosissa, mutta niiden mekanismit ja vaikutukset ovat yhä epäselvät. Tässä tutkimuksessa keskityimme kahteen Gimap-perheen jäseneen: Gimap3 ja Gimap5. Ne ovat keskenään homologisia proteiineja ja omaavat myös yhteisiä toiminnallisia piirteitä; molemmat säätelevät T-solujen positiivista valintaa. B-solu lymfooma (Bcl-2) proteiiniperheen jäsenet ovat tärkeimpiä apoptoosin säätelijöitä vaikuttaen mitokondrion ulkokalvon läpäisyvyyteen ja sitä kautta sytokromi c:n vapautumiseen mitokondriosta. Gimap3 ja Gimap5 proteiinit pystyvät vuorovaikuttamaan joidenkin Bcl-2 proteiinien kanssa. On mahdollista että Gimap3 ja Gimap5 proteiinit toimivat apoptoosin säätelijöinä Bcl-2 proteiinien kautta.

Tässä tutkimuksessa aloimme selvittämään Gimap3 ja Gimap5 proteiinien toimintaa apoptoosin aikana. Gimap3 proteiini prosessoidaan kun apoptoosia stimuloitiin staurosporiini (STS) tai tuumorinekroositekijä-alfa (TNF-α) käsittelyllä. Tulosten perusteella Gimap3 luultavasti hajotetaan apoptoosissa. Gimap5:ssä ei näkynyt vastaavia muutoksia lainkaan. Ilmeisesti sekvenssi joka määrää proteiinin käsiteltäväksi apoptoosin aikana sijaitsee proteiinin solukalvon läpäisevässä osassa, jossa suurin osa Gimap3 ja Gimap5 proteiinien sekvenssieroista sijaitsee.
Gimap3 prosessoitiin apoptoosin aikana myös kun kaspaasien toiminta estettiin. Gimap3 prosessointi tapahtuu siis ennen kaspaasien aktivointia, joka sopisi olettamukseen että se toimii apoptoosissa Bcl-2 proteiineihin sitoutumalla. Kuitenkaan emme nähneet apoptoosilta suojaavaa eikä selvästi sitä stimuloivaa vaikutusta kummaltakaan Gimap proteiinilta TNF-α aktivoitua apoptoosia vastaan. Myöskään Gimap3 proteiinin ekpressiotasossa ei ollut eroa inaktiivisten ja aktivoitujen T-solujen välillä.
Tutkimuksessa selvisi että Gimap3 prosessoidaan solussa STS ja TNF- α aktivoidun apoptoosin aikana, mutta sillä ei ole pro-/antiapoptoottista vaikutusta eikä sen ekspressiossa ole merkittävää vaihtelua. On yhä epäselvää miksi Gimap3 hajotetaan apoptoosin aikana ja mikä on tapahtuman mekanismi.

Avainsanat: Gimap, IAN, apoptoosi

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Abbreviations

GIMAP	GTPase of Immunity-Associated Proteins
GTP	Guanosine Triphosphate
IAN	Immune-Associated Nucleotide-binding protein
mtDNA	Mitochondrial Deoxyribonucleic Acid
STS	Staurosporine
TNF	Tumor Necrosis Factor
TCR	T-cell Receptor

Introduction

Apoptosis

Apoptosis is a term for controlled cell suicide, it is a general mechanism to regulate cell populations by getting rid of cells which are harmful, damaged or not wanted. In contrast to necrosis where cells undergo uncontrolled death induced by extracellular factors, apoptosis is an important natural process in controlling cell cycle, development and other functions in organisms. This phenomenon has been described almost a hundred years ago (Majno and Joris, 1995) and the term apoptosis (from Greek = falling off) proposed by Kerr and Searle in 1972 established its place for common use (Kerr et al., 1972). Apoptotic cells undergo structural changes including shrinkage and fragmentation of nucleus, chromatin condensation, membrane blebbing and DNA fragmentation (Kerr, 1971; Arends et al., 1990). These apoptotic bodies are eventually phagocytosed and degraded by macrophages or neighboring cells without generating an inflammatory response (Kerr et al., 1972). Naturally occurring apoptotic death of cells differs from pre-mature necrotic death. The structural changes during necrosis are different; characteristic to necrotic cells is swelling, loss of nucleus, loss of plasma membrane integrity and DNA is not cleaved like in apoptosis (Figure 1). Necrosis is always detrimental, the release of cells contents (such as lysosomal enzymes) into the surroundings may damage its neighboring cells leading to inflammatory response. Necrotic cells are not removed by phagocytosis by macrophages. In contrast in apoptosis damaged cells are removed to minimize the damage. Apoptosis functions not only in reducing damage, it has an important role in maintaining tissue homeostasis, development, maturation of nervous and immune system and tumor regression. Therefore it is predictable that incorrect function of genes regulating apoptosis may lead to several diseases such as cancer, acquired immune deficiency syndrome (AIDS), autoimmunity and neurodegenerative disorders (Kerr, 1971; Ellis and Horvitz, 1986; Trauth et al., 1989; Loo et al., 1993; Nossal, 1994).

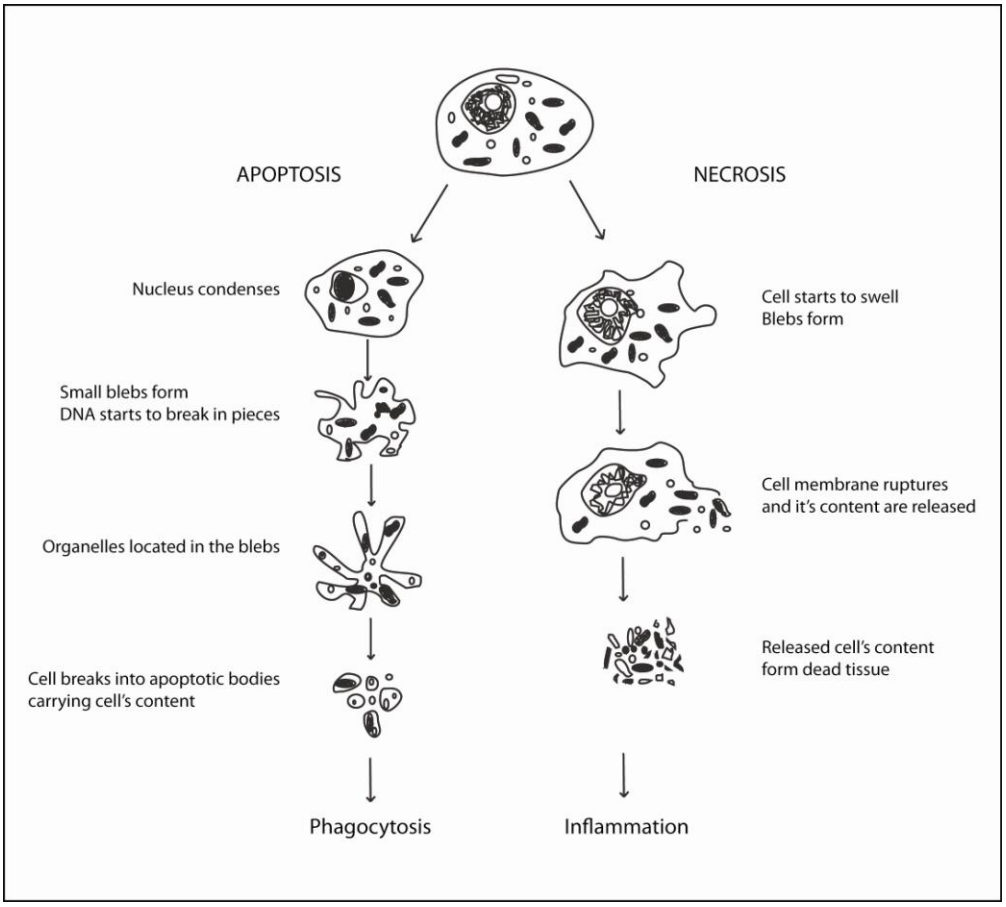

Figure 1: Apoptosis and necrosis are different kinds of mechanisms of cell death. In apoptosis the cell shrinks, the nucleus condenses and chromatin starts to break in small pieces. Cell membrane form blebs where pieces of DNA and organelles locate. Separate membrane-enclosed bodies are formed from blebs carrying cells content. These apoptotic bodies are phagocytosed and material inside is recycled. Apoptosis is highly controlled by the cell itself.

In necrosis there are no similar nuclear changes. Instead of shrinking, the cell swells. Blebs are formed but it leads to breakage of cell membrane and release of cell contents. The dead material released by necrosis causes inflammation reaction.

T-cell development & apoptosis

T-cell maturation occurs through positive and negative selection in thymus. Selection is based on the function of receptors on the thymocyte surface called T-cell receptors (TCRs) which recognizes antigens presented by major histocompatibility complexes (MHCs) (Townsend et al., 1985; Babbitt et al., 1985). In positive selection survival of T-

cells is permitted when TCRs recognize self-antigen molecules displayed by MHC on antigen presenting cells such as macrophages, dendritic cells and B-cells. If a self-antigen is not recognized the cell undergoes apoptotic death. In contrast if self-antigen reacts too strongly with the TCR, a T-cell will undergo apoptosis. The latter phenomenon is called negative selection. The T-cell repertoire is randomly generated by recombination of TCR-genes and all not wanted T-cell candidates are removed by apoptosis. The positive selection acts as the first checkpoint that the T-cell has a functional TCR. Negative selection is essential to remove strongly self reactive T-cells preventing autoreactivity against self (Jameson and Bevan, 1998; Jameson et al., 1995).

Hematopoietic stem cells are produced in bone marrow where they migrate to the thymus and mature into T-cells. Maturation stage is determined according to the expression of co-receptor molecules CD4 and CD8. The earliest pre-cursors, are double negative ($CD4^-$ $CD8^-$) cells which produce neither of the proteins. The large immature pool of thymocytes produced in thymus are double positive ($CD4^+CD8^+$) cells. These immature T-cells undergo selection and only a fraction of them survives and develops into mature T-cells. Mature T-cells are released from the thymus to peripheral tissues. At the next stage the cells are single positive, either $CD4^+CD8^-$ or $CD4^-CD8^+$. These two classes of T-cells have different function, CD8 are cytotoxic while CD4 are helper cells. Cytotoxic CD8 cells secrete molecules such as Fas, perforin and granzymes which can induce apoptosis and destroy the cell to which MHC presented antigen they have bound. CD4 cells activate cell-mediated immune response by binding to antigen presented by antigen-presenting cells like macrophages and dendritics cells. They can also bind to antigen presented by B cells activating antibody-mediated immunity (Jameson et al., 1995; Kisielow and von Boehmer, 1995).

Simplified pathways of apoptosis and relation to mitochondria

Mitochondria are found in every eukaryotic cell, they have double membrane which encloses the organelle. Inner membrane is folded increasing the surface of the inner membrane on which cellular respiration occurs. The main energy source of the cell, adenosine triphophate (ATP), is produced in this process. Oxidative phosphorylation

requires $FADH_2$ and NADH which are produced utilizing breakdown products of macronutrient from glycolysis and citric acid cycle. Electron transport chain of oxidative phosphorylation consists of several protein structures embedded in the inner mitochondrial membrane, which utilize electrons from FADH2 and NADH and pumps H^+ across the inner mitochondrial membrane. Formed protein gradient activates the last protein complex of electron transport chain, ATP synthetase, which produces ATP from ADP by adding another phosphate group into the molecule. Most of the cellular energy supply is generated in mitochondria. The efficiency of mitochondrial ATP production variate depending on the energy demand of the cell. Production of ATP can be regulated by several mechanisms to match the energy demand (Benard et al., 2010). In addition to ATP production mitochondria have other functions such as urea cycle, fatty acid metabolism, heme biosynthesis, calcium homeostasis and regulation of apoptosis (Ballard and Whitlock, 2004).

Mitochondria evolved from aerobic bacteria engulfed by anaerobic prokaryotes leading to endosymbiotic relationship. Aerobic bacteria consumed the oxygen harmful to the anaerobic host and gained nutrients from the host. Therefore both provided a better environment to each other giving rise to eukaryotic cells (Margulis, 1996). Mitochondrial function in apoptosis is thought to have similarities with some bacterial mechanisms in defending or competing against other organisms; they form pore complexes on the membrane to give passage for cell death inducing proteins (Martinou and Green, 2001). It would support the idea that controlled cell death in eukaryotic cells evolved due to mitochondrial endosymbiosis (Frade and Michaelidis, 1997). Even though it has been shown that mitochondrial DNA is not essential for apoptosis (Jacobson et al., 1993), it provides several important molecules which act in the activation and regulation of apoptosis (Zamzami and Kroemer, 2001; Simon et al., 2000; Li et al., 1997).

There are two main independent apoptosis pathways, extrinsic and intrinsic pathways, also called death receptor and mitochondrial pathways respectively (Figure 2). In the death receptor pathway, signals for apoptosis originate from tumor necrosis factor (TNF) family ligands (such as TNF-α) interacting with their receptors on the cell surface. The

best characterized receptors for these ligands are tumor necrosis factor receptor 1 (TNFR1) and CD95 (also known as FasR, Apo-1 and TNFRSf6). Signal from the receptor activates a cysteine protease (caspase) called caspase-8 (and also caspase-10) via adaptor protein such as FADD (Krammer, 2000). Caspase family members exist typically in inactive form and are cleaved in order to be activated. Activation of caspase-8 can lead to cleavage of other downstream caspases triggering apoptosis. Caspase-8 activation can also interact with the intrinsic pathway resulting in some crosstalk between intrinsic and extrinsic pathways of apoptosis (Figure 2) (Luo et al., 1998; Li et al., 1998).

A variety of known stimuli can provoke an intrinsic apoptosis pathway independent of death receptor pathway. These include e.g. chemotherapeutic agents, staurosporine (STS), stress molecules (reactive oxygen and nitrogen species) and growth factor withdrawal (Simon et al., 2000; Bertrand et al., 1994; Atabay et al., 1996; Assefa et al., 2000; Matsko et al., 2001). After the cell receives an external death signal, many apoptogenic molecules are released from mitochondria. One of these is a member of electron transfer chain called cytochrome c. The protein play a part in production of ATP but it is also an important factor in regulation of apoptosis (Liu et al., 1996). In the cytoplasm cytochrome c activates a member of cysteine protease (caspase) family called caspase-9 (Alnemri et al., 1996), by forming a protein complex “apoptosome” with adaptor apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 (Li et al., 1997). Activation of caspase-9 leads to activation of caspase-3 and other downstream caspases which leads to cleavage of their target proteins. This event disables important cellular functions and results in morphological changes characteristic for apoptosis (Lazebnik et al., 1994; Earnshaw et al., 1999).

Before cytochrome c is released from mitochondria, it has to pass outer mitochondrial membrane. The molecular basis of mitochondrial membrane permeabilization is not completely understood but one suggestion is that apoptosis regulating proteins translocate on the outer mitochondrial membrane and interact with mitochondrial permeability transition pore complexes resulting in release of apoptosis triggering factors like cytochrome c from mitochondria (Zamzami and Kroemer, 2001). Most well studied

apoptosis regulating proteins are members of B-Cell Lymphoma 2 (Bcl-2) -family, which might function in the mechanisms of mitochondrial outer membrane permeabilization (Shimizu et al., 2001; Shimizu et al., 1999; Marzo et al., 1998). In addition some Bcl-2 proteins are able to form channels that might be an exit for apoptosis promoting proteins from mitochondria. (Minn et al., 1997; Schendel et al., 1997).

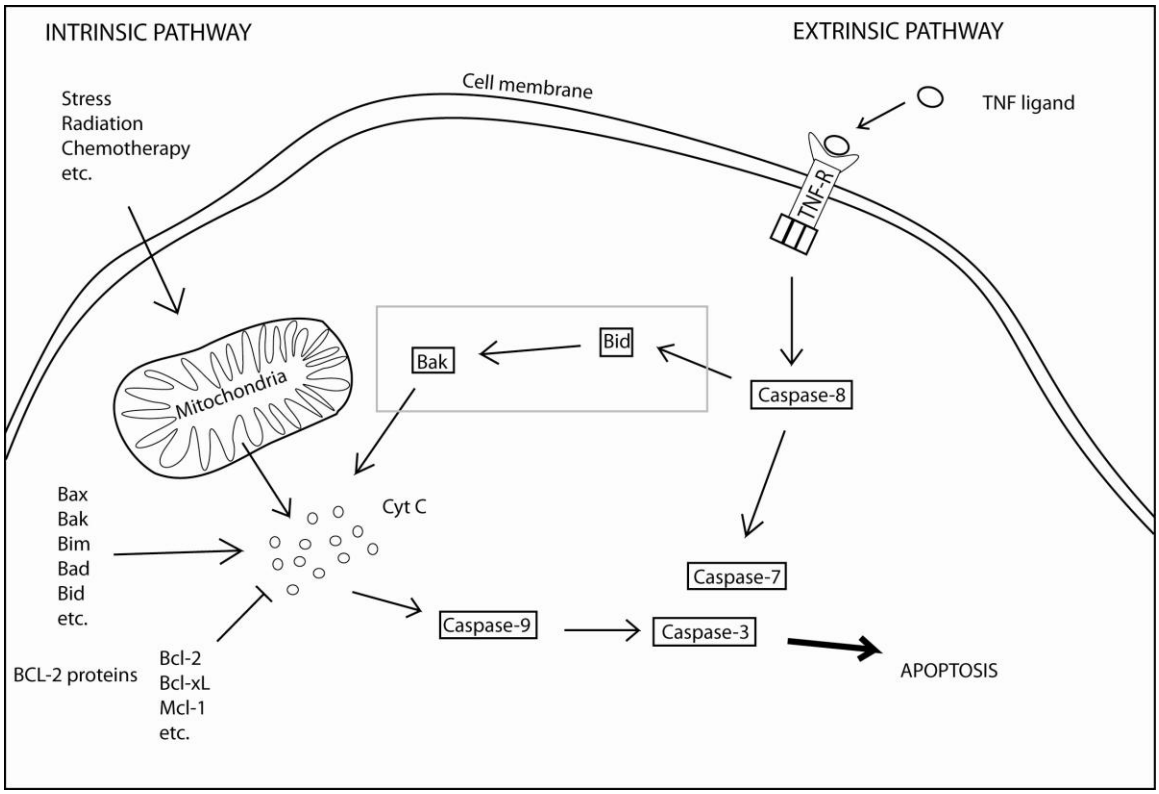

Figure 2: Simplified main pathways of apoptosis in eukaryotic cell. Intrinsic pathway can be triggered by extracellular stimuli resulting in cytochrome c release from mitochondria. B-cell lymphoma 2 (Bcl-2) proteins regulate cytochrome c release; apoptotic Bcl-2 proteins such as Bax, Bak, Bad and Bid induce and anti-apoptotic such as Bcl-2, Bcl-xL and Mcl-1 inhibit cytochrome c release. Released cytochrome c activates caspase-9 and Apaf-1 in the cytosol forming apoptosome which cleaves caspase-3 and other downstream caspases leading eventually to apoptosis. Extrinsic pathway is triggered by a ligand binding to a death receptor (e.g TNF-R) resulting in caspase-8 activation. Like caspase-9 in intrinsic pathway, caspase-8 activates it's downstream caspases including caspase-7 and caspase-3 resulting in apoptosis. Caspase-8 can also activate intrinsic pathway through pro-apoptotic Bcl-2 family member proteins Bid and Bak which induce cytochrome c release from mitochondria.

Bcl-2 family

Bcl-2 family proteins were discovered to operate as regulators of apoptosis by regulating the release of cytochrome c from mitochondria (Shimizu et al., 1999; Kelekar and Thompson, 1998; Kuwana and Newmeyer, 2003). Bcl-2 family count in pro-apoptotic and anti-apoptotic members either by inducing or resisting apoptosis respectively (table 1). In which group the protein belongs is largely defined by the Bcl-2 homology domains (BH1, BH2, BH3 and BH4) (Kelekar and Thompson, 1998; Kuwana and Newmeyer, 2003). BH3 domain is common for all the family members. Anti-apoptotic members are always multidomained and have at least BH1, BH2 and BH3 domains. Members which have only BH3 domain are pro-apoptotic and are activated by interaction with multidomained pro-apoptotic Bcl-2 proteins (Lindsten et al., 2000; Cheng et al., 2001; Wei et al., 2001). Bid carries only BH3 domain and has to interact with Bak to trigger apoptosis (Wei et al., 2000). Bid induces apoptosis through intrinsic pathway but Bid itself is activated by caspase-8, a typical activator in extrinsic pathway (figure 2). Therefore the activation of extrinsic pathway can activate also the intrinsic pathway (Luo et al., 1998; Li et al., 1998).

Many Bcl-2 proteins are found to localize on mitochondrial outer membrane and ER in the cell and some pro-apoptotic members translocate on mitochondrial membrane during apoptotic stimuli (Kuwana and Newmeyer, 2003; Akao et al., 1994). One possibility how Bcl-2 proteins regulate apoptosis is by interacting with mitochondrial porin, also called voltage dependent anion channel (VDAC), which is one of the proteins forming permeability transition pore complex on mitochondrial membrane (Shimizu et al., 1999; Shimizu et al., 1998). Some Bcl-2 proteins can also form channels on membranes alone (Minn et al., 1997; Schendel et al., 1997) and Bax is one of them able to form a channel just big enough to pass cytochrome c (Saito et al., 2000). Anti-apoptotic members can regulate mitochondrial membrane permeability by inhibiting pro-apoptotic Bcl-2 proteins or interacting directly with permeability transition pore complexes preventing cytochrome c release (Marzo et al., 1998; Shimizu et al., 1998).

Table 1: Categorization of some Bcl-2 family proteins as pro-apoptotic and anti-apoptotic members. Pro-apoptotic members are sub-categorized according to how many BH domains they have. Model for the table: (Kuwana and Newmeyer, 2003).

Pro-apoptotic		Anti-apoptotic
Multidomain:	BH3-only:	
Bax	Bid	Bcl-2
Bak	Bad	Bcl-xL
Bok/Mtd	Bim	Bcl-w
Bcl-xS	Bmf	Mcl-1
	Bik	A1
	Hrk/DP5	Boo/Diva
	Blk	
	Nip3	
	BNip3/Nix	
	Puma	
	Noxa	

Many Bcl-2 members are regulated during thymocyte maturation and they are thought to regulate thymocyte survival by interrupting with apoptotic mechanisms. For example Bcl-xL protein expression is upregulated when double negative T-cells differentiate into double positive cells (Ma et al., 1995). Bcl-xL has anti-apoptotic activity and induces the survival of double positive cells. In single positive cells Bcl-xL expression is negligible and does not affect the survival of the cells (Ma et al., 1995). In contrast Bcl-2 is upregulated when double positive cells differentiate into single positive cells. While Bcl-xL promotes double positive cell survival, Bcl-2 has the same role in single positive cells (Linette et al., 1994). Bim again is pro-apoptotic and functions by inhibiting anti-apoptotic functions of Bcl-xL (Bouillet et al., 2002), Bcl-2 and Bcl-w (O'Connor et al., 1998). Pro-apoptotic Bax is translocated to mitochondria after TCR stimulation which is thought to have a role in the release of cytochrome c (Yoshino et al., 2001).

Gimap protein family

GTPases of the immunity-associated proteins (Gimap) (originally called immune-associated nucleotide-binding proteins (IANs)) are found in vertebrates and higher plants (Nitta et al., 2006). The first Gimap family member found, AIG1, was found in *Arabidopsis thaliana* (Liu et al., 2008; Reuber and Ausubel, 1996). All members of GIMAP protein family have a GTP binding motif which is also called AIG1 domain after the prototype member of *Arabidopsis.* Gimaps are a gene clusters in the genome and therefore are encoded by contiguous sequences of the genome (Nitta et al., 2006; Krucken et al., 2004; MacMurray et al., 2002). Arabdidopsis thaliana carries a gene cluster of 13 *gimap* genes (Liu et al., 2008; Reuber and Ausubel, 1996), mouse has a cluster of 9 genes and a cluster of 8 different genes have been found in human (Krucken et al., 2004; Krucken et al., 2005).

The function of Gimap family proteins is still widely unclear. In higher plants Gimap proteins function in self-defense response when attacked by pathogenic micro-organism (Reuber and Ausubel, 1996). In vertebrates Gimaps are thought to have a role in thymocyte maturation, most of the Gimap family member protein expression is enriched in immune cells and tissues (Nitta et al., 2006; Reuber and Ausubel, 1996; MacMurray et al., 2002; Cambot et al., 2002; Krucken et al., 1997; Poirier et al., 1999; Zenz et al., 2004; Daheron et al., 2001; Hornum et al., 2002). Same kind of regulation during thymocyte maturation is seen also in Bcl-2 family members and some Gimaps are known to localize at similar compartments as Bcl-2 proteins and to interact with Bcl-2 family proteins (Nitta et al., 2006). Some of the Gimap family members appear to have a role in cell survival; Gimap4 has a pro-apoptotic effect (Nitta et al., 2006; Schnell et al., 2006) when Gimap1, Gimap3, Gimap5 and Gimap8 are considered as anti-apoptotic proteins (Nitta et al., 2006; MacMurray et al., 2002; Krucken et al., 2005; Saunders et al., 2010; Pandarpurkar et al., 2003; Sandal et al., 2003).

(a) Gimap3 & Gimap5

Gimap3 and Gimap5 are highly homologous, they share 83.8% identity in total 291 amino acid sequence and 88.9% identity in open reading frame nucleotides in 873 base pairs (Nitta 2006). Both Gimap3 and Gimap5 also have C-terminal hydrophobic region but the sequences distinct (More detailed in discussion part, Figure 12). Gimap5 has a part of a protein called helix α7 near C-terminal hydrophobic segment which plays part in dimerization as the protein; removal of helix α7 induces dimerization (Schwefel et al., 2010). This region of the protein is almost identical with Gimap3 sequence suggesting that Gimap3 has a same kind of dimerization mechanism. Between mouse and human, *gimap3* and *gimap5* sequence localization and orientation within *gimap* gene cluster are conserved and it's been suggested that they emerged by gene duplication (Krucken et al., 2004). In human *Gimap3* gene only exists as a pseudogene but it is functional in mice (Krucken et al., 2004).

Gimap5 expression is highly elevated in leukemia cells (Zenz et al., 2004). Knockout of Gimap5 is known to result in T lymphopenia, hepatic extramedullary hematopoiesis, weight loss, and intestinal inflammation and it disrupts development and regulation of natural killer cells, B-cell and granulocytes (Pandarpurkar et al., 2003; Schulteis et al., 2008; Barnes et al., 2010). Gimap5 regulates positive selection of double positive T-cells (Nitta et al., 2006) and it is suggested to be the most important regulator of thymocyte survival amongst Gimap family proteins (Rutledge et al., 2009).
Like Gimap5, Gimap3 expression is also elevated in leukemia cells (Zenz et al., 2004; Daheron et al., 2001). Gimap3 regulates thymocyte development as well although the function in regulating thymocyte survival seems to differentiate from Gimap5 depending on the activation status of thymocytes; when Gimap5 regulates positive selection of double positive T-cells, Gimap3 regulates positive selection of single positive T-cells (Nitta et al., 2006).

Localization of Gimap3 and Gimap5 was thought to be similar with each other but there has been a lot of unclarity in the results of localization experiments. Previously Gimap3 has been reported to localize on mitochondrial outer membrane (Daheron et al., 2001)

and ER (Nitta et al., 2006). Gimap5 has been reported to localize on similar heavy membrane fractions such as mitochondria and ER (Nitta et al., 2006; Zenz et al., 2004). One paper also claimed centrosomes and golgi as Gimap5 localization targets (Sandal et al., 2003). Experiments were done by overexpressing Gimap5 or by using C-terminal HA- or EGFP-tag which might disturb the localization. Later on results were against ER and mitochondrial localization (Keita et al., 2007). A recent paper, which used endogenously expressed Gimap5 and a specific anti-body, reports it to localize on lysosomes and multivesicular compartments but neither on ER or mitochondria (Wong et al., 2010). Therefore Gimap3 and Gimap5 might have different cellular localization. They are thought to localize at the same sub-cellular compartments with some Bcl-2 family members. Both Gimap3 and Gimap5 can interact with several anti-apoptotic Bcl-2 family proteins including anti-apoptotic Bcl-2 and Bcl-xL and pro-apoptotic Bax, Bak, Bad and BimEL (Nitta et al., 2006). This suggests that they play a role in regulation of apoptosis via Bcl-2 family proteins. Also their expression pattern in T-cell maturation seems similar with Bcl-2; Gimap3 and Gimap5 expression is upregulated during the development of double positive T-cells into single positive T-cells (Nitta et al., 2006)

Gimap5 is reported to have anti-apoptotic effect. Gimap5 can protect against apoptosis induced by cytokine withdrawal or by γ-radiation and okadaic acid (Sandal et al., 2003). Expression of Gimap5 is increased after stimulation with apoptotic cytokines in monocytes which could be related with anti-apoptotic response (Hellquist et al., 2007). Gimap5 is thought to be essential for T-cell survival by maintaining mitochondrial membrane integrity (Pandarpurkar et al., 2003). There seems to be some connection between Gimap5 and maintenance of ER homeostasis regulation in primary T-cells which might be a possible mechanism how Gimap5 regulates apoptosis (Pino et al., 2009) and support for that is impaired calsium signaling seen in Gimap5 deficient cells (Ilangumaran et al., 2009).

Gimap5 is considered to be anti-apoptotic (Nitta et al., 2006; Sandal et al., 2003) but however it’s role only as an anti-apoptotic protein is more or less unclear since it’s been shown that Gimap5 induces apoptosis in naive T-cells (Dalberg et al., 2007). Then again the pro-apoptotic effect is not seen in activated T-cells and it has been suggested that

Gimap5 might have an anti-apoptotic function in one occasion and pro-apoptotic in another (Nitta et al., 2006; Dalberg et al., 2007).

Gimap3 is less studied than Gimap5, the function and mechanism of the protein in the cell is still widely unclear for us. We know that Gimap3 has a function in regulating thymocyte maturation; the expression on Gimap3 is increased during positive selection of T-cells and loss of Gimap3 results in reduction of mature T-cells (Nitta et al., 2006). Gimap3 has been reported to have anti-apoptotic effect on cells (MacMurray et al., 2002; Pandarpurkar et al., 2003). In addition Gimap3 regulates mitochondrial DNA segregation (Jokinen et al., 2010). Although Gimap3 and Gimap5 are highly similar in structure and sequence, they have slightly different functions.

Aim of the Study

In this study we wanted to explore the function and mechanism of Gimap3 and Gimap5 proteins in apoptosis. The study is based on Dr. Battersby's observations that there were changes in Gimap3 level when apoptosis is induced. We wanted to find out what happens to the protein during apoptosis and if the protein affects the cell survival. Since Gimap5 and Gimap3 are thought to have similar functions in the cell and they share highly homologous structure, we concentrated on this particular pair of Gimap family proteins in our experiments.

Materials and Methods

T-cell isolation

T-cells were freshly isolated from female FVB mouse spleens. Spleens were homogenized in Dulbecco's modified eagle's medium (DMEM) (EuroClone) supplemented with 5% fetal bovine serum (FBS) and cells were filtered twice through 40μm filter. T-cells were separated from total leukocytes by using Pan T Cell Isolation Kit (Miltenyi Biotec) based on magnetic labeling and separation by magnetic column according to manufacturer's instructions.

Cell lines and culture

Apoptosis inductions were carried out for approximately 1.5 $x10^6$ EL4 – cells (mouse lymphoma cell line) on 6-well plate, 70-90% confluent NIH3T3 cells (mouse embryonic fibroblast cell line) on 6-well plate or $2x10^6$ T-cells (isolated from female FVB mouse spleen) plated on 24-well dish. Growth conditions were at 37°C and 5% CO. DMEM supplemented with 10% fetal bovine serum (FBS) and 1% glutamax (Invitrogen) was used as media for NIH3T3 and EL4 cells. T-cells were cultured in RPMI (10% FBS, 1xGlutamax (Invitrogen), 1% glutamine and 100 U/ml Penicillin and Streptomycin (Bio Whittaker)).

Apoptosis inductions

Apoptosis was induced for different durations between 1 and 24 hours with 1μM Staurosporine (STS) (Sigma) or 10 ng/ml tumor necrosis factor alpha (TNF-α) (Sigma) with 2,5 μg/ml of cycloheximide (Sigma-Aldrich). T-cells were treated after 1 and 3 days of stimulation.

T-cell stimulation

$2x10^6$ T-cells were stimulated with 10μl Dynabeads mouse CD3/CD28 T cell expander (Invitrogen) and 10U IL2 for 1-4 days on 24-well plate.

Caspase blocking in NIH3T3 cells

Gimap3 overexpressing NIH3T3 cells were grown on 6-well plate to reach 80-90% confluency on the day of experiment. Caspase inhibitors Z-VAD-FMK and Z-DQMD-FMK (Sigma) were used in concentrations of 10 μM and added 2 hours before apoptosis induction treatment. 16h of STS treatment and 8h of TNF-a treatment were used as apoptotic triggers as described earlier and after the treatment cells were proceeded to immunoblotting.

Immunoblotting

Cells were collected or washed in 1ml of phosphate buffered saline (PBS) and centrifuged at 2000xg for 5 minutes at room temperature (Beckman Coulter – Microfuge

18 Centrifuge). Solubilization buffer of PBS with 1% dodecyl maltoside (DDM), 0.1 mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor tablet (Complete Mini: Protease inhibitor cocktail tablet, Roche) was added on the cell pellet (amount depending on the pellet size) and incubated on ice for 20 minutes. Insoluble material was separated by centrifugation at 20000xg at +4°C for 20 minutes (Beckman Coulter – Allegra X-22R Centrifuge). Protein concentrations of samples were measured by Bradford protein assay (Bradford, 1976) with SpectraMax 190 spectrophotometer. Samples with equal amount of protein (10µg-20µg) were prepared for SDS-PAGE. Volumes of the samples were adjusted equal with the solubilization buffer. Proteins were separated by SDS-PAGE in running buffer (25mM Tris base, 192 mM glycine, 0,1% SDS, pH 8.8) using 12% or 15% polyacrylamide gel. PageRuler Prestained Protein ladder (Fermentas, Cat. #SM0671) was used as a size marker. Proteins were transferred to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences) by semi-dry transfer. Nitrocellulose membranes were stained with Ponceau S –dye (Fluka) to estimate if the protein transfer was successful. Membranes were blocked for antibody staining with 5% BSA in Tris-Buffered Saline containing 0.1% Tween 20 (TBST) for 1h at room temperature. When Gimap3 antibody was used on the membrane, 1% milk blocking in TBST was used instead. Blocked membranes were incubated at 4°C overnight with primary antibody. Membranes were washed with TBST three times for 20 minutes. For detection, HRP-conjugated secondary antibody was added and incubated for 1h at room temperature. Membrane was washed again with TBST as previously and signal was detected using ECL LumiGlo -reagent (Cell Signaling Technology). Films were developed with Kodak X-OMAT 2000.

Used antibodies are listed on table 2. Recognition sequences of Gimap3 antibody are listed in appendix 1.

Table 2: Antibodies used in immunoblotting analysis**.**

Anti-body	**Info, dilution**
Gimap3	1:1000
Anti-HA	Sigma-Aldrich, 1:1000
Anti - c-myc	Roche, 1:1000
Anti-GFP	Clontech. 1:200
Cleaved caspase-3	Cell Signaling, 1:200
Anti-Cops5/Jab1	BD Biosciences, 1:1000
Anti-β-tubulin	Cell Signaling Technology, 1:1000
SDHA 70 kDa	Invitrogen, 1:5000
anti-calnexin	Stressgen, 1:4000
Tom 40	Santa Cruz Biotechnology, 1:1000

Cloning of Gimap3 constructs

(a) Production of *Gimap3* sequence with att-sites

Two separate PCR-reactions were needed. Contents of both reactions (total volume 50 μl): Expand High Fidelity PCR System –polymerase mixture 2.6U / reaction (Roche), 200 μM of primers, 200 μM of each dNTP (Roche) and 100 ng of template.

In the first PCR-reaction, forward primer began replication from the beginning of *Gimap3*-gene leaving HA-tag out and both forward and reverse primers produced Gateway-compatible att-adaptor sites at the ends of sequence. Primers used in the first reaction are listed in table 3. PCR cycle conditions: denaturation step at 95°C for 1 min, followed by 10 cycles of 94°C 15 s, 55°C 30 s and 68°C 1 min 30 s, and a final extension period at 68°C for 7 minutes.

In the second PCR-reaction att-adaptor sites were completed to make Gimap3-gene with full att-sites at the ends. Primers used in the reaction are listed in table 3. Second PCR cycle conditions: denaturation step at 95 °C for 1 min, 5 cycles of 95°C 15 s, 45°C 30 s

and 68°C 1 min 30 s, 5 cycles of 94°C 15 s, 55°C 30 s and 68°C 1 min 30 s, and then cooled down to 4°C.

Table 3: Primers of two reactions used to produce att-flanked *Gimap3* sequence.

First reaction primers:	
Forward	5'-AAAAAGCAGGCTACCATGGAAACACTTCAGAATGTTGTAACAGGCGGAAA-3'
Reverse	5'-AGAAAGCTGGGTAAAATGCAGAGCATTAAACATAAAA-3'
Second reaction:	
Forward	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'
Reverse	5'-GGGGACCACTTTGTACAGAAAGCTGGGT-3'

Products were run on ethidium bromide stained 1% agarose gel in 0.5 x Tris-Edta (TE) buffer using bromophenol blue dye and GeneRuler 1kb DNA ladder Plus (Fermentas, SM#1138). Gimap3 DNA-band was extracted and purified from the gel by using NucleoSpin Extract II –kit (Macherey-Nagel).

(b) Producing Entry Clone

Att-flanked Gimap3 gene was cloned into pDONR201-vector (Invitrogen) which has an *E. Coli* growth inhibiting ccdB-gene in the middle of att-sites. When recombination took place, ccdB-gene got replaced by Gimap3-gene. Thus ccdB-gene and kanamycin resistance gene of pDONR201 functioned as selection markers.

Reaction components: att-flanked Gimap3 PCR-product 10ng/µl, pDONR201 15ng/µl, TE-buffer pH8.0 and 0.2µg/µl BP Clonase II Enzyme Mix (containing integrase and integration host factor) (Invitrogen). Reaction mixture was incubated at room temperature for 1 hour and after that at +37°C for 10 minutes with 200 ng/µl of proteinase K (Invitrogen) to stop the reaction. Construct was introduced into heat competent E.Coli DHB10B cells by a 42°C heat shock of duration of 30 seconds. Culture was put under selection of 50 µg/ml of kanamycin. Plasmid was purified from the small scale culture

using NucleoSpin Plasmid –kit (Macherey-Nagel). Plasmid construct was verified by sequencing and comparing the sequence with Gimap3 sequence using MultAlin software (Corpet, 1988).

(c) Producing Expression Clone

Retroviral vector pBABE was used to transduce genes into cells. Vector had ampicillin resistance cassette as a prokaryotic selection marker and puromycin cassette as an eukaryotic selection marker and also a modified Gateway compatible cassette at the multiple cloning site (MCS) to make site-specific recombination possible.

Gimap3 –gene was cloned into pBABE by recombination between pDONR-Gimap3 and a target vector equipped with gateway suitable cloning site. Reaction mixture consisted of 12ng/µl of pDONR-Gimap3, 15ng/µl of pBABE vector, LR Clonase Enzyme Mix 0.2µg/µl (containing integrase, integration host factor and excisionase) (Invitrogen) and TE buffer pH8.0. Reactions were incubated at room temperature for 1 hour. Reaction was terminated by adding proteinase K in the concentration of 200 ng/ml (Invitrogen) and incubated at +37°C for 10 minutes. Construct was introduced into heat competent E.Coli DHB10B cells and selected with ampicillin 100 µg/ml in LB-medium. Plasmid was purified from small scale cultures by using NucleoSpin Extract II –kit (Macherey-Nagel), samples were digested using BsrGI –restriction enzyme 200 U/ml (New England Biolabs) which cleaved the sequence next to att-recombination site. Digested samples were run on 0,7% agarose gel to verify the success of recombination. Purification of large scale culture was made using NucleoBond Xtra Midi –kit (Macherey-Nagel).

Production of GIMAP3 expressing cell lineages

Phoenix packaging cell lineage (Nolan Lab) was used to produce retroviral particles. Phoenix cells are based on a human embryonic kidney cell line (293T) which are expressing gag-polymerase and envelope proteins required for virus synthesis and packaging of ecotropic and amphotropic viruses (Pear et al., 1993). DMEM was used as a media in growth conditions of 37°C and 5% CO. Cells were grown on 6-well plate to reach confluency of 70-80% at the time of transfection. Transfection was carried out by

Ca_2PO_4 mediated method (Nolan Lab). 10μg of DNA construct was mixed with 1ml of 1x HBS (8.0 g NaCl, 6.5 g HEPES, 10 ml Na_2HPO_4; pH 7.0) and with 0.12M $CaCl_2$ to produce DNA bound $CaPO_4$ particles. Chloroquine was added with a concentration of 25μM on Phoenix cells before transfection to inhibit lysosomal DNases by increasing lysosomal pH (Hasan et al., 1991). After 24 hours of transfection, fresh medium was changed and after 48 hours supernatant containing viral particles was collected and centrifuged at 750 xg for 5 minutes to pellet cell debris.

About $5x10^5$ of target cells on 6-well plate were infected with 1 ml of viral supernatant and polybrene was added with final concentration of 5μg/ml on the culture. After 24 hours from infection viral supernatant was changed into fresh one and after 48 hours cells were put under selection. Selection was done in 1.5μg/ml of puromycin (Sigma) for minimum of 3 days. Constructs were verified by immunoblotting with Gimap3 and Anti-HA antibodies.

Finding the cleavage site of Gimap3 during apoptosis

Several different cell lineages carrying different lengths of Gimap3 C-terminal sequence conjugated with YFP were made using transient transfections. Structures of transfected plasmids in pcDNA3 –vector are shown on figure 3. Amino acid sequences of Gimap3 part of constructs are shown in appendix 1.

Two transfections of each construct were made, one to be treated with STS and one as a control.

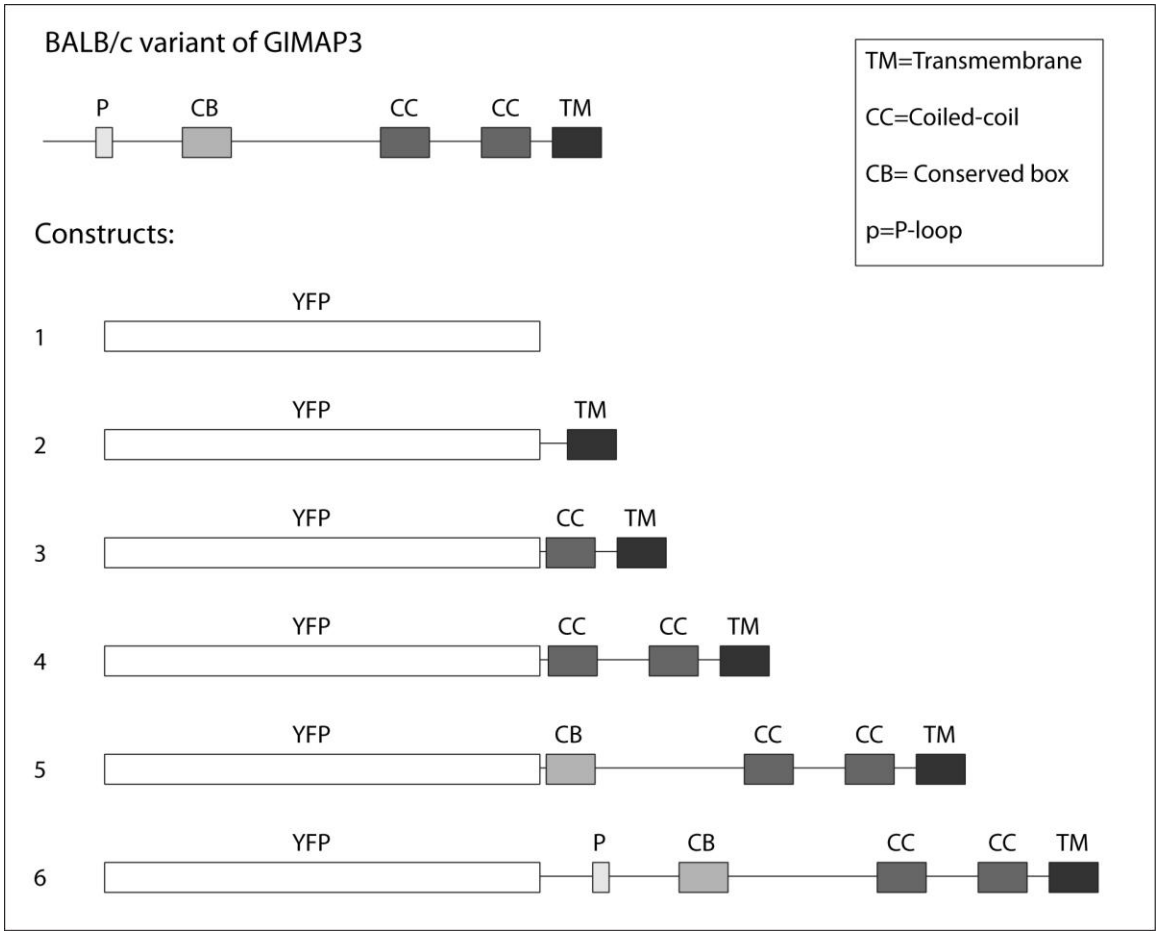

Figure 3: Topmost picture show the primary structure of Gimap3 (BALB/c variant). Abbreviations of structures: TM = transmembrane, CC = coiled coil, CB = conserved box, P = P-loop. Constructs marked with numbers 1-6 were in pcDNA3 –vector and used in transient transfections to produce NIH3T3 cell lineages expressing proteins carried by plasmids.

NIH3T3 cells were grown to reach ~50% confluency on 6-well plate. Cells were trypsinized and resuspended in 2ml of DMEM supplemented with 10% FBS, 1x glutamax (Invitrogen) and 100 U/ml Penicillin and Streptomycin (Bio Whittaker). Cells were transfected in suspension by using JetPrime DNA transfection reagent (Polyplus-Transfections). Transfection mixture per construct was made as follows: 200µl of JetPrime buffer and 2µg of plasmid-DNA were mixed and vortexed, 4 µl of JetPrime DNA transfection reagent was added and finally the mixture was vortexed 10 seconds and incubated 10 min in room temperature. Transfection mixture (200µl) was added on cells and incubated for 24h at +37°C and 5% CO. Success of transfection was checked

with fluorescence microscopy to detect YFP fluorescence. Fresh media containing 1μM STS was added on cells and incubated for 16h. Only fresh media was added on control cells. Cells were collected after the treatment and proceeded to immunoblotting.

Scoring of apoptosis

Cell death detection was made to test if expression of Gimap3 or Gimap5 provides cells protection against apoptosis. Cell lineages Gimap3 and Gimap5 expressing NIH3T3 cells were used. NIH3T3 containing empty pBABE vector was used as a control.

(d) ELISA Cell Death detection

10^4 cells were seeded on a 96-well plate per well and were grown over night before the experiment. On the day of experiment, NIH3T3-Gimap3 cells seemed to be about 10%-20% more confluent than other lineages when observed by light microscope. As an apoptosis inducing agent TNF-α was used in concentration of 10 ng/ml with 2.5 μg/ml of cycloheximide. Incubation timepoints were 0, 1, 2, 4, 6 and 8 hours. Triplicates were made from every time point. Cell deaths were assayed by using Cell Death Detection ELISA^{PLUS} –kit (Roche) according to manufacturers instructions. Assay is based on a sandwich staining of mono- and oligonucleosomes (which are formed during apoptosis) with peroxidase conjugated antibody. After color reaction between peroxidase and it's substrate, average absorbance (A_{405nm}-A_{490nm}) of parallel samples were measured with SpectraMax 190 spectrophotometer. Absorbance of initial point (0h) of every cell lineage was considered as a blank sample

(e) DeadEnd Fluorometric TUNEL System

$4x10^5$ cells were seeded on a 6-well plate containing coverslip on the bottom of the well and grown over night. On the experiment day cells were growing in confluency of about 40-50 % and treated with 10 ng/ml of TNF-α with 2.5 μg/ml of cycloheximide. Two time points of the treatment were done, 6 and 8 hours. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) at the end of the treatment. Fragmented DNA was stained and detected according to DeadEnd Fluorometric TUNEL System Protocol (Promega).

Amount of total cells counted per sample was >300 and only nucleus with bright intensity of fragmented DNA fluorescence were considered as dead cells.

Results

Gimap3 and Gimap5 does not protect from TNF-α induced apoptosis

It's been reported that Gimap3 and Gimap5 might have protective effect on T-cell survival (Nitta et al., 2006). Since Gimap5 is known to protect against external apoptotic factors such as cytokine withdrawal, γ-radiation and okadaic acid (Sandal et al., 2003) it would be expected to have anti-apoptotic functions against other apoptotic stimuli as well. We wanted to see if Gimap3 and Gimap5 would have anti-apoptotic effect on TNF-α induced apoptosis. Two separate experiments were done to test this by using Cell Death Detection ELISA^{PLUS} –kit (Roche) and DeadEnd Fluorometric TUNEL System Protocol (Promega). Experiments were done on NIH3T3 cells expressing Gimap3 or Gimap5. NIH3T3 cell lineage carrying an empty pBABE vector was used as a control. Apoptosis was stimulated using 10ng/ml TNF-α with 2.5µg/ml cycloheximide for 1-8 hours.

In ELISA Cell Death –assay indicator of intensity of cell death was based on sandwich staining of mono- and oligonucleosomes which are formed in apoptosis. Absorbance (A_{405nm}-A_{490nm}) of samples were measured and compared with a blank sample value (time point 0h). (Figure 4)

In TUNEL DeadEnd –protocol, number of dead cells were counted by staining fragmented DNA with fluorescent antibody. Nucleus stain DAPI was used to see overall amount of cells. Percentage of dead cells among over 300 cells of each sample was counted. (Figure 5)

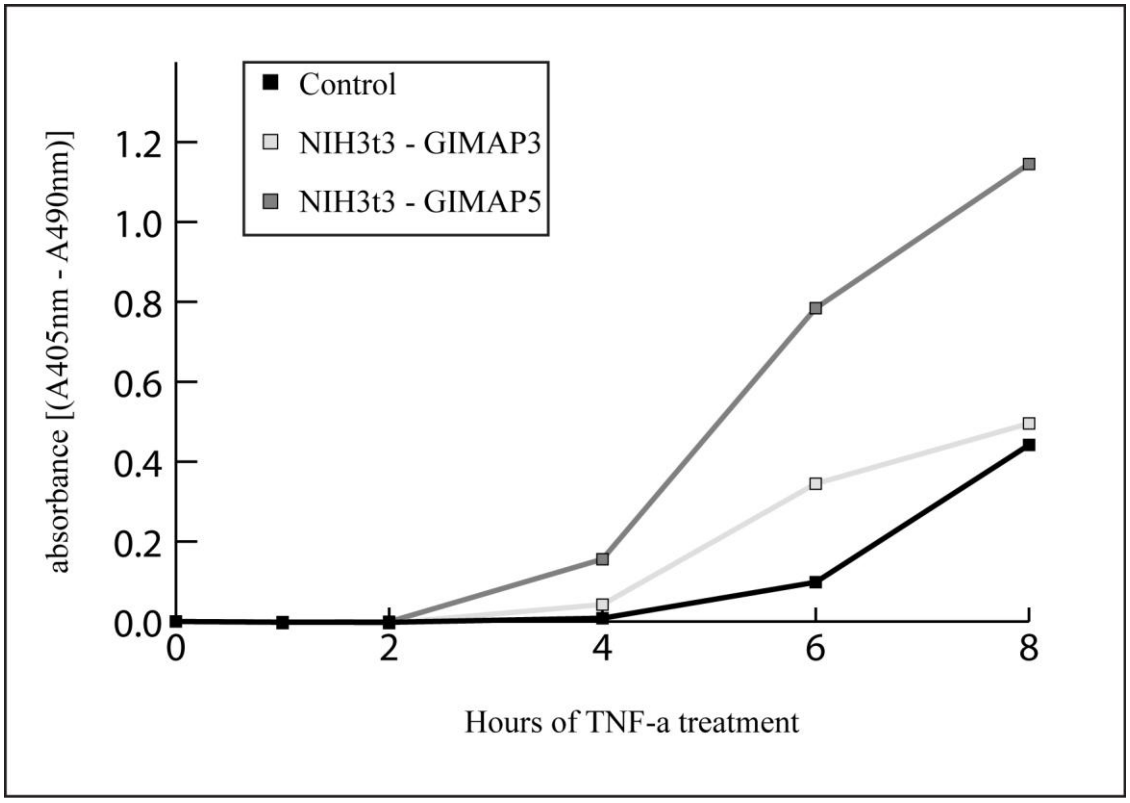

Figure 4: ELISA Cell Death Assay on NIH3T3 cells expressing either Gimap3 or Gimap5. Intensity of absorbance (y-axis) indicates the intensity of cell death. Apoptosis was induced by 10 ng/ml TNF-α with 2.5 µg/ml cycloheximide and samples were collected at different time points (0, 1, 2, 4, 6 and 8 hours). Positive control sample value (A_{405}-A_{490}) was 2.6. Black boxes = Control cells (NIH3T3 with empty pBABE –vector), light grey boxes = NIH3T3–Gimap3, dark grey boxes = NIH3T3-Gimap5. Result shows that the control and Gimap3 samples have about the same amount of dead cells. Gimap5 sample again has slightly more dead cells compared to control.

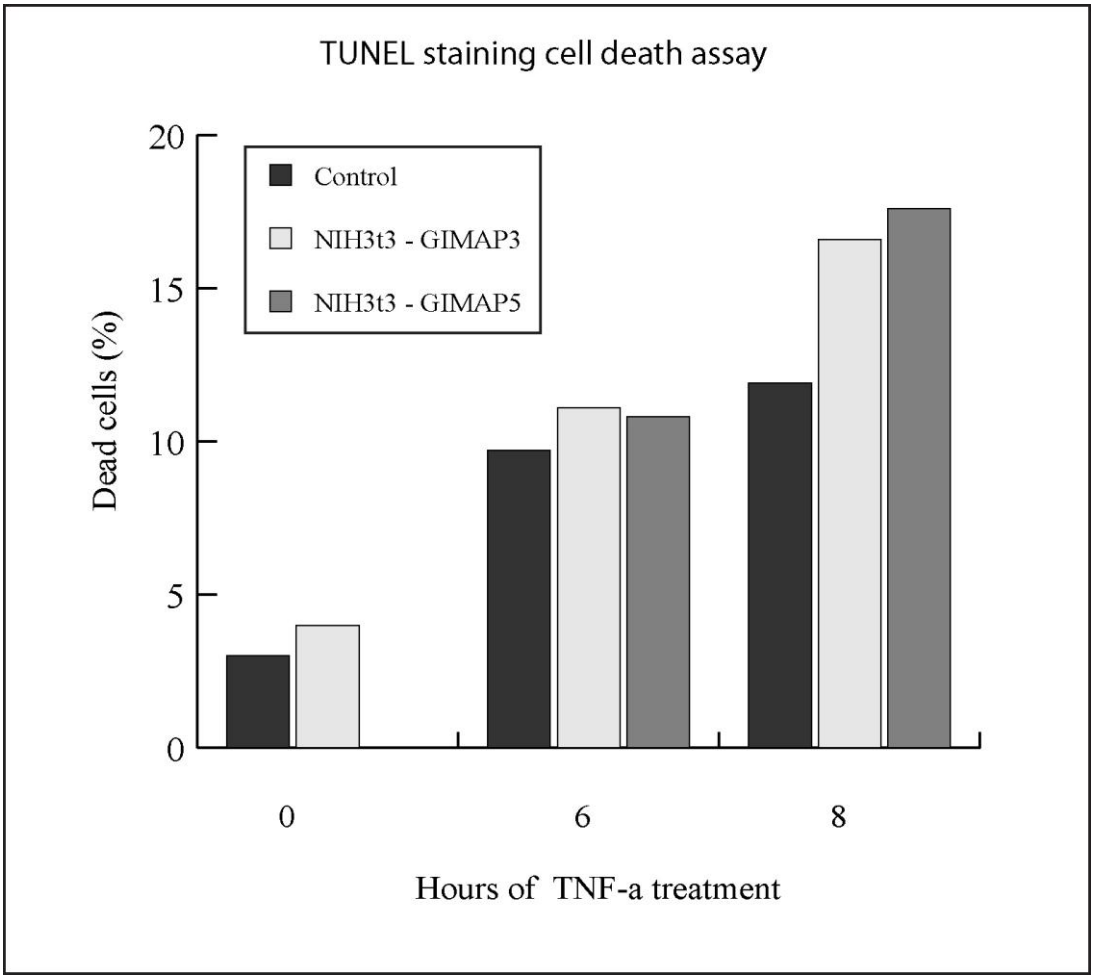

Figure 54: TUNEL staining of NIH3T3 cells expressing Gimap3 or Gimap5. Assay is based on staining of fragmented DNA with fluorescent antibody. Apoptosis was induced by 10 ng/ml of TNF-α with 2.5 µg/ml cycloheximide for 6 and 8 hours. The minimum of 300 cells from each sample were counted and percentage of dead cells among those are reported on y-axis. Only cells showing high intensity of fluorescence were considered as dead cells. Black column = Control cells (NIH3T3 with empty pBABE), light grey column = NIH3T3-Gimap3, dark grey column = NIH3T3-Gimap5. Result shows that neither Gimap3 or Gimap5 protects from TNF-α induced apoptosis, actually the number of dead cells was slightly lower in control sample.

In both of cell death measuring assays the results were congruent with each other. There was no protective effect seen despite GIMAP3 or GIMAP5 was expressed. In fact it seems like intensity of apoptosis increased slightly in cell lineages which expressed GIMAP3 or GIMAP5 (Figures 4 and 5).

HA-tag gets cleaved during TNF-α induced apoptosis

In the experiment we used NIH3T3 cells expressing HA-tagged Gimap3. Apoptosis was induced by treatment with TNF-α and samples were taken from different time points. Two similar western blots were made with a difference that other membrane was stained with Gimap3 antibody and other one with anti-HA.

On anti-HA blot a disappearance of HA-signal occurs during apoptosis induction. When stained with Gimap3 antibody, a small transition of the protein size is seen after 4 hours. After this surprising result we found out that it's been previously reported that HA-tag gets cleaved by caspase 3/7 during TNF-α induced apoptosis because it has a caspase 3/7 cleavage site (DVPD sequence) (Schembri, 2007) (Figure 6).

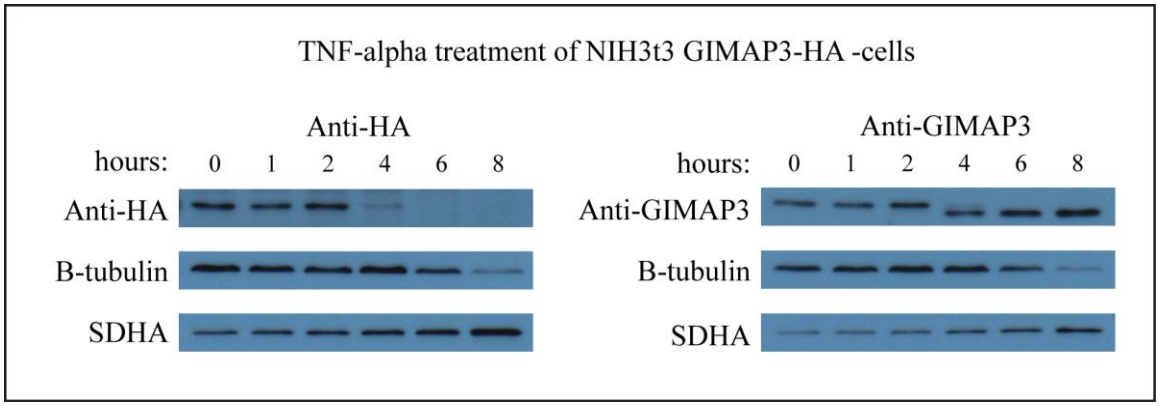

Figure 6: NIH3T3 cells expressing Gimap3-HA were treated with 10ng/ml TNF-α with 2.5µg/ml cycloheximide for 0, 1, 2, 4, 6 and 8 hours. Two similar blots were made, other one stained with HA antibody (left) and other one with Gimap3 antibody (right). SDHA and β-tubulin antibodies were used as loading controls.

Signal of HA-antibody vanishes after 4 hours of TNF-α treatment. When stained with Gimap3 antibody, a small transition in protein size occurs which seems to be due to HA-tag cleavage/degradation.

Gimap3 loses immunoreactivity during apoptosis

Since HA-tag gets cleaved during TNF-α induced apoptosis, we used Gimap3 with no tag in the following experiments. This way we could avoid the size transition due to HA-tag cleavage. Gimap5 is known to function in apoptosis regulation against extracellular apoptotic factors (Sandal et al., 2003) and due to it's homology with Gimap3 they might

share same kind of regulatory mechanism. Therefore Gimap5 was included to the experiment to see if they would show similar pattern in antibody stainings. NIH3T3 cells overexpressing c-myc conjugated Gimap5 were used in the experiment because we had no endogenous antibody at the time.

NIH3T3 cells were used and treated for 0, 4, 8 and 16 hours with 1µM STS or 10 ng/ml TNF-α with 2.5 µg/ml of cycloheximide. Gimap3 and c-myc antibodies were used to detect Gimap-proteins. Since caspase-3 activation (cleavage) is essential for apoptotic structural changes (Janicke et al., 1998), we used cleaved caspase-3 antibody to determine if apoptosis was properly induced.

Both STS and TNF-α induced apoptosis seem to decrease the amount of Gimap3 significantly. Clear effect can be seen in STS treated cells after 16 hours of incubation and in TNF-α cells already after 8 hours. TNF-α treatment appears to be also more fatal to cells since β-tubulin signal almost vanishes after 16 hours suggesting that cytoskeleton gets disintegrated. In contrast to dramatic effect of apoptosis on Gimap3 signal, Gimap5 instead decreases only slightly or not at all. (Figure 7)

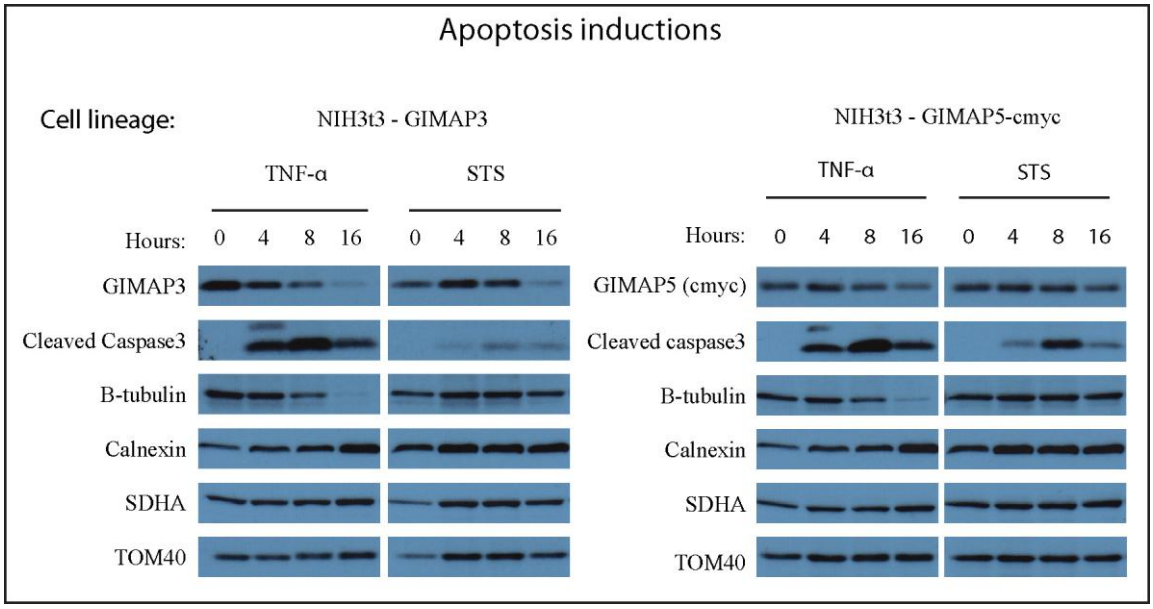

Figure 7: Apoptosis induction with 1µM STS or 10 ng/ml TNF-α with 2.5µg/ml cycloheximide were done for NIH3T3 cells expressing Gimap3 and Gimap5-cmyc. Treatment lengths were 0, 4, 8 and 16 hours. Blots were stained with Gimap3 and c-myc antibodies to detect Gimap3 and Gimap5 respectively. Cleaved caspase-3 antibody was used to indicate induction of apoptosis. Antibodies for SDHA, Calnexin, TOM40 and β-tubulin were used as loading controls.

Gimap3 signal decreases significantly during apoptosis but Gimap5 instead does not show much of a change. Signal of β-tubulin decreases most like due to disintegration of cytoskeleton caused by toxicity of TNF-α.

Overexpression of Gimap3 or Gimap5 might affect the normal function of the protein (Keita et al., 2007) and neither of the proteins are normally not expressed in NIH3T3 cells. Therefore the decrease of Gimap3 signal was also tested on primary T-cells, which naturally express Gimap3 protein. Primary T-cells were treated with TNF-α or STS for 0, 4, 20 and 24 hours with the same concentrations as NIH3T3 cells were treated previously. Decrease of Gimap3 occurred also in primary T-cells. Signal of cleaved caspase-3 at the 0 hour time point is due to personal mistake of keeping cell pellets too long in PBS in room temperature (Figure 8). Unfortunately we could not test Gimap5 response for apoptosis in primary T-cells because of the lack of specific antibody but the previous

results performed on NIH3T3 (Figure 7) would suggest that Gimap5 is not affected by TNF-a induced apoptosis or the effect is weak.

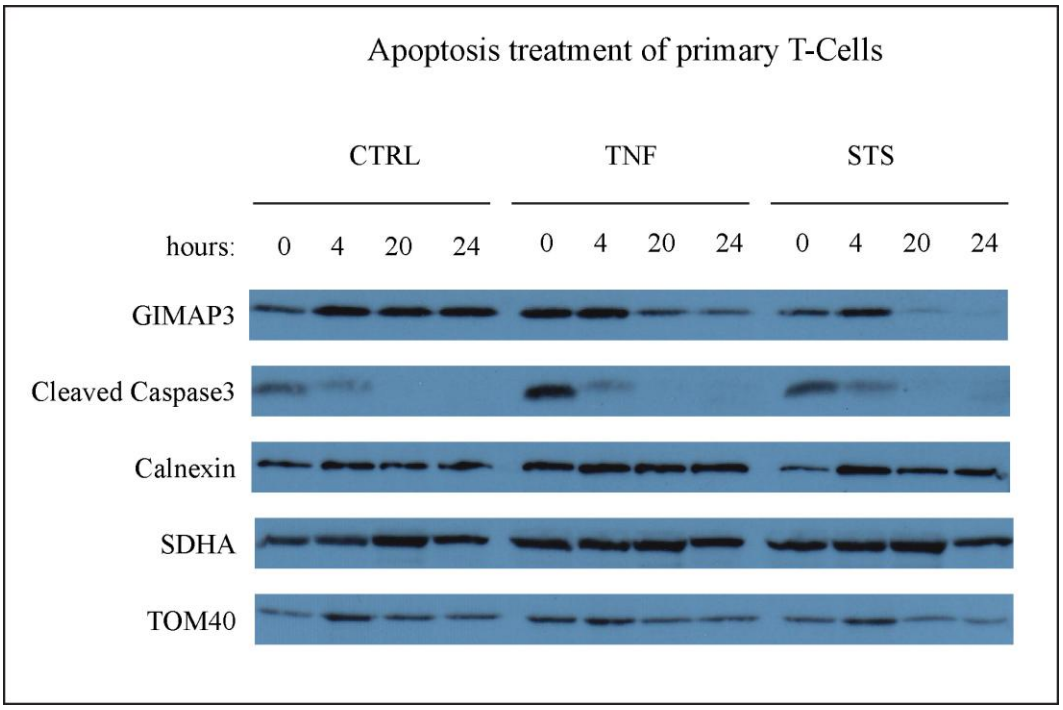

Figure 8: Apoptosis of primary T-cells were induced by 1μM STS and 10 ng/ml TNF-α with 2.5 μg/ml cycloheximide treatments. Samples were collected at different time points of treatment: 0, 4, 20 and 24 hours. Antibodies for Calnexin, SDHA and TOM40 were used as loading controls.

When compared to control, signal of Gimap3 antibody decreases during both TNF-α and STS treatment. Signal of caspase-3 activation at 0 hour time points is due to personal mistake in treating the cells pellets.

Gimap3 is known to function in T-cell maturation as expression rises during development of double positive cells into single positive (Nitta et al., 2006). If the processing of Gimap3 happens in non-activated T-cells, would we get the same result using activated T-cells? Also we could see if normal Gimap3 expression would get elevated or reduced during T-cell proliferation. We used CD3/CD28 Dynabeads (Invitrogen) to activate and proliferate T-cells, they provide primary and co-stimulatory signals required for T-cell activation and expansion without the need for present antigen. Primary T-cells were proliferated for total 4 days and 24h STS treatments were made after 1 and 3 days of stimulation. Gimap3 and cleaved caspase-3 antibodies were used and antibodies for calnexin, SDHA and TOM40 were used for loading controls. (Figure 9)

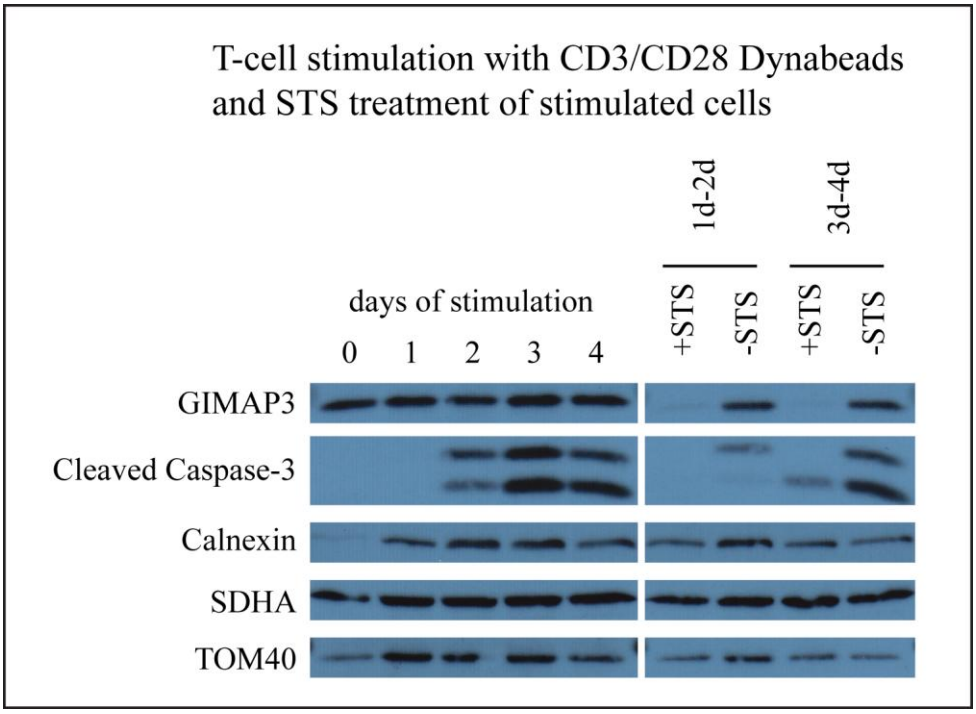

Figure 9: Primary T-cells were kept alive and proliferating by stimulation with CD3/CD28 Dynabeads for 4 days. After 1 and 3 days cells were treated with 1µM STS for 24 hours to induce apoptosis. Antibodies for calnexin, SDHA and TOM40 were used as loading controls. Caspase-3 activation can be seen during T-cell proliferation and Gimap3 signal stays stable. After 24 hours of apoptosis induction with STS, both Gimap3 and cleaved caspase-3 signals vanishes.

Signal of caspase-3 activation was seen during primary T-cell proliferation and Gimap3 signal is held stable but when apoptosis was induced with STS, Gimap3 and also cleaved caspase-3 signals vanishes. The result indicates that Gimap3 gets processed in both primary and activated T-cells during apoptosis and there were no difference in Gimap3 expression level during proliferation. (Figure 9).

Gimap3 decrease is caspase independend process

Decrease of Gimap3 might be a result of degradation, cleavage or modification of Gimap3 interfering with apoptotic pathways. If Gimap3 processing would occur downstream of caspases, it would be restrained by inhibiting caspase activation. To test this, caspase inhibitors z-VAD-fmk and z-DQMD-fmk (10µM) were used on NIH3T3 cells overexpressing Gimap3. Z-VAD-fmk is a pan-caspase inhibitor which inhibits

several caspases such as upstream caspase-8. Z-DQMD-fmk is specific inhibitor for caspase-3. Apoptosis was induced only for the needed time when clear effect on Gimap3 cleavage was seen (Figure 7), 10 ng/ml TNF-α for 8 hours and 1 µM STS for 16 hours. Treatments were made with either of caspase inhibitors and also with no caspase inhibition. Incubations without apoptosis inducing factors were made as controls on Gimap3 expression. Antibodies for Gimap3 and cleaved caspase-3 were used. Antibodies for calnexin and SDHA were used as loading controls. (Figure 10)

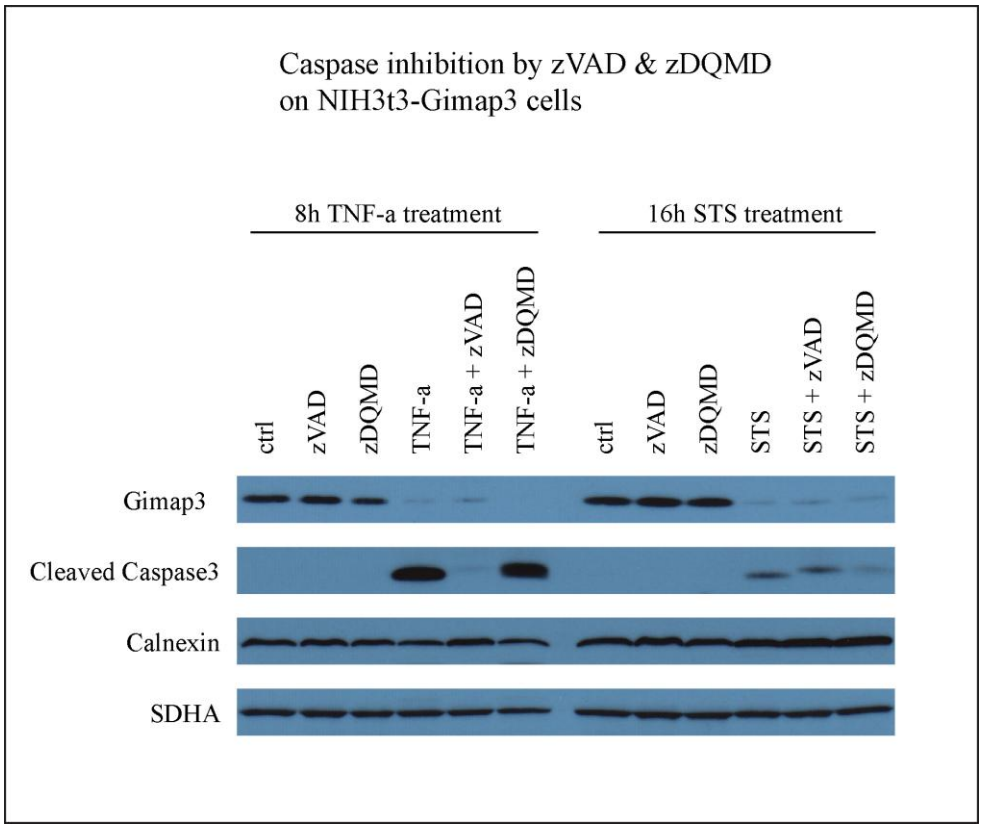

Figure 10: Apoptosis inductions with 10 ng/ml TNF-α with 2.5 µg/ml cycloheximide (for 8 hours) and 1µM STS (for 16 hours) were made on NIH3T3-Gimap3 cells in the presence of caspase inhibitors z-VAD-fmk and z-DQMD-fmk (10µM). Treatment with apoptosis inducing factor alone were done as a control. Treatments with only caspase inhibitor were done as a control for Gimap3 expression level to be compared with untreated sample. Samples were ran on 12% SDS-PAGE gel and immunoblotted. Antibodies for SDHA and calnexin were used as loading controls. Gimap3 signal decrease occurs also when cleaved caspase-3 signal is not present which is most clearly seen on TNF-α + z-VAD-fmk treatment. Cleaved caspase-3 signal in TNF-α + z-DQMD-fmk sample is most likely due to too low concentration of caspase inhibitor.

Gimap3 decrease was seen also when caspase inhibitors were used and when caspase-3 activation could not be seen indicating that Gimap3 decrease is caspase independent. In TNF-α treatment with z-DQMD-fmk there is still caspase-3 activation to be seen which is probably due to too low concentration of the caspase inhibitor. Higher amount of z-DQMD-fmk might have been toxic to the cells because of the DMSO concentration increase. (Figure 10)

Gimap3 has a degradation/modification signal near the C-terminal

Gimap3 is processed during apoptosis but it is unclear if it's degraded, cleaved or modified. Whatever the process is there should be a signaling sequence on the amino acid sequence which targets Gimap3 to get processed. If the signaling sequence would be deleted from the Gimap3 protein, we wouldn't see any decrease in the signal of Gimap3 antibody in apoptotic treatment. We tested this by producing NIH3T3 cell lineages expressing several different lengths of Gimap3 conjugated with YFP (Figure 3) and checking the Gimap3 antibody signal after

16 hours of 1μM STS treatment. Also control transfections were made from all the constructs which were incubated for 16h hours without STS. Two similar western blots were made from the same samples with a difference that other one was stained with Gimap3 antibody and other one with GFP antibody. (Figure 11)

STS treatment did not have an effect on YFP alone compared to untreated sample. Constructs 2 does not contain the Gimap3 peptide sequences that our Gimap3 antibody recognizes. Constructs 3-6 were detected with Gimap3 antibody and after STS treatment the signal decreased notably. All the constructs with a part of or the whole Gimap3 sequence (constructs 2-6) shows decrease also in GFP signal. These results might suggest that there is a recognition site in the sequence of the C-terminal transmembrane part of the protein which signals it to be degraded or modified so that it can't be recognized neither by Gimap3 or GFP antibodies (Figure 11).

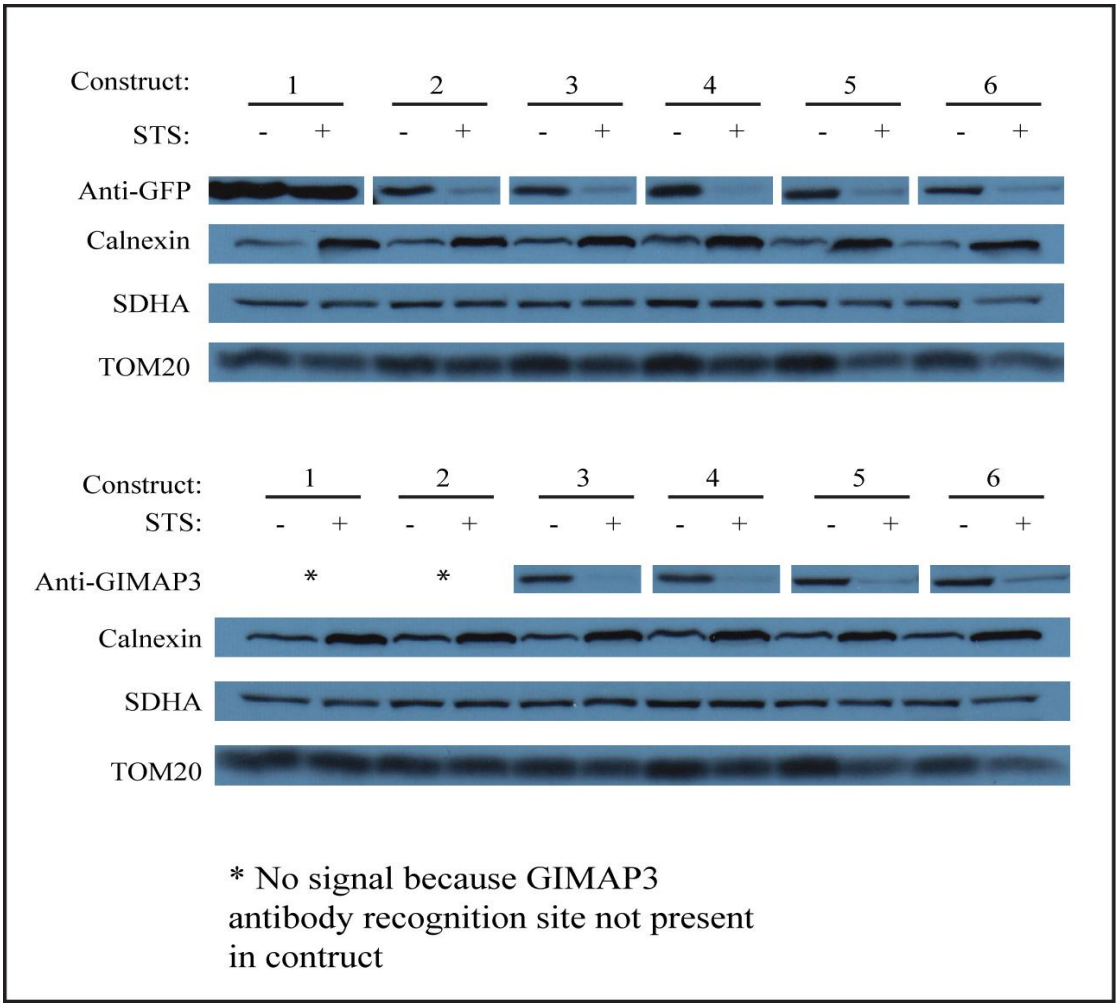

Figure 11: NIH3T3 cell lineages expressing YFP conjugated Gimap3 constructs (listed in the figure 3) were made and apoptosis was induced by 1µM STS for 16 hours. Untreated cells were used as controls. GFP antibody was used to detect YFP and calnexin, SDHA and TOM20 were used as loading controls. Gimap3 signal can't be seen in construct 2 because antibody recognition site for Gimap3 antibody is not present. In constructs 3-6 Gimap3 signal shows decrease during apoptosis. In addition also GFP signal decreases in constructs 2-6, in other words in constructs having any part of Gimap3 sequence.

Discussion

In our experiment Gimap3 got actively processed during apoptosis. The processing of Gimap3 occurred in all the cell lineages we used; overexpressing NIH3T3 lineage (Figure 7), primary T-cells (Figure 8) and activated T-cells (Figure 9). Surprisingly there was no protection against apoptosis by Gimap3 (or Gimap5) (Figures 4&5). Instead seems like the amount of dead cells was slightly increased when compared to cells carrying only control vector. Also when the cells were observed under microscope in TUNEL cell death assay, many of the Gimap3 and Gimap5 expressing cells showed shrinkage of nucleus visualized by DAPI staining (although they did not show any signal of DNA fragmentation) while in control cells most of the nucleus were normal. Since Gimap3 processing happens during the apoptosis it could be a required event for apoptosis to proceed. This contradicts to previous reports about Gimap3 being anti-apoptotic (MacMurray et al., 2002; Pandarpurkar et al., 2003). The result could also mean that regulation of apoptosis by Gimap3 is cell type dependent and the effect is different in one occasion to other. A relative protein Gimap4 is known to regulate apoptosis depending on the cell type (Cambot et al., 2002). Gimap5 is also reported to have cell type dependent function as both pro-apoptotic and anti-apoptotic protein (Dalberg et al., 2007).

If processing of Gimap3 would be a downstream process of caspases, it would be restrained by inhibiting caspase function. We tested this by using pan-caspase inhibitor and a caspase-3 specific inhibitor. Despite caspase inhibitors Gimap3 got processed which excludes the possibility that Gimap3 processing is a caspase downstream process (Figure 10). In activated T-cells activated caspase-3 decreased notably during apoptosis induction compared to control and at the same time Gimap3 signal decreases (Figure 9). This suggests that there might be some connection between Gimap3 processing and caspase-3 activation. The function of Gimap3 has to be at earlier point than downstream of caspases since caspase inhibition did not affect the fact that Gimap3 gets processed. The ability of Gimap3 to interact with Bcl-2 proteins would also suggest that it functions before caspase activation (Nitta et al., 2006). One chance might be that it has a direct effect on caspases. For example another anti-apoptotic protein family, IAPs (inhibitors of

apoptosis), can act by directly inhibiting activated caspase-3 function until it is degraded by proteasomes (Yang et al., 2000). Still there are no reports that Gimap3 would interact with caspases. If Gimap3 processing is a required event for apoptosis to happen, it could be an upstream event of caspase activation. Gimap3 might also regulate leakage of lysosomal membrane which leads to caspase activation. This far we don't know if Gimap3 localizes on lysosomal membrane like Gimap5 (Wong et al., 2010).

The Gimap3 processing involves some kind of modification so that antibody can't recognize the protein. For example Bcl-2 is phosphorylated when it regulates lysosomal membrane integrity in apoptosis (Zhao et al., 2001). Gimap3 might have same kind of mechanism of modification during apoptosis. Modification could lead to structural changes in the protein which would repress the binding of antibody to the recognition site. Gimap5 has an ability to dimerize when C-terminal helix α7 is cleaved off changing the structure of the protein or exposing essential amino acid sequence making oligomerization possible (Schwefel et al., 2010). This helix α7 region (amino acids 240-260) of the protein sequence is almost identical with Gimap3 with a difference of only one amino acid. One possibility might be that the processing of Gimap3 involves similar mechanism as Gimap5 dimerization; during apoptosis Helix α7 might get cleaved off which allows Gimap3 to dimerize. Gimap3 processing could be also interaction with another protein which binds to it, for example it's interaction with Bcl-2 might hide the recognition site of the antibody. Helix α7 might have a role not only in oligomerization but also in binding to other proteins (Schwefel et al., 2010).

To narrow down the sequence which is essential for Gimap3 processing, we made several cell lineages expressing different protein constructs of Gimap3 conjugated with YFP seen in figure 3. We found out that all of YFP-Gimap3 constructs showed decrease in both YFP and Gimap3 signal during apoptosis (Figure 11). This shows that both Gimap3 and YFP sequences gets affected in apoptosis. In the case of modification or cleavage it would take large scale structural changes to cover both antibody recognition sites. The most likely situation is that the whole protein sequence gets degraded including Gimap3 and YFP, which leads to disappearance of antibody signals.

Experiment also shows that signaling site for assumed degradation of Gimap3 lays in the sequence included in the shortest construct 2 since also that was affected (Figure 12). From amino acid sequence alignment of Gimap3 and Gimap5, we can see that they have differences on the sequence included in the YFP-Gimap3 construct 2 (Figure 12). Gimap3 sequence contains many charged amino acids which are not present in Gimap5 sequence, for example there is an additional lysine (K) residue which could be essential for ubiquitination (Figure 12). There are also additional glutamic-acid (E) and aspartic acid (D) present in the region, those could function in signaling Gimap3 for degradation. Additional charged amino acids might also explain the difference between Gimap3 and Gimap5 localization and behavior during apoptosis. The result makes clear that the highly homologous regions of Gimap3 and Gimap5 are not required for Gimap3 processing; for example previously mentioned helix α7 region is also excluded from the construct 2.

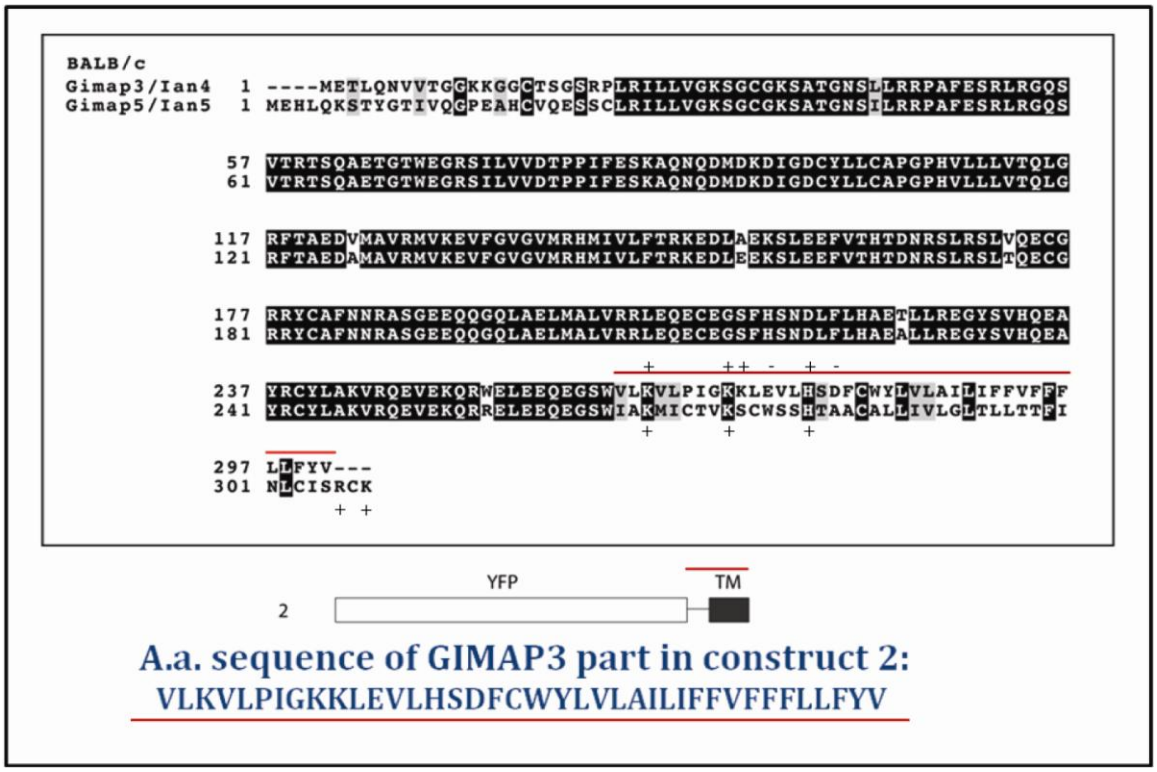

Figure 5: Amino acid sequence alignment of Gimap3 and Gimap5 with the primary structure and amino acid sequence of protein construct 2 below. Red line shows the part of Gimap3 resided in construct 2. Sequence of this construct differs highly between Gimap3 and Gimap5 and in addition there are differences

between charged amino acids (charges of amino acids marked with -/+). Those charged amino acids might play a role in signaling. Charge differences near the transmembrane part might explain the differences in localization between Gimap3 and Gimap5 and also it might be essential in recognition of Gimap3 for processing during apoptosis.

There are known mechanisms related to apoptosis where a key protein gets ubiquitinated and degraded leading to immune response and induction of apoptosis. One of them includes anti-apoptotic Bcl—2 family member Mcl-1 where it gets phosphorylated during apoptotic stimuli leading to uniquitination and degradation of Mcl-1 (Maurer et al., 2006). Loss of Mcl-1 facilitates cytochrome c release and apoptosis. Similar mechanisms are also found in plants. Research on Arabidopsis has revealed a group of pathogen effector proteins which function in defence response against microbial pathogens (Hann et al., 2010). They interact with disease-resistant proteins leading to immune response. For example an effector protein AvrRpt2 has cysteine protease activity and is able to target disease-resistant protein RIN4 for degradation (Kim et al., 2005a; Kim et al., 2005b). Loss of RIN4 leads to enhanced immune response and typically apoptosis.

Perhaps Gimap3 shares a similar degradation pattern with Mcl-1 and RIN4 proteins in regulation of immune response and apoptosis. Even though we did not see protective effect against apoptosis by Gimap3 it could still function as a regulator in the activation of apoptosis. Now we know that Gimap3 gets most likely degraded during apoptosis but the exact recognition site and essential amino acids for the process in the sequence of C-terminal are still unrevealed. Further research is needed to understand the actual function of Gimap3 and the mechanism how it gets degraded during apoptosis.

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Appendix 1
Amino acid sequences of GIMAP3 part of YFP-conjugated constructs.

Construct 2

VLKVLPIGKKLEVLHSDFCWYLVLAILIFFVFFFLLFYV*

Contruct 3

YKYLAKVRQEVEKQRWELEEQEGSWVLKVLPIGKKLEVLHSDFCWYLVLAILIF
FVFFFLLFYV*

Construct 4

VQVSGEEQQGQLAELMALVRRLEQECEGSFHSNDLFLHAETLLREGYSVHQEAY
RCYLAKVRQEVEKQRWELEEQEGSWVLKVLPIGKKLEVLHSDFCWYLVLAILIF
FVFFFLLFYV*

Construct 5

TSTYLLCAPGPHVLLLVTQLGRFTAEDVMAVRMVKEVFGVGVMRHMIVLFTRK
EDLAEKSLEEFVTHTDNRSLRSLVQECGRRYCAFNNRASGEEQQGQLAELMALV
RRLEQECEGSFHSNDLFLHAETLLREGYSVHQEAYRCYLAKVRQEVEKQRWELE
EQEGSWVLKVLPIGKKLEVLHSDFCWYLVLAILIFFVFFFLLFYV*

Construct 6

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLP
VPWPTLVTTFGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYK
TRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGI
KVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKR
DHMVLLEFVTAAGITLGMDELYKMETLQNVVTGGKKGGCTSGSRPLRILLVGKS
GCGKSATGNSLLRRPAFESRLRGQSVTRTSQAETGTWEGRSILVVDTPPIFESKAQ
NQDMDKDIGDCYLLCAPGPHVLLLVTQLGRFTAEDVMAVRMVKEVFGVGVMR
HMIVLFTRKEDLAEKSLEEFVTHTDNRSLRSLVQECGRRYCAFNNRASGEEQQG
QLAELMALVRRLEQECEGSFHSNDLFLHAETLLREGYSVHQEAYRCYLAKVRQE
VEKQRWELEEQEGSWVLKVLPIGKKLEVLHSDFCWYLVLAILIFFVFFFLLFYV

Peptide sequences for Gimap3 antibody generation (highlighted grey).

#1 amino acids 2-16 : - ETLQNVVTGGKKGGC -
#2 amino acids 259-272 : - C*-EGSWVLKVLPIGKK –
*(Cysteine added for conjugating purposes)