

Identification of interaction partners of the cytosolic polyproline region of CD95 ligand (CD178)¹

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Abstract The CD95/Fas/Apo-1 ligand (CD95L, CD178) induces apoptosis through the death receptor CD95. CD95L was also described as a co-stimulatory receptor for T-cell activation in mice *in vivo*. The molecular basis for the bidirectional signaling capacity and directed expression of CD95L is unknown. In the present study we identify proteins that precipitate from T-cell lysates with constructs containing fragments of the CD95L cytosolic tail. The determined peptide mass fingerprints correspond to Grb2, actin, β -tubulin, formin binding protein 17 (FBP17) and PACSIN2. Grb2 had been identified as a putative mediator of T-cell receptor-to-CD95L signaling before. FBP17 and PACSIN2 may be associated with expression and trafficking of CD95L. When overexpressed, CD95L co-precipitates with FBP17 and PACSIN. Protein–protein interactions are mediated via Src homology 3 (SH3) domain binding to the polyproline region of CD95L and can be abolished by mutation or deletion of the respective SH3 domain. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

CD95 ligand (CD95L, Apo-1L, FasL, CD178) is a 40 kDa type II transmembrane receptor of the tumor necrosis factor (TNF) family of death factors [1]. Its function is best documented in the context of activation-induced cell death (AICD) in the T-cell compartment where it plays a pivotal role in the regulation of thymic or peripheral selection and tolerance and immune response termination [2–7]. In addition, cytotoxic activity of T-cells and NK cells is associated at least in part with

the release of CD95L as an integral component of Golgi-derived secretory lysosomes to act as an effector molecule on CD95-positive target cells [8–10].

Several reports employing CD95L- and CD95-defective mouse strains indicated that membrane-bound CD95L might also act as a co-stimulatory molecule for T-cells. It appears that ligation of CD95L interferes with TCR signaling and coordinates cell cycle progression of T lymphocytes. Interestingly, CD95L ligation seems to affect CD4⁺ and CD8⁺ T-cell responses differently. While CD4 cells are inhibited in cell cycle progression and enter apoptosis, CD8 cells need the CD95L co-stimulus to progress through the cell cycle, to proliferate and acquire cytolytic effector function [11–12].

Several other members of the TNF family have been assigned 'bidirectional functions' as co-stimulatory receptors and as death factors for different cell types [13–24]. Yet, the available information concerning the molecules involved in reverse signal transduction is limited. Six members of the TNF family (CD27L, CD30L, CD40, CD137L, TNF α , CD95L) have a putative casein kinase I (CKI) motif (–SXXS–). Only for TNF α but not for CD95L, a role of CKI phosphorylation in reverse signal transduction has been demonstrated [25].

Increasing evidence suggests that the basis for CD95L signaling and function is located within the cytosolic polyproline stretch which forms an ideal docking region for proline-interacting Src homology 3 (SH3) or WW domains (Fig. 1). Peptides corresponding to the proline-rich region of the murine CD95L were reported to interact with the SH3 domain of the Src kinase p59^{fyn(T)} and it was suggested that CD95L membrane expression is primarily regulated through an association with the kinase [26]. Our recent studies indicate that in addition to Fyn, CD95L reverse signaling might involve other molecules of the Src family, Grb2-related adapter proteins, PI-3 kinase, Nck, p47phox and also WW domain proteins such as FE65 or formin binding protein 11 (FBP11) [27]. Moreover, deletion of the proline-rich region of CD95L completely alters the subcellular localization of the molecule [8].

To search for unknown interactors of CD95L that might help to understand the regulation of transport and function, we expressed the cytoplasmic portion of CD95L and used this to precipitate binding partners. As a result, actin, β -tubulin, Grb2, FBP17 and PACSIN2 were identified. With a focus on the latter three proteins, we demonstrate that CD95L binding is mediated by SH3 domains in all cases. This was confirmed

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Abbreviations: aa, amino acids; AICD, activation-induced cell death; CD95L, CD95 ligand; FBP, formin binding protein; FCH, Fes/Fer/CIP4 homology; GST, glutathione S-transferase; MALDI, matrix-assisted laser desorption ionization; SH, Src homology; TNF, tumor necrosis factor

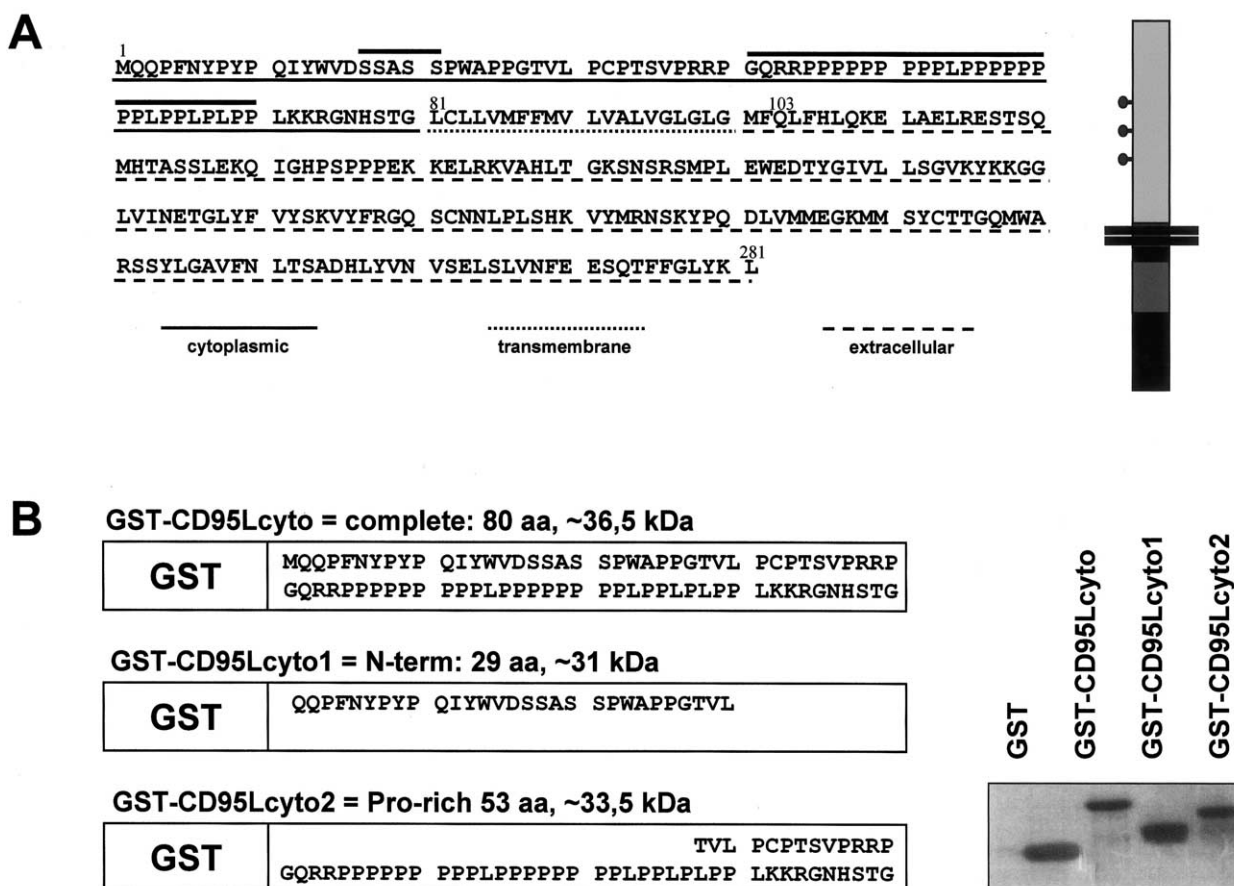


Fig. 1. A: CD95L is a member of the TNF superfamily of transmembrane type II death factors and consists of 281 aa with a cytoplasmic tail of 80 aa, a transmembrane section of 22 aa and a glycosylated (three sites indicated) extracellular part of 179 aa. The intracellular portion contains a CKI motif (aa 17–21) and a unique proline-rich stretch. B: The following GST fusion proteins were used to identify CD95L binding proteins: GST-CD95Lcyto contains the complete cytoplasmic tail, GST-CD95Lcyto1 the N-terminal region and GST-CD95Lcyto2 the proline-rich part of the molecule. All purified proteins were routinely analyzed by protein staining.

in pull-down assays using point mutations of PACSIN2 and in co-precipitation studies with SH3 domain-lacking FBP17.

2. Materials and methods

2.1. Cells

The human T-cell lines HUT78 and Jurkat E6.1 and the stable CD95L transfectants KFL-9 [28] were propagated in RPMI 1640 with 5% (v/v) fetal bovine serum and antibiotics at 37°C in a humidified atmosphere with 5% CO₂. T-cell blasts were established from Ficoll-separated human mononuclear cells by stimulation with phytohemagglutinin A (PHA) as described in [27]. Around day 14, the cells were stimulated with phorbol ester and ionomycin for 5 h to express CD95L.

2.2. Fusion proteins

The full length CD95L cytosolic region (CD95Lcyto, 80 aa), the N-terminal part with low proline content (CD95Lcyto1, 29 aa) and the membrane-proximal portion (CD95Lcyto2, 53 aa) were expressed as glutathione *S*-transferase (GST) fusion proteins (Fig. 1). Similarly, GST fusion proteins containing SH3 domains of human PACSIN2 (aa 429–486, identical to aa 388–445 of the hypothetical protein gi:12053195, accession AL136845.1), FBP17 (aa 615–672, identical to aa 524–581 of the 'unnamed protein'; gi:10435680, accession AK023681.1) and Grb2 (full length, N-terminal SH3 domain corresponding to aa 3–57 and C-terminal SH3 domain corresponding to aa 157–213) were generated. In addition, GST fusion proteins containing full length murine PACSIN3 and full length and SH3 mutated murine

(P478L) PACSIN2 were used. Of note, the SH3 regions including the critical proline at position 478 of PACSIN2 are highly conserved between mouse and man.

2.3. Precipitation and Western blotting

For immunoprecipitation or precipitation with fusion proteins, cells were lysed in Triton X-100 or Nonidet P-40 (NP40) buffers as described [27]. Supernatants were incubated for at least 90 min rotating at 4°C with antibody and protein A (i.e. anti-CD95L mAb clones G-247.4 or NOK1 from PharMingen, anti-myc from Cell Signaling Technology) or GST fusion protein and glutathione sepharose beads (Amersham/Pharmacia Biotech). Then, the beads were pelleted, washed, boiled in sample buffer and the precipitated proteins electrophoresed on SDS-polyacrylamide gels. Whole cell lysates were run with 50 µg/lane. Separated proteins were transferred to nitrocellulose membranes and blots developed with anti-CD95L (PharMingen), anti-Grb2 (Santa Cruz Biotechnology) or anti-myc (Invitrogen) and ECL detection reagents.

2.4. Two-dimensional (2D) gel electrophoresis and peptide mass fingerprinting by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

For 2D separation of CD95L-associated proteins, 10⁹ HUT78 cells were lysed in 10 ml of NP40 lysis buffer. The lysate was precleared for 90 min with GST and glutathione beads. The supernatant was then split in three aliquots and incubated with the individual CD95Lcyto fusion proteins and glutathione beads for another 90 min. The beads were then washed, dried by aspiration and dissolved for 30 min at 50°C in 50 µl of sample buffer with urea, NP40, 2β-mercaptoethanol and carrier ampholytes. Then, 210 µl of rehydration buffer were

added and incubation was prolonged for 30 min. Supernatants were recovered and transferred on Immobiline DryStrips (pH 3–10 L) and focused on IPGphor equipment (Amersham/Pharmacia Biotech) with standard conditions. After isoelectric focusing, the strips were equilibrated and the second dimension was carried out by standard SDS-PAGE on 11% gels. The gels were stained with Coomassie brilliant blue R 250. Single gel spots were punched out for in-gel digestion with trypsin (Promega) for 16 h. The samples were dissolved in aqueous TFA/acetonitrile (2:1) for mass spectrometrical analysis. The mass spectra were recorded by using a time-of-flight delayed extraction MALDI mass spectrometer (Voyager-Elite, Perseptive Biosystems). 50 mg/ml 2,5-dihydroxybenzoic acid in 0.3% aqueous TFA/acetonitrile (2:1) was used as matrix. The spectra were obtained in the reflection mode by summing 70–200 laser shots. Proteins were identified by using the peptide mass fingerprinting analysis software ProFound (<http://canada.proteometrics.com/prowl/cgi/ProFound.exe?FORM=1>). The NCBI database with the species human was used for the searches by considering at maximum one missed cleavage site, oxidation of methionine, acetylation of the N-terminus, modification of cysteines by acrylamide and a mass accuracy of 100 ppm for monoisotopic masses.

2.5. Transfection

Human 293T cells were maintained in DMEM with 10% (v/v) FBS. 6×10^5 cells were seeded in 10 cm dishes 1 day before transfection with 5 μ g of DNA using the calcium-phosphate method. Transiently transfected cells were analyzed after 24 h. As expression vectors, we used full length FBP17, FBP17(–SH3) (lacking the SH3 domain) in pcDNA3.1D/V5-His (Invitrogen), PACSIN2 in pMyc-CMV (Clontech) or the closely related PACSIN1 with a myc-tag in pcDNA3.

The pcDNA3-based CD95L expression plasmid PL217s was kindly provided by Pascal Schneider and Margot Thomé from Lausanne.

3. Results

3.1. Identification of CD95L interacting proteins by 2D-electrophoresis

CD95L fusion proteins were used in pull-down experiments to search for interacting proteins in lysates from HUT78 leukemic cells (Fig. 2). GST was used to preclear the lysates from GST-associating material (Fig. 2A). GST–CD95Lcyto1 containing only a few proline residues precipitated very low amounts of proteins (Fig. 2B). In contrast, the GST–CD95Lcyto2 fusion protein with the proline-rich fragment (Fig. 2D) and the GST–CD95Lcyto fusion protein containing the complete cytosolic tail (Fig. 2C) precipitated an overlapping set of proteins visualized by Coomassie staining and suitable for analysis by MALDI time-of-flight MS. Using high stringency criteria, seven out of eight spots detected in GST–CD95Lcyto precipitates and three out of three spots from GST–CD95Lcyto2 precipitates were identified (Fig. 2C–E). The peptide masses of the proteins excised from GST–CD95Lcyto2 precipitates were identical to the respective spots in GST–CD95Lcyto precipitates and corresponded to an unknown protein of 72 kDa (accession AK023681.1) and

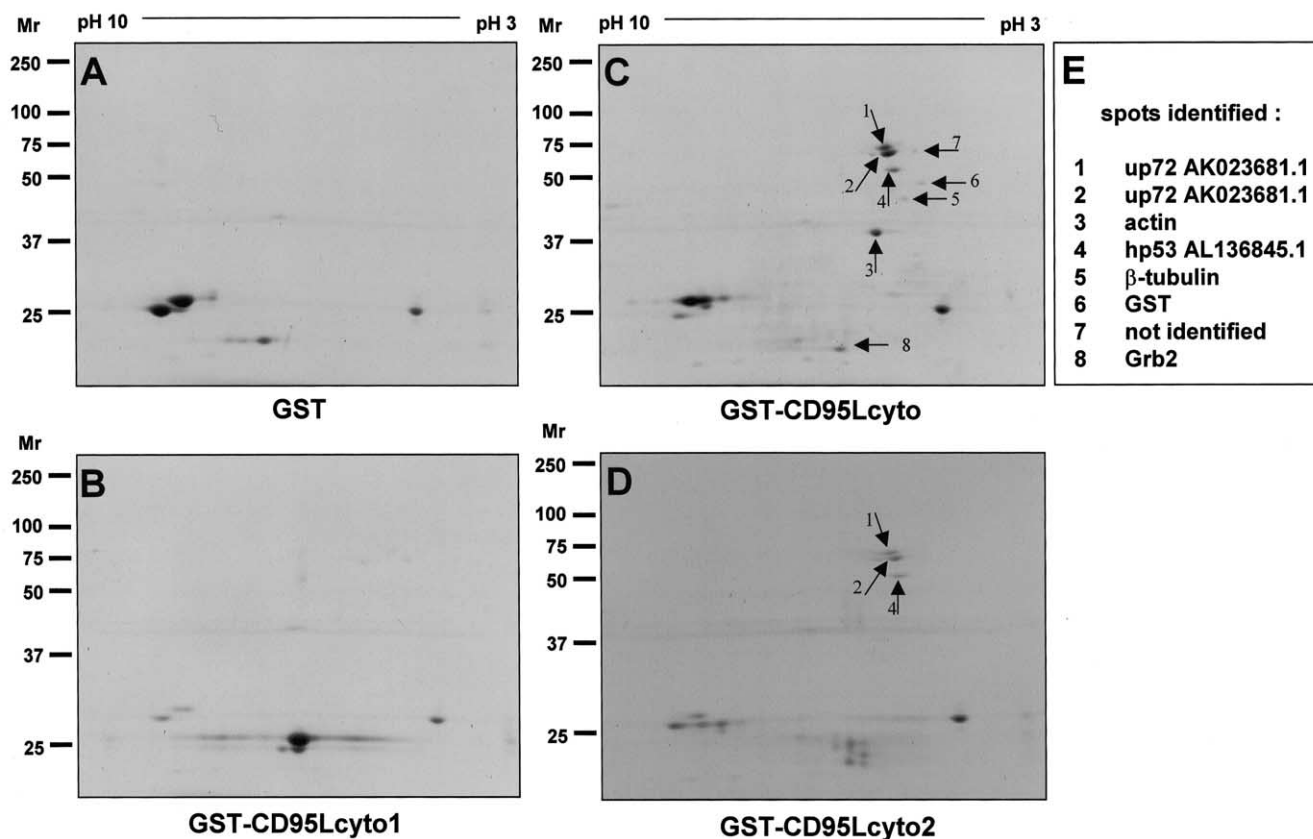


Fig. 2. GST–CD95L fusion proteins were used for precipitation from HUT78 cells. Precipitated material was separated by 2D gel electrophoresis with isoelectric focusing (pH 3–10) in the first and SDS-PAGE in the second dimension. A: GST served as a control to visualize background staining with Coomassie blue. B: Precipitation with (GST–CD95Lcyto1) did not yield any excisable spots above background. C: With GST–CD95Lcyto, a large number of additional spots were detected by Coomassie staining. D: GST–CD95Lcyto2 delivered an overlapping pattern of binding proteins. Three spots that were further analyzed corresponded to respective spots in (C). E: Eight spots of (C) were subjected to tryptic digestion and peptide mass fingerprint. Spots 1 and 2 yielded identical peptide mass fingerprints and were identified as an unnamed protein of 72 kDa (up72, AK023681.1), spot 3 as actin, spot 4 as a hypothetical protein of 53 kDa (hp53, AL136845.1), spot 5 as β -tubulin, spot 6 as GST and spot 8 as Grb2.

a hypothetical protein of 53 kDa (AL136845.1), respectively. In addition, from GST-CD95Lcyto pull-downs, actin and β -tubulin were identified as well as GST and the adapter protein Grb2. Homology searches with the sequences of the ‘unknown protein of 72 kDa’ and the ‘hypothetical protein of 53 kDa’ yielded that these proteins might in fact be identical to the FBP17 and PACSIN2, respectively.

3.2. Western Blot detection of Grb-2 associated with CD95L fragments

We recently suggested that Grb2 and other members of the Grb2/Gads/Grap family of adapter proteins might interact with CD95L [27] and thereby provide the link to TCR signaling. In Western blotting experiments, we were able to visualize (co-)precipitated Grb2 using our CD95L constructs for precipitation from different T-cell lines such as Jurkat or HUT78 (Fig. 3A). However, using different anti-Grb2 antibodies to co-immunoprecipitate CD95L from stable transfectants (KFL-9), we obtained inconsistent results. Only a single batch of a polyclonal antiserum co-precipitated CD95L as a doublet typical for these cells whereas all other anti-Grb2 antibodies or antisera failed to do so (Fig. 3B).

3.3. Association of Grb2, FBP17 and PACSIN2 with CD95L depends on SH3 binding

From our previous studies we knew that a number of SH3 domains strongly bind to the proline-rich region of CD95L

[27]. Thus, we focused on the three candidate binding partners that contain SH3 regions (Fig. 4A). We expressed full length Grb2 and both SH3 domains separately as GST fusion proteins and analyzed whether the purified fusion proteins were capable of binding to overexpressed CD95L. Clearly, the full length Grb2 fusion protein and the C-terminal SH3 domain strongly associated with CD95L in vitro (Fig. 4B). Similar results were obtained with PHA blasts where we also detected some binding to the N-terminal SH3 domain but the strongest interaction with the complete molecule (not shown).

FBP17 and PACSIN2 belong to a family of structurally related proteins that have been associated with lysosomal transport processes and signal transduction. Both proteins share an overall composition with an N-terminal FCH (Fes/Fer/CIP4 homology) domain, a central coiled-coil region and a C-terminal SH3 domain. Clearly, only fragments containing the respective SH3 domains were able to precipitate significant amounts of CD95L from KFL-9 cells (Fig. 4C) or T-cell blasts (not shown). We also generated GST fusion proteins containing SH3 domains of other members of this protein family and employed their capability to interact with CD95L. In vitro pull-down assays revealed that the SH3 domains of most proteins of this family precipitate CD95L from transfectants and T-cell blasts. Tested proteins include the closely related PACSIN1 and PACSIN3, the CD2 binding protein 1 (CD2Bp1, SH3 domain identical to the one of the PEST phosphatase interacting protein homolog), the Rho-

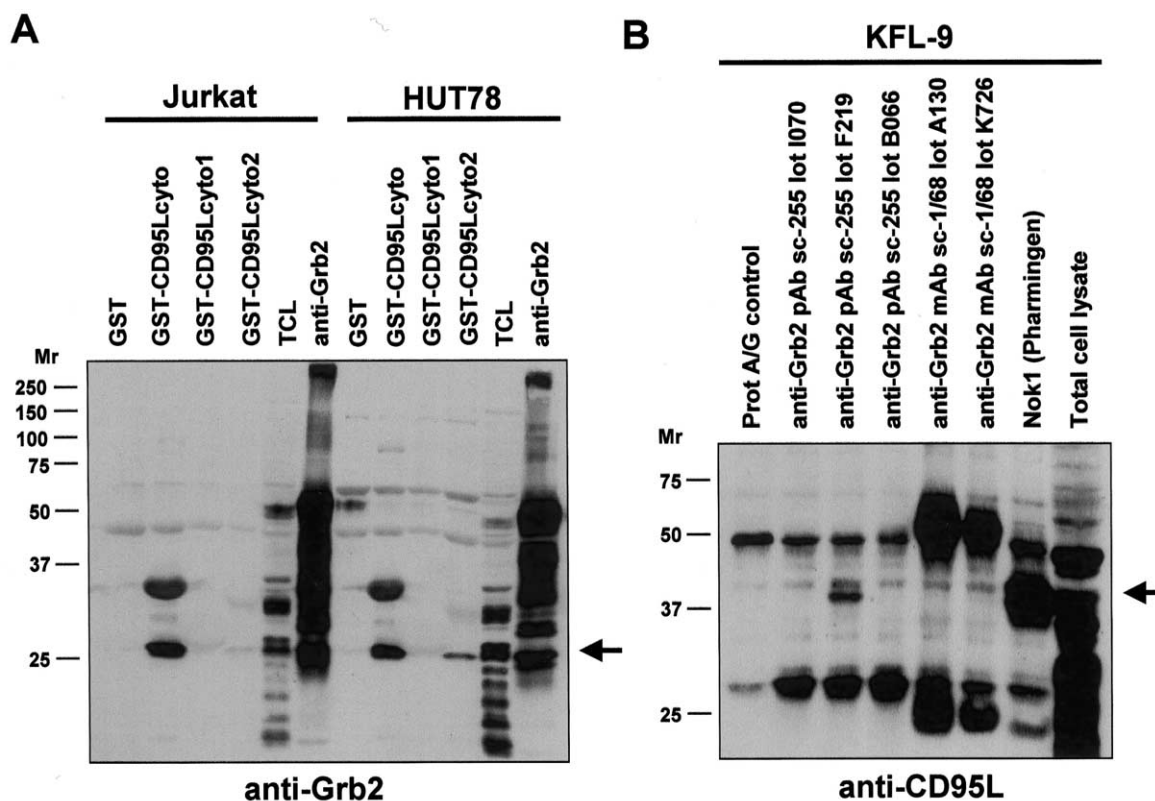


Fig. 3. A: The association of Grb2 with the proline-rich portion of CD95L was verified by Western blot analysis. GST-CD95Lcyto and GST-CD95Lcyto2 constructs precipitated Grb2 from lysates of Jurkat and HUT78 cells (see arrow). B: Failure to co-precipitate CD95L with Grb2 from CD95L transfectants. Having learned during the previous analyses of CD95L-interacting SH3 proteins that a certain batch of Grb2 antibody (polyclonal anti-Grb2 (rabbit) from Santa Cruz SC255-lot F219) in fact might co-precipitate CD95L, we used a number of other anti-Grb2 antibodies to confirm this result. Obviously, none of the other batches of this antibody nor any of the other tested antibodies was able to co-precipitate CD95L from KFL-9 transfectants. The CD95L doublet precipitated with mAb NOK-1 from KFL9 cells served as a positive control.

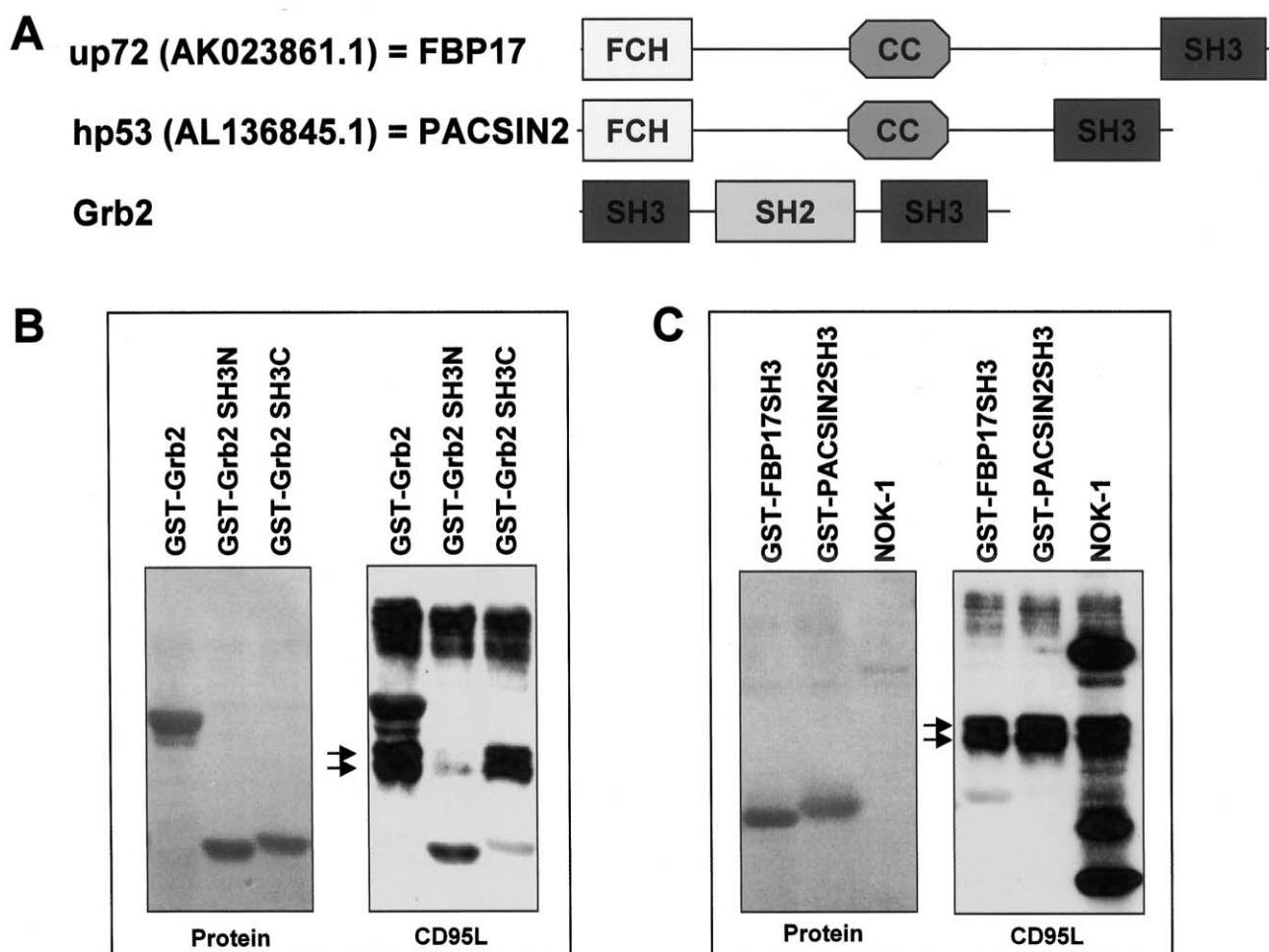


Fig. 4. A: Data bank searches revealed that the unnamed protein up72 corresponds to the FBP17 and hp53 to PACSIN2. The overall domain composition of these molecules and of Grb2 is shown. B,C: We expressed different parts of FBP17, PACSIN2 and Grb2 as fusion proteins to determine the individual CD95L-interacting domain. As expected, only the SH3 domains were able to precipitate CD95L from KFL-9 cells. In the case of Grb2, the full length fusion protein (protein staining and anti-CD95L probing are shown) and the C-terminal SH3 domain precipitated substantial amounts of CD95L (B). Also for FBP17 and PACSIN2, the isolated SH3 domains expressed as GST fusion proteins showed strong binding of CD95L in pull-down experiments (C).

GAP C1 and the RhoGAP C1-related protein with comparable domain structure KIAA0456, the FLJ00007 protein and the CDC42-interacting protein CIP4 (O15184) (data not shown). The specificity of SH3-mediated CD95L interactions with full length Grb2 and the SH3 domains of PACSIN2 and FBP17 was demonstrated in titration experiments with KFL-9 lysates (Fig. 5B).

Having shown that the SH3 domain of PACSIN2 interacts with CD95L, we were next interested to analyze whether a single amino acid exchange mutation within the SH3 domain (P478L) would result in an abrogation of binding. To this end, PACSIN2 fusion proteins were used to precipitate CD95L from stimulated PHA blasts. As shown in Fig. 6, full length (murine) PACSIN2 and PACSIN3 were as efficient in such pull-down experiments as isolated (human) SH3 domains. Importantly, the single point mutation (P478L) within the PACSIN2 SH3 domain completely abrogated CD95L binding (Fig. 6).

3.4. CD95L co-precipitates with FBP17 and PACSIN2

FBP17, FBP17-SH3 and PACSIN2 or PACSIN1 were transiently transfected with or without CD95L in 293 T-cells.

NP40 lysates of single or co-transfectants were used to precipitate CD95L with anti-CD95L mAb or the putative interactors with anti-myc mAb. Protein expression was analyzed in whole cell lysate with anti-CD95L or anti-myc mAb, respectively. Single and double transfection was successful in all cases although the amount of protein expressed varied to some extent (upper panel of Fig. 7A). Importantly, anti-myc immunoprecipitation followed by CD95L staining of the Western blot revealed co-precipitated CD95L only in cases where the expressed proteins contained SH3 domains but not when the FBP17 construct without SH3 domain was co-transfected. Interestingly, when CD95L was precipitated from double transfectants, only PACSIN2 but not FBP17 or PACSIN1 was detected as co-precipitated myc-tagged material (Fig. 7B).

4. Discussion

Recent studies with mutant mice indicate that CD95L serves not only as a death factor and ligand for CD95 but also as a co-stimulatory receptor and modulator of T-cell activation in vivo. For CD4-positive cells, CD95L engagement was reported to inhibit TCR-driven cell cycle progression,

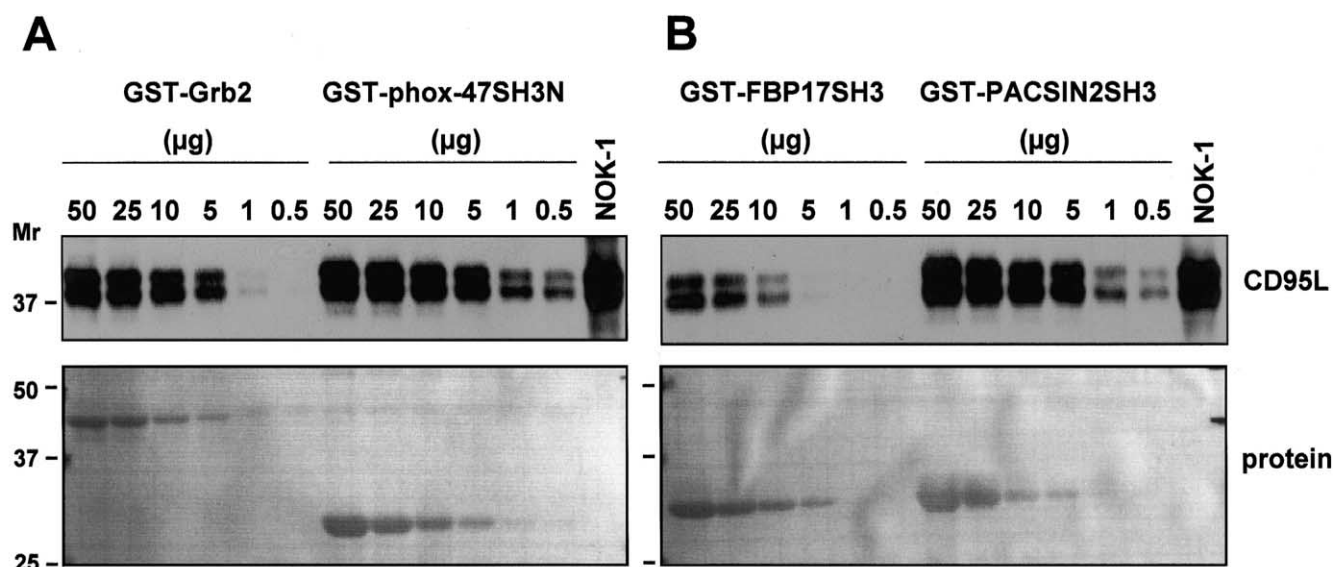


Fig. 5. The specificity of interaction with full length Grb2 and the previously identified CD95L interacting N-terminal SH3 domain phox47 (A) as well as the SH3 domains of FBP17 and PACSIN2 (B) were tested. Titration of the fusion proteins (50–0.5 μg) is nicely seen by the parallel titration of precipitated CD95L from 5×10^6 KFL-9 cells. Precipitates with anti-CD95L mAb NOK-1 of the same lysate served as a control and as an indication for 'CD95L-loading' of the transfectants. Fusion proteins were visualized by Ponceau S-staining and CD95L was detected with mAb G247-4.

associated with increased rates of apoptosis [11,12,29]. Interestingly, a population-specific bias of CD95L action was suggested by several reports showing that under similar conditions, CD8-positive cells enter the cell cycle and proliferate when CD95L is engaged together with the TCR [12,29,30].

The molecular basis for the reverse signaling of CD95L remained unclear.

Biochemically most relevant, the membrane-proximal portion of the cytoplasmic tail of CD95L contains a polyproline stretch that is likely to allow binding of proline-reactive SH3

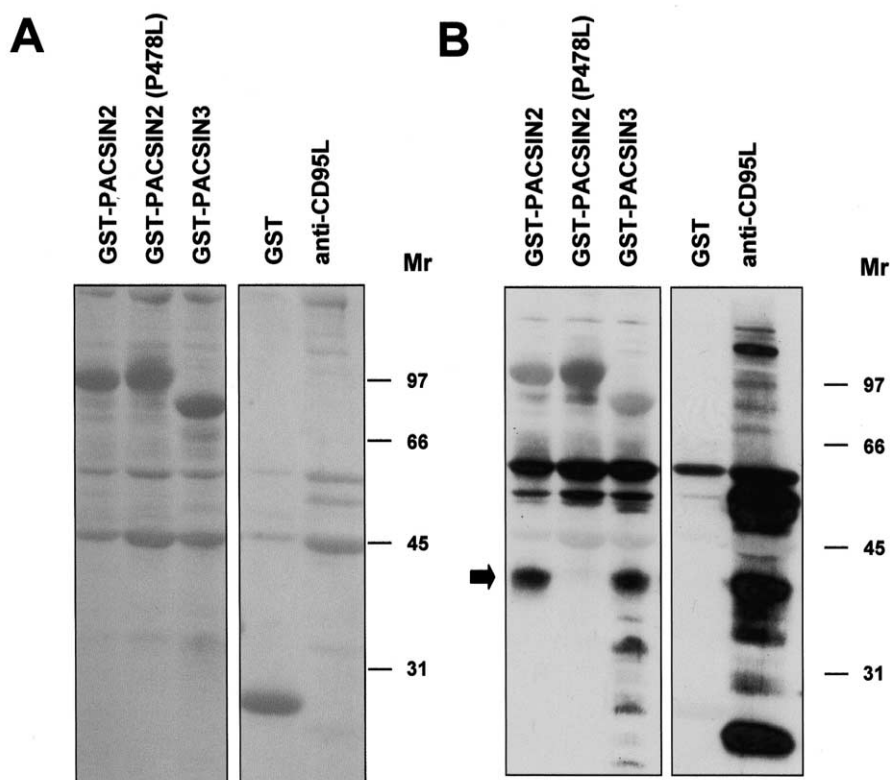


Fig. 6. GST-PACSIN fusion proteins were used to precipitate CD95L from lysates from human PHA blasts stimulated with PMA and ionomycin for 5 h. For each precipitation, 90×10^6 T-cell blasts were lysed in 1 ml lysis buffer. Precipitates with anti-CD95L mAb of the same lysate served as a positive and with GST as a negative control. Ponceau S staining (A) and anti-CD95L staining (B). Clearly, a single point mutation (P478L) within the PACSIN2 SH3 domain abrogates the CD95L-interaction.

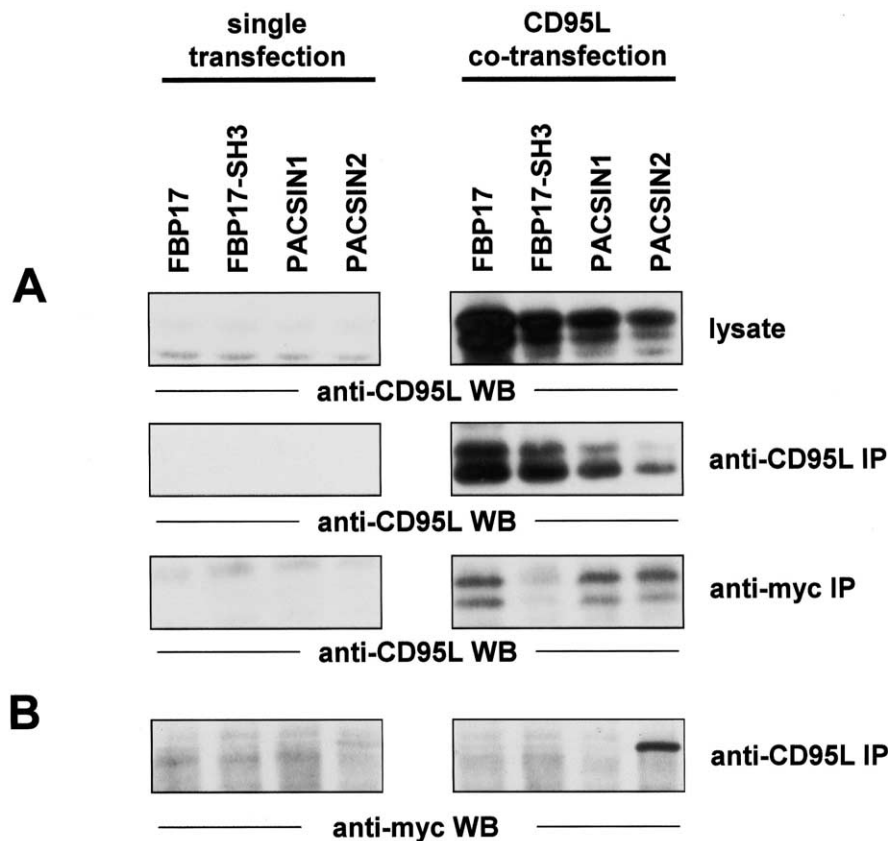


Fig. 7. FBP17, FBP17-SH3, PACSIN1 and PACSIN2 were transiently transfected in 293 T-cells as myc-tagged fusion proteins alone or co-transfected with CD95L by the calcium phosphate method. NP40 lysates were made 24 h after transfection. The expression of each protein was analyzed in 50 μ g of whole cell lysate, e.g. with anti-CD95L mAb (A, upper panel). The lysates were split into two aliquots for parallel immunoprecipitation (IP) with anti-CD95L or anti-myc mAb as indicated. Proteins were transferred to nitrocellulose and analyzed with anti-CD95L and anti-myc antibodies. A: Anti-myc immunoprecipitation followed by CD95L staining revealed co-precipitated CD95L in the presence of intact SH3 domains. B: When CD95L mAb was used to precipitate from double transfectants, only the myc-tagged PACSIN2 was stained although the amount of CD95L was clearly lower in these cells.

or WW domains [31,32]. In 1995, binding of the SH3 domain of the Src kinase p59^{lyn(T)} to CD95L-derived peptides was reported [26]. In 1996, Hachiya deposited several protein fragments in the NCBI database that they found to interact with CD95L in a yeast two-hybrid screen. All these Fas-ligand associated factors (FLAF1–3) contained either WW or SH3 domains. Although no further results were published it is now clear that the FLAFs correspond to FBP11, the c-Cbl-associated protein, and the BAI1-associated protein 2 β , respectively. Recently, we showed that a number of other SH3 or WW modules are also capable of interacting with full length CD95L [27]. Among these, Src kinases, Grb2-related adapter proteins, PI-3 kinase, Nck, p47phox, FE65 or FBP11 mediate critical steps in activation associated with antigen receptor, adhesion molecule or co-stimulatory downstream signaling.

In the present report, we identify proteins that interact with the proline-rich cytoplasmic portion of CD95L. Our results provide further evidence for the reverse signaling capacity of CD95L and strongly suggest that the expression and transport of CD95L seems to be regulated in a coordinated and complex fashion via the proline-rich region of the molecule. Importantly, we confirmed our previous result showing that Grb2 precipitates with proline-containing CD95L constructs. Grb2 is a well-defined adapter protein that plays a pivotal role in growth factor receptor as well as TCR signaling. After

TCR-activation the Grb2–Sos complex is recruited to the phosphorylated transmembrane adapter, linker for activated T-cells (LAT), thereby triggering the Ras/MAPK pathway and leading to transcription of a whole range of proteins needed for T-cell activation and differentiation [33]. An interaction of Grb2 and CD95L could thus point to a crosstalk between CD95L and the TCR.

PACSIN2 is a cytosolic adapter protein that links clathrin-dependent endocytosis to the actin cytoskeleton and is involved in the regulation of vesicular traffic [34–38]. All PACSINs contain phosphorylation sites for CKII and protein kinase C (the name PACSIN refers to 'protein kinase C and CK2 substrate in neurons'). Moreover, it was reported that PACSIN isoforms specifically interact with Sos, thereby regulating actin dynamics via MAPK signaling [38]. In the context of CD95L, PACSIN2 could be involved in the reorganization of the cytoskeleton and in trafficking of cytoplasmic vesicles leading to compartmentalization and regulation of extrusion of CD95L.

FBP17 belongs to the heterogeneous group of FBPs. In association with the various formins, these proteins regulate the organization and assembly of the actin cytoskeleton and are involved in orchestrating cell motility, adhesion and cytokinesis [39]. FBP17 contains a C-terminal SH3-domain that interacts with CD95L. FBP17 is widely expressed in a variety

of human tissues [40]. FBP17 interacts with sorting nexin 2 (SNX2) and thus provided a link between MLL, a gene connected to myelogenous leukemia, and the epidermal growth factor receptor (EGFR) pathway. In general, SNX proteins are also involved in protein trafficking and lysosomal targeting. Therefore, association of CD95L with FBP17 could once again indicate a mode of action of lysosomal transport of CD95L and also point to a signaling crosstalk between the death factor and the EGFR pathway.

We also found actin and β -tubulin in CD95Lcyto precipitates. This could be more evidence for the association of CD95L with lysosomes and/or cytoskeletal elements. Thus, β -tubulin is a major component of microtubules, arranging the mitotic spindle during cell division and regulating axonal transport and organelle positioning [41]. In addition, it was reported that tubulin plays an important role in G-protein-mediated signal transduction [42]. Obviously, even though tubulins are structural proteins, they participate in cellular signaling albeit through physical forces. In this scenario, it is discussed whether tubulins modulate the conformation of receptors or G-proteins leading either to a change in receptor–ligand interaction and/or influencing the G-protein-mediated signal.

An interesting phenomenon associated with CD95L upon TCR-activation in the course of AICD and during cytotoxicity mediated by cytolytic T-cells is that CD95L surface expression as seen on CD4-positive T-cells [43,44,29] and lysosomal expression and transport associated with cytotoxic (CD4⁺ or CD8⁺) T-cells and NK cells [8–10,45,46] may be regulated differentially and thus require distinct sets of CD95L binding proteins. In both cases, the association with regulatory elements seems to depend, however, on the proline-rich region of the cytosolic portion of the molecule [8]. It has been suggested that the sorting of the CD95L from the Golgi to the secretory lysosome may provide a general mechanism for controlling the cell surface appearance of proteins involved in immune regulation. In that sense, the finding that all tested SH3 domains of proteins characterized by FCH and SH3 domains are capable of interacting with CD95L suggests that this protein family plays a key role in directed transport in cells.

The association of membrane-associated or intracellular CD95L with a multitude of adapter proteins or enzymes may therefore not only explain the crosstalk between a death factor and antigen receptors or growth factor receptors. The present observations may also help to finally identify the biochemical basis for the functional sorting of CD95L during expression either on cell surfaces or in secretory lysosomes.

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