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Slowly getting a clue on CD95 ligand biology

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Abstract

Since the ligand for the death factor CD95 (CD95L) was identified almost a decade ago, it has been established that this molecule (CD95L, FasL, Apo-1L, CD178, TNFSF6, APT1LG1) has multiple immunoregulatory and pathophysiologically relevant functions. CD95L does not only act as a death factor when externalized with secretory lysosomes on cytotoxic T and NK cells or when expressed on $CD4^+$ T cells in the course of activation-induced cell death, it is also a key molecule for the establishment of immune privilege or tumor cell survival and may serve as a costimulatory molecule during T cell activation. Moreover, alterations of expression or shedding of different forms of CD95L are associated with many diseases including various malignancies, HIV infection, autoimmune disorders (systemic lupus erythematodes, rheumatoid arthritis), acute myocardial infarction, traumatic injury and many others. In most cases, however, the physiological link between altered CD95L expression and pathophysiology is unknown. Given the potency of the molecule to regulate death and survival of many different cell types, the control of CD95L production, transport, storage, shedding and release is of tremendous biological and clinical interest. This commentary aims at briefly summarizing the current knowledge, hypotheses and controversies about CD95L as a multifunctional ligand and receptor. It touches upon the complex networks of intracellular dynamics of protein transport and trafficking and the potential bidirectional signal transduction capacity of CD95L with a focus on molecular interactions that have been worked out over the past years.

Keywords: Costimulation; Reverse signal transduction; CD95 ligand; Protein transport; Secretory lysosomes; Death factor

1. CD95L is more than just a death ligand expressed on T lymphocytes

The CD95L (FasL, Apo-1L, CD178) is a 281 amino acid long (approximately 40 kDa) type II transmembrane protein of the TNF family of death factors (Fig. 1) [1]. Its death-inducing function is best documented in the context of AICD in T cells [2]. Outside the lymphocyte compartment, although this is object of a still-unresolved controversy, CD95L expression has been associated with the establishment of immune privilege and tumor survival [3]. Thus, constitutive membrane expression of CD95L was shown for cells within such immune privileged tissues including the eye [4], testis [5], uterus and placenta [6], lung [7], thyrocytes [8], $CD68^+$-histiocytes in the dermis [9] and intervertebral discs [10]. Under physiological conditions FasL is implicated in the control of erythroid differentiation [11], angiogenesis in the eye [12], skin homeostasis [13] and CTL-mediated killing of virally infected or transformed cells [14]. If not controlled properly, this function can degenerate into tissue destruction in autoimmune diseases. Defects in CD95L are a cause of autoimmune lymphoproliferative syndrome (ALPS), also known as Canale–Smith syndrome (CSS), a childhood syndrome involving hemolytic anemia and thrombocytopenia with massive lymphadenopathy and splenomegaly in man [15] that resembles the phenotype observed in gld mice carrying a spontaneous point mutation in the CD95L gene that leads to the production of a nonfunctional protein (Phe 273 to Leu 273) [16].

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Abbreviations: aa, amino acids; AICD, activation-induced cell death; CD95L, CD95 ligand; FBP, formin binding protein; GST, glutathione-*S*-transferase; MALDI-TOF, matrix-assisted laser disorption ionization time-of-flight; PACSIN, protein kinase C and casein kinase substrate in neurons; SH, Src homology; TCR, T cell receptor; TNF-R, tumor necrosis factor receptor.

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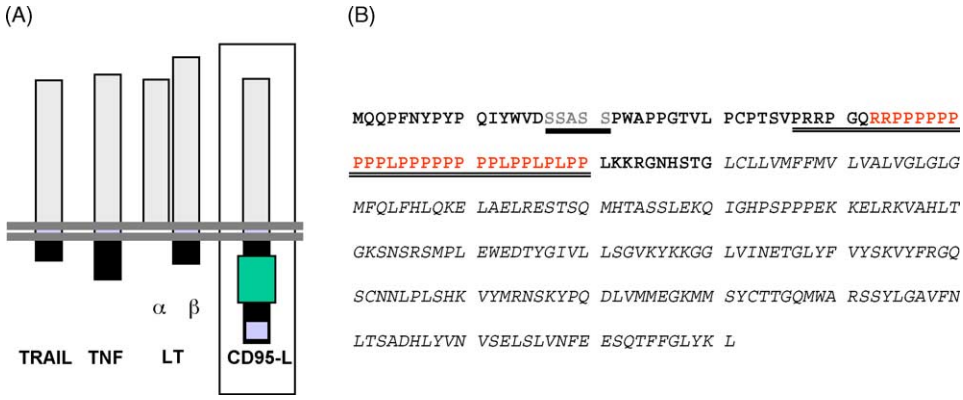

Fig. 1. Pecularities of a death factor. (A) When compared to several other members of the TNF family, the transmembrane CD95L has an extremely long cytoplasmic tail of about 80 amino acids which is highly conserved among species. (B) The amino acid sequence of CD95L offers features indicative for signal transduction and function. First, with several other members of the TNF family, it shares a casein kinase I substrate motive (solid line). However, in the case of CD95L, even this motif is special because it is a so-called "double" CKI motive that includes additional regulatory elements. Second, a very prominent proline-rich domain (PRD, double line) spanning amino acids 37–70 forms a docking site for SH3- and WW domain proteins.

2. Variants of CD95L and its receptors

The default receptor for CD95L is CD95 [17]. Human CD95L-expressing cells require direct cell-to-cell contact to generate a death-signal via the CD95 receptor. Binding to an alternative decoy receptor 3 (DcR3, TNFRSF6B) [18], or its soluble variant [19], does not result in the initiation of apoptosis. Soluble CD95L (sCD95L), a proteolytically cleaved or differently spliced form of membrane-bound CD95L (mCD95L), is most probably less capable of inducing apoptosis at normal physiological levels [20–22]. Nevertheless, for both the membrane and the soluble form, a spontaneous trimer formation has been suggested to be required for the death-signal via multimers of CD95 [23]. However, under certain conditions high concentration of the soluble forms can be observed locally that in turn result in increased apoptosis. Therefore, some recent reports suggest an alternative mechanism for the establishment of immune privilege upon local release of sCD95L in tissues where no mCD95L is expressed.

3. CD95L in T lymphocytes

During T cell development and maturation, CD95L is involved in negative selection. In this context, CD95L is supposed to act as an effector molecule (Fig. 2A) leading to the deletion of autoreactive thymocytes [24]. In mature T cells, CD95L is expressed differentially in individual T cell subsets. On $CD4^+$ cells, CD95L appears as a membrane-bound receptor on T helper1 cells following TCR stimulation by antigen, anti-TCR/CD3 antibodies, bacterial

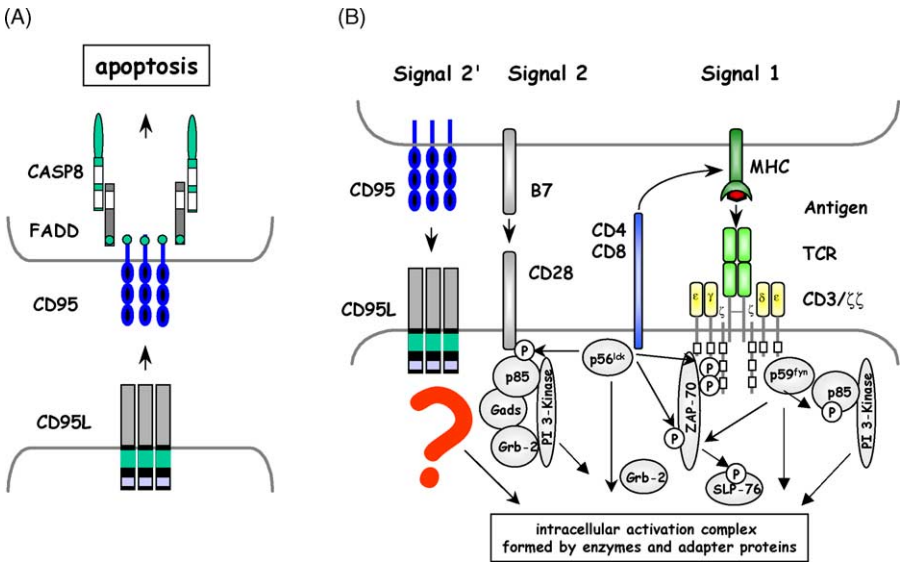

Fig. 2. Dual function of CD95L. (A) CD95L trimers function as death factors to trigger CD95-induced apoptosis via FADD and caspase activation. This is the key event during activation-induced cell death, it plays a major role in target cell cytolysis, in the establishment of immune privilege, and during tumor counterattack. (B) CD95L alters the TCR/CD3 stimulus during T cell activation. It may serve as a costimulatory molecule to either directly interfere with signal 1 given by the TCR or by interfering with signal 2 given by classical costimulatory molecules such as CD28.

superantigens or other stimuli such as phorbol esters and calcium ionophore treatment. In these cells, CD95L is a major effector molecule in the course of AICD [25–27] thereby regulating the termination of T cell responses and preventing autoimmunity. For $CD4^+$ T helper2 cells, a reduced expression or absence of CD95L under similar conditions has been reported that explains resistance of such clones to AICD [28]. However, further mechanisms have been suggested to render these cells unresponsive to CD95-mediated cell death. Some reports indicate a role for FAP1, the Fas-associated phosphatase 1, others a role of FLIP(short) or Bcl_{xl} in the generation of Th2-resistance [2]. In cytotoxic T cells (and NK cells), CD95L is expressed and synthetized upon primary stimulation and then directed to and stored in so-called secretory lysosomes [29]. Besides CD95L, this type of lysosomes also contains perforins and granzymes. Upon contact with e.g. virally infected target cell, the TCR-derived signal will lead to a redistribution of cytoskeletal elements leading to the extrusion of the cytotoxic molecules into the immunological synapse [30], surface expression of CD95L and binding to CD95 on the target cell or shedding of CD95L in the presence of a metalloproteinase.

4. CD95L as a costimulator for T cells

A series of reports employing mice defective for CD95L (*gld*) or Fas (*lpr*; lpr^{cg}) suggest a costimulatory function for CD95L in both $CD8^+$ and $CD4^+$ T cell [31–34]. While $CD4^+$ cells show a reduction in TCR/CD3-induced cell cycle progression and entered apoptosis, $CD8^+$ cells require the CD95L-costimulation to rapidly progress through the cell cycle, resulting in proliferation and acquisition of cytolytic effector function [31–34] (Fig. 2B). Although the molecular basis for the reverse costimulatory signaling of CD95L remains unknown (Fig. 2B), two motifs present in the cytosolic region of CD95L are likely to be involved in the regulation of a receptor crosstalk: a casein kinase I (CKI) substrate motif and a proline-rich domain (PRD) (Fig. 1B).

5. CD95L as a member of the TNF family of bidirectional signal transducers with a CKI substrate motif

Ligands of the TNF superfamily are transmembrane proteins that share structurally related extracellular TNF homology domains (THDs) which bind to cystein-rich domains (CRDs) of their specific receptors (of the TNF-R superfamily). Ligand/receptor interactions of members of these families orchestrate the organization and function of many facettes of the immune system. Thus, they regulate key events in cellular activation, proliferation, differentiation, cell death and survival of immune cells and other tissues [35]. Since most of the TNF family members act as membrane-bound factors and require direct cell-to-cell contact, it seems obvious that signaling upon ligation might not simply be a one-way action, but rather a bidirectional crosstalk between cells and both membrane receptors involved. Although substantial *in vivo* evidence for the physiological role of reverse signaling has only been shown for CD40L [36], bidirectional (i.e. costimulatory) functions have also been described for other members of the TNF superfamily in T cells, B cells and monocytes. Thus, the ligand–receptor complexes of CD70/CD27 (TNFSF7/TNFRSF7) [37], CD153/CD30 (TNFSF8/TNFRSF8) [38,39], CD154/CD40 (TNFSF5/TNFRSF5) [36,40–42], 4-1BB-L/CD137 (TNFSF9/TNFRSF9) [43,44], OX40L/CD134 (TNFSF4/TNFRSF4) [45], TRANCE/RANK (TNFSF11/TNFRSF11) [46] LIGHT/LIGHTR (TNFSF14/TNFRSF14) [47–49] and the death factors TNF [50], TRAIL (TNFSF10) [51] and CD95L (TNFSF6) [31–34] were reported to convey retrograde signals. Of interest, so far the bidirectional signaling capacity has been associated with 6 out of 16 TNF family members (CD27L, CD30L, CD40L, CD137L, TNF-α and CD95L) that contain a putative CKI substrate motif (-SXXS-). Since such phosphorylation sites are not present in any of the remaining TNF family members, it seems likely that reverse signaling depends on this domain [52]. However, CKI-dependent serine phosphorylation of the corresponding residues upon receptor activation has exclusively been shown for TNF-α but none of the other ligands. In case of CD95L, the membrane distal N terminus in fact contains a unique 'double' CKI substrate site (amino acids 17–21 in man (-SSASS-) and 17–22 in mice (-SSATSS-)) (Fig. 1). Although the presence of such a motif (-S(P)XXS-) indicates a role for an additional kinase to regulate sensitivity of this site for CKI [52] by phosphorylation of the first serine residue, so far no experimental data are available to prove the involvement of any kinase or phosphatase in the regulation of CD95L reverse signal transduction.

6. The proline-rich domain as a unique protein-docking site of CD95L

What is quite clear by now, however, is that the proline-rich domain (PRD, spanning amino acids 37–70 of the human CD95L, Fig. 1) which is absolutely unique among all members of the TNF family, has a major functional relevance for the regulation of trafficking and expression and maybe also for reverse signal transduction. First, it was found several years ago that peptides corresponding to the proline-rich region of the murine CD95L (aa 40–77) were able to selectively interact with the SH3 domains of the Src-related kinase $p59^{fyn(T)}$ [53]. From this it was suggested that CD95L membrane expression is primarily regulated through an activation-independent

SH3-mediated association with $p59^{fyn(T)}$. In 1996, Hachiya and coworkers deposited several protein fragments in the NCBI database that were found to interact with CD95L in a yeast two-hybrid screen. Although these Fas-ligand associated factors (FLAF1-3; AAB93495, AAB93496, AAB93497) contained either a WW domain (FLAF1) or one or more SH3 domains (FLAF2,3) and were supposed to regulate CD95L stability, no further results were published. Sequence comparison reveals that FLAF1 forms part of the FBP11 (also called the huntingtin-interacting protein HYPA), FLAF2 is part of the c-Cbl-associated protein SH3P12 = 'sorbin and SH3 domain containing 1' (containing 3 SH3 domains), and FLAF3 represents a portion of the BAI1-associated protein 2 (BAP2)-beta (with a single SH3 domain). Our own studies indicated that CD95L might interact with several other molecules in addition to Fyn and the poorly characterized FLAFs [54,55]. Some of the putative interactors are proteins involved in signal transduction, others seem to be associated with transport processes, reformation of the cytoskeleton and maybe targeting to secretory lysosomes in cytotoxic T cells and NK cells.

7. Association of proteins with proline recognition domains: what we know from SH3-pull-downs

In order to identify proteins that interact with the proline-rich region of the CD95L cytoplasmic tail, we initially used two different protein-based screening approaches. First, we checked a large panel of SH3 and WW domains expressed as GST fusion proteins for their ability to precipitate CD95L from stable transfectants and T lymphoblasts [54]. Second, we constructed fusion proteins that contain the cytosolic part of CD95L with or without proline stretch and used these for pull-down assays from T cell lysates [55]. Both strategies seemed successful and the results have been published in detail elsewhere. Our analyses revealed that in addition to Fyn SH3 domains, a number of other SH3 or WW modules are also capable to effectively and specifically interact with the full length CD95L overexpressed in transfectants and inducibly expressed in stimulated human T lymphocytes. The identification of various other Src-related kinases and different adapter proteins of the Grb2-family, PI 3-kinase, NCK and Phox-47 and the WW domain proteins FE65, FBP11 and dystrophin as putative CD95L-interacting molecules, opens new insights into the potential of CD95L to regulate and to modulate activation processes in T cells and other CD95L expressing cells.

8. Association of proteins with proline recognition domains: what we know from CD95L-pull-downs

We also used a more direct approach to identify proteins that interact with the proline-rich cytoplasmic portion of CD95L. To this end, we constructed different variants of the intracellular region of human CD95L and used these to precipitate protein from T cell lysates. The precipitates were then separated by two-dimensional gel electrophoresis and sequence information obtained by MALDI-TOF-based peptide finger print analyses. The results obtained provided some more evidence for the reverse signaling capacity of CD95L but once again suggest that the proline-rich region is really critical for the expression and transport of CD95L [55]. Importantly, we verified the results of the previous screening showing that Grb2 precipitates with proline-containing CD95L constructs. Besides, with PACSIN2 and FBP17 we identified two structurally related proteins that may be involved in protein trafficking and cytoskeletal reorganization and also may relate to the presence of beta-tubulin and actin in the precipitates.

9. 'Novel' interactors for CD95L

PACSIN2 is a 53 kDa human splice variant of the rat Syndapin II, a member of a family of cytosolic adapter proteins that link clathrin-dependent endocytosis to the actin cytoskeleton and are involved in the regulation of vesicular traffic [56–60]. To date, three PACSINs are known, all of which contain phosphorylation sites for casein kinase II and protein kinase C (the name PACSIN refers to 'protein kinase C and casein kinase substrate in neurons'). In contrast to the ubiquitously expressed PACSIN2, PACSIN1 so far was not detected in cells other than neurons. Further, it was recently reported that PACSIN isoforms specifically interact with Sos, thereby regulating actin dynamics via MAP kinase signaling [61]. In the context of CD95L, PACSIN2 could be involved in the reorganisation of the cytoskeleton and in trafficking of cytoplasmic vesicles leading to compartimentalization and regulation of extrusion of CD95L. The potential association of PACSINs with the GEF Sos might also suggest a role of this interaction in downstream signaling to MAP-kinases. Most interestingly, the activity of the sheddase ADAM13 that contains three proline rich repeats in its long cytoplasmic tail, was shown to be efficiently inhibited by the SH3 domain of PACSIN2 *in vivo* [62]. However, due to the availability of only one SH3 domain per PACSIN2 molecule, both CD95L and ADAM13 either compete for this domain or are connected via multimerization of PACSIN2, probably involving the coiled-coil domain. In the competing model, the molecular switch suggests an upregulation of ADAM13 activity in cells that highly express CD95L whereas the latter hypothesis supports the opposite case. Further investigation will elucidate the precise regulatory mechanism of the ADAM13 and CD95L sorting procedure.

FBP17, a 72 kDa protein, belongs to the large heterogeneous family of formin binding proteins. 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 [63]. Interestingly, PACSINs and FBP17 are very similar with regard to their overall modular composition. Both contain a C-terminal SH3-domain that interacts with CD95L and an N-terminal FCH (Fes/CIP homology) domain that shares homology e.g. to regulators of the actin cytoskeleton in yeast. Fuchs *et al*. [64] showed that FBP17 is widely expressed in a variety of tissues in human. They demonstrated the interaction of FBP17 with sorting nexin (SNX2) and thus provided a link between MLL, a gene connected to myelogenous leukemia, and the epidermal growth factor receptor pathway [65]. In general, SNX-proteins are also involved in protein trafficking and lysosomal targeting. Therefore, association of CD95L with FBP17 could represent an alternate mode of lysosomal transport of CD95L and also point to a signaling crosstalk between the death factor and the EGFR-pathway. As mentioned, we also found actin and β-tubulin in CD95L precipitates. Although we did not yet formally prove the interactions, 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 [66]. In addition, it was reported that tubulin plays an important role in G-protein mediated signal transduction [67]. 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.

10. Manifold but still selective interactions with CD95L

In the context of reverse signaling the long and—as we already now know—by far not complete list of putative CD95L interacting proteins opens the field for speculations and models. However, one should make several points very clear: not all SH3 or WW domains bind to CD95L. First, if there is more than one SH3 domain in a given protein, binding is never seen for all SH3 domains. Second, many SH3 domains that we tested in our screening assay did not show any binding to CD95L (whether this correlates with different binding to types I or II SH3 binding regions is under investigation). Moreover, not all SH3 domain containing proteins that we found to interact with CD95L *in vitro* are present e.g. in T cells indicating a cell-type specific set of interactors and interactions regulating CD95L biology in a given cell type.

11. Speculations about reverse signaling in T cells

For T cells, the role of Src-related kinases (i.e. Lck, Fyn and Yes), PI 3-kinase, adapter proteins including Grb2, Gads, and Nck-1 in T cell activation has been extensively investigated over the past two decades (Fig. 2B). Most of these molecules mediate critical steps in the assembly of an activation complex associated with antigen receptors, adhesion molecules and costimulatory downstream signaling mediators. Since we found Grb2 in precipitates with CD95L fusion proteins, this interaction may serve as a model scenario for CD95L-to-TCR crosstalk. Grb2 is a well defined adapter protein that plays a pivotal role in signaling of the insulin receptor, growth factor receptors (e.g. EGF-R, PDGF-R and others), the TCR and others. TCR-ligation rapidly results in recruitment of the Grb2–Sos complex to the phosphorylated transmembrane adapter LAT (linker for activated T cells), thereby triggering the Ras/MAPK pathway, leading to transcription of a number of proteins required for T cell activation and differentiation (reviewed in ref. [68]). An interaction of Grb2 and CD95L could therefore point to either a competitive or agonistic crosstalk between CD95L and the TCR. This might partly explain the proliferation described for $CD8^+$ cells *in vivo* [32,34] via a Ras-Raf-MEK-MAPK pathway. Since the Grb2–Sos complex is well known to recruit the small G-protein Ras and since spatial proximity between Ras and the p110 subunit is crucial for PI 3K activation, Grb2 might not only directly provide a proliferation stimulus (like known from the EGF-R pathway) but also facilitate PI 3K activation [69,70]. The PI 3K product PIP3 was shown to activate both PDK-1 and PKB/AKT, resulting in anti-apoptotic signals in skin tumor cells [71] via phosphorylation of Bad [72] or the transcription factor FOXO3a [73]. Taken together, Grb2 and p85, both binding to the PRD via their SH3 domains, might generate both a proliferating (via Ras and MAP-kinase) and anti-apoptotic (via PKB) signal at the same time, regardless whether the CD95L extracellular domain is cleaved from the surface or not.

Our own and other laboratories' experiments to prove a more or less direct involvement of CD95L in the regulation of TCR/CD3 responses as a costimulatory molecule turned out to become really frustrating. We have to admit that so far we failed to consistently detect changes due to a direct action of CD95L in any parameter that we have analyzed (tyrosine phosphorylation, Erk activation, AKT activation). We also failed to detect changes in TCR/CD3 or phorbolester/ionophore stimulation of activated T cells. However, we know that the antibodies that we used in our studies and the FasFc fusion protein that we produced are functional at least in blocking AICD in the very same clones and lines. Thus, the exact experimental conditions to prove such kind of signaling capacity of the CD95L still need to be worked out. The analyses are further hampered by the fact that it turns out that CD95L expression is regulated far more complex and dynamically than believed and that $CD8^+$

and $CD4^+$ or Th1 and Th2 cells seem to differ in their expression, etc. Therefore, our next experiments will focus on the use of stably transfected CD95L T cell lines established in our laboratory and on crosslinking of CD95L.

12. NCK-linking CD95L, the cytoskeleton and the TCR/CD3 complex

We recently described the interaction of two of the three isolated SH3-domains of NCK-1 with the PRD of CD95L [54]. These data were confirmed by recent co-transfection, co-precipitation and co-localization studies with wildtype and mutants of NCK-1 in different cell lines (Qian *et al.*, manuscript in preparation, data presented at 'Apoptosis 2003'). NCK-1, is a small adapter protein of 377 amino acids which is built of one C-terminal SH2 and three SH3 domains. It was shown to constitutively associate with the active γ2 isoform of the serine/threonine CKI-γ2 [74] and therefore might associate the kinase with the putative CKI-motif in CD95L. As mentioned before, Watts *et al.* described the CKI-dependent phosphorylation of a corresponding motif in the TNF-R [52]. One might speculate that a similar serine-phosphorylation by a casein kinase, recruited by NCK-1, is responsible for reverse signaling through CD95L. Immunocomplex kinases assays hopefully will prove this concept soon. NCK-1 was implicated in regulation of the cytoskeleton through interaction with the Wiskott–Aldrich syndrome protein WASP [75], the WASP interacting protein WIP [76], WASP-family verprolin homologous protein WAVE1 [77], the Centaurin-α family of PIP3 binding proteins [78], the p21-activated kinase PAK1 [79], the ε-chain of CD3 [80] and others. Furthermore, Nck-1 appears to interfere directly with protein translation [81] whereas NCK-2, a protein with high homology to NCK-1, has recently been mentioned in the context of cell motility by interacting with the focal adhesion kinase FAK [82].

Taken together, although not proven yet, the interactions with SH3 domains of Src kinases, PI 3-kinase, Grb2 and NCK-1 adapter proteins strongly suggest that at least if CD95L is co-expressed with one of the many receptors that utilize these proteins for signal transduction, there might be a crosstalk via the CD95L cytoplasmic tail that influences the stimulation through the other receptor.

13. Targeting of CD95L to secretory lysosomes and the immunological synapse

An interesting phenomenon associated with CD95L upon TCR-activation in the course of AICD and during cytotoxicity mediated by cytolytic T cells is that the CD95L surface expression as seen on $CD4^+$ T cells [2,28,32] and lysosomal expression and transport associated with cytotoxic ($CD4^+$ or $CD8^+$) T cells and NK cells [29,30,83,84] may be regulated differentially and thus require distinct sets of CD95L binding proteins (Fig. 3). In both cases, the association with regulatory elements seems to depend on the proline-rich region of the cytosolic portion of the molecule [84]. It has been suggested that sorting of CD95L from the Golgi network to secretory lysosomes may provide a general mechanism for controlling the cell surface appearance of proteins involved in immune regulation. The association of membrane-bound or intracellular CD95L to proline-interacting adapter proteins or enzymes may therefore not only explain the crosstalk between the death factor and other surface receptors. Of note, also in this context important functions of the small SH3 adapters of the Grb2 family and especially NCK-1 include their involvement in the formation of the immunological synapse upon TCR ligation and the link to cytoskeletal reorganisation [80].

14. Secretory lysosomes-loaded weapons for the "kiss of death"

As mentioned before, in activated cytotoxic T cells, CD95L is sorted to secretory lysosomes [83]. These specialized lysosomal compartments (in man) contain granzymes A, B, H, K and M, pore forming perforin-monomers and, as the only transmembrane component described so far, CD95L (Fig. 3). Upon interaction with a target cell, the lipid bilayer of the secretory lysosomes, fuses with the plasma membrane, thereby releasing the soluble lysosome components and presenting mCD95L on the cell surface. This trigger of target cell apoptosis has been referred to as the "kiss of death" [85]. Unlike other proteins that are sorted to the lysosomal compartment by di-leucine or tyrosine-based sorting motifs [86,87], Griffiths and colleagues convincingly showed that CD95L is targeted to secretory lysosomes via the PRD. This study proposed a new model for targeting pathways in cells with secretory lysosomes compared to conventional lysosomes. Upon transfection, the presence of the PRD is sufficient to sort CD95L to secretory lysosomes whereas deletion of the PRD, but not of tyrosine or di-leucine motifs in the tail of CD95L (7Y-9Y-13Y and 29V-30L), results in surface expression of the protein. Over the past years, various tumors have been suggested to constitutively express CD95L on their cell membrane as a mechanism to effectively kill tumor infiltrating lymphocytes (TILs) by a mechanism termed the "Fas counterattack" [88–93]. One example that also highlights the targeting of CD95L to specific subcellular lysosomal compartments is the identification of CD95L-loaded melanosomes. In these structures FasL colocalizes with both melanosomal and lysosomal antigens. Isolated melanosomes express FasL and exert Fas-mediated apoptosis in Jurkat cells. Unfortunately, it has so far not been investigated what leads to expression and storage or secretion of CD95L in

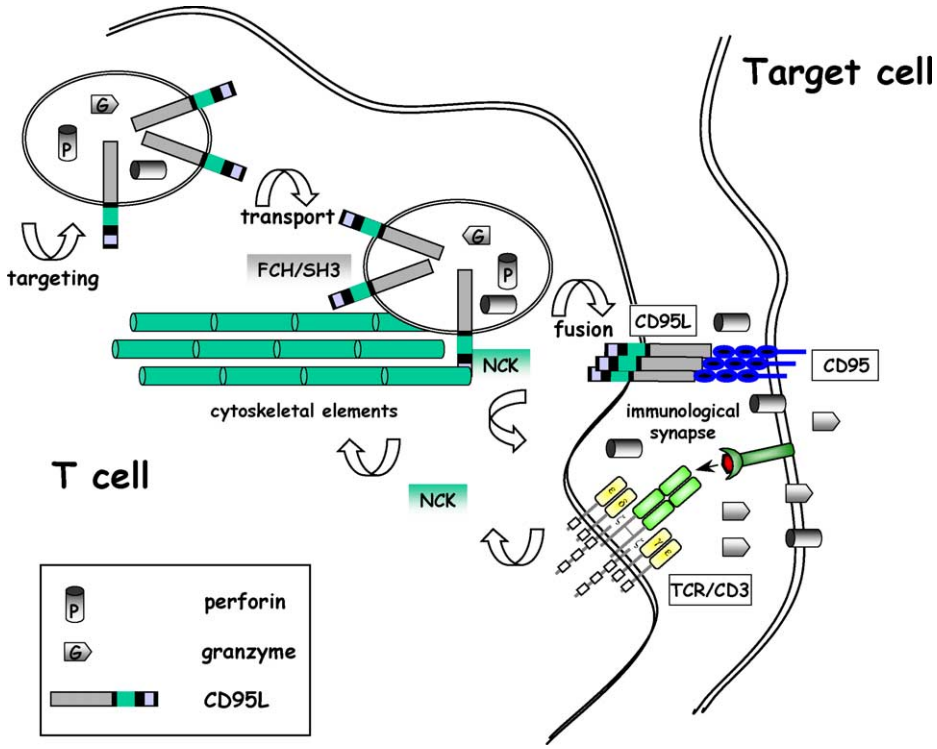

Fig. 3. Secretory lysosomes-loaded for the "kiss of death?" Recent work by Gillian Griffiths and colleagues demonstrated that in cytotoxic cells, CD95L is directed to the lysosomal compartment and that this targeting strictly depends on the poly-proline region within the cytoplasmic tail of CD95L. With regard to the different molecular interactions that we and others have shown over the past years, one might speculate that the proline-rich domain is not only required for lysosomal targeting but also for transport and release of these lysosomes into the immunological synapse. In such a scenario, protein–protein interactions with members of the FCH/SH3 family (including FBP17, PACSINs) and small adapter proteins like Grb2 and NCK would play a role in the assembly and the routing of cytotoxic lysosomes that contain granzymes, perforins and the CD95L.

such tumor cells and it will be interesting to find out alterations or mutations occurring in those malignancies might be related to mutations in the PRD of CD95L or any of the transport proteins involved. But, although a number of possible interaction partners of CD95L have been identified, regulatory molecules guiding CD95L either to the secretory lysosomes or the plasma membrane remain unknown.

15. Other regulators of CD95L transport?

As mentioned, by two-dimensional gel electrophoresis and peptide mass analyses, we recently identified two CD95L interacting molecules that had not been addressed in the context of T cell activation and function before: PACSIN2 and FBP17. Both molecules belong to a larger family of structurally related proteins which are in most cases associated with lysosomal transport processes, cytoskeletal reorganization and signal transduction. They share an overall composition with an N-terminal FCH (Fes/CIP4 homology) domain, a central coiled-coil region and a C-terminal SH3 domain. Only fragments with intact SH3 domains precipitate CD95L from T cell lines. Furthermore, SH3 domains of the family members PACSIN1 and PACSIN3, CD2 binding protein 1 (CD2BP1 = PSTPIP), RhoGAP C1 and RhoGAP C1-related protein, FLJ00007 protein and CDC42-interacting protein CIP4, bind to CD95L *in vitro*[2,3]. Future experiments will address the possibility that these proteins regulate protein (CD95L) transport in different cells differently.

16. Conclusions

Although the CD95L has been identified 10 years back, this commentary should have stressed that we are still far from understanding the complex biology of the molecule. CD95L is found in both immunologically active and immune privileged tissues and in immune cells as well as in tumor cells. The regulation of expression and the function seem to be cell-type-specific. This concerns the cytotoxic function and the potential co-stimulatory signaling capacity. To monitor a reverse signaling *in vitro* proved to be a frustrating experience, however, as has been demonstrated for TNF, the upcoming analyses of the CK1 substrate motif may shed light on this function that has been clearly demonstrated *in vivo*. In addition, the role of the soluble form is unclear and it is not known under which (pathological) conditions it is produced. In terms of

[2] Qian *et al.*, unpublished.
[3] Baum and Zörnig, unpublished.

CD95L transport and storage, we think that the identification of the PRD interacting proteins will help us to position the molecule in the complex cytoskeleton and transport network.

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