

# Multiple interactions of the cytosolic polyproline region of the CD95 ligand: hints for the reverse signal transduction capacity of a death factor<sup>1</sup>

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**Abstract** The CD95/Fas/Apo-1 ligand is expressed on activated lymphocytes, NK cells, platelets, certain immune-privileged cells and some tumor cells and induces apoptosis through the death receptor CD95/Fas/Apo-1. In murine T cells, membrane-bound CD95L (Fas ligand) also acts as a costimulatory receptor to coordinate activation and function in vivo. The molecular basis for this reverse signal transduction is yet unknown. In the present report, we identify individual interaction domains of enzymes and adapter molecules that selectively interact with full-length CD95L from transfectants and human T cells. These results may help to explain the costimulatory capacity of CD95L. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** CD95 ligand; Signal transduction; Src homology 3 domain; WW domain; Protein–protein interaction; T lymphocyte

## 1. Introduction

Over the past few years, various members of the tumor necrosis factor (TNF) superfamily of type II integral membrane proteins have been assigned functions as (co-)stimulatory receptors for different cell types. CD27 ligand (CD27L/CD70) [1], CD30 ligand (CD30L/CD153) [2,3], CD40 ligand (CD40L/CD154) [4–6], CD137L [7,8], OX40L [9] and TRANCE (RANK) [10] were reported to convey retrograde signals following engagement with their respective receptors. Importantly, also transmembrane forms of the so-called death ligands of this family including TNF $\alpha$  [11,12] and CD95 ligand (CD95L) (FasL, Apo-1L) [13–16] are able to signal bidirectionally. Utilizing CD95 and/or CD95L mutant mice, it was demonstrated that signals transduced through CD95L

regulate activation of CD4- and CD8-positive T cells in vivo. Upon stimulation with T cell receptor (TCR) agonists in the presence of CD95, cell cycle progression of CD4-positive cells was found to be inhibited [14–16], while CD8-positive cells were activated to proliferate [13–16]. The molecular basis for this differential, subset-specific, retrograde signaling remains to be elucidated. In six of the 15 known members of the TNF family (CD27L, CD30L, CD40, CD137L, TNF $\alpha$ , CD95L) a putative casein kinase I (CKI) motif (-SXXS-) has been identified and for TNF $\alpha$ , a role of CKI in phosphorylation of the cytoplasmic tail of membrane TNF has been demonstrated [17]. The role of a CKI-dependent serine phosphorylation of the corresponding motifs (amino acids 17–21 in man (-SSASS-) and 17–22 in mice (-SSATSS-), Fig. 1A) in CD95L signaling, however, remains open.

Besides the CKI motif, the 80 amino acid long cytoplasmic tail of the CD95L has another prerequisite for a signal transducing transmembrane ‘docking’ protein (Fig. 1A). The membrane-proximal portion of the molecule contains a polyproline stretch (amino acids 44–70) indicative of allowing interactions with proline-specific interaction domains such as Src homology 3 (SH3) or WW domains. In this context, it has been reported earlier that peptides corresponding to the polyproline stretch of the murine CD95L were able to selectively interact with the SH3 domain of the T cell-specific tyrosine kinase p59<sup>Fyn</sup>(T) (but not with p56<sup>lck</sup> and ras-GAP, or only weakly with SH3 domains of Grb2) suggesting that Fyn may be involved in the regulation of CD95L transport processes and/or membrane expression in T cells [18].

In the present report, we demonstrate that in addition to Fyn, a number of other SH3 or WW modules interact with the functionally active full-length CD95L molecule overexpressed in transfectants and inducibly expressed in human T lymphocytes. The identification of various Src-related kinases and different adapter proteins of the Grb2, phosphatidylinositol 3-kinase (PI 3-kinase), Nck and Phox families and also of the different WW group 1 and group 2 domain proteins (dystrophin, FE65, formin binding protein) as putative CD95L-interacting molecules opens the field to establish further insights into the molecular mechanisms of how CD95L regulates and modulates activation processes in CD95L expressing cells.

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**Abbreviations:** CD95L, CD95 ligand; CKI, casein kinase I; SH, Src homology; TCR, T cell receptor; TNF, tumor necrosis factor

## 2. Materials and methods

### 2.1. Cells

KFL-9 cells [19] are stable CD95L transfectants derived from the erythroleukemia line K562 which express low levels of CD95 and are resistant to CD95-mediated death. The cells are propagated in RPMI 1640 with 5% (v/v) fetal bovine serum (FBS, Biochrom, Berlin, Germany), antibiotics (penicillin at 100 U/ml and streptomycin at 100 µg/ml), L-glutamine (2 mM) and HEPES buffer solution (10 mM) and periodically checked for high CD95L expression. T cell blasts were established from Ficoll-separated mononuclear cells stimulated with phytohemagglutinin A (PHA; 0.5 µg/ml, Wellcome, Burgwedel, Germany). After 3 days, the cells were washed and further expanded in the presence of human recombinant interleukin (IL)-2 (10 U/ml, EuroCetus, Frankfurt, Germany) for up to 2 weeks in IL-2-supplemented culture medium (RPMI with 5% FBS and antibiotics). Dead cells were removed by Ficoll gradient centrifugation when necessary. All cells were grown at 37°C in a humidified atmosphere with 6% CO<sub>2</sub>.

### 2.2. Fusion proteins

Many of the glutathione S-transferase (GST) fusion proteins used in this study have been described and characterized [20–22]. In order to confirm the results from the initial experiments, SH3 domains of candidate proteins were generated using primers that are strictly confined to the SH3 domain-relevant sequences by unidirectional insertion of cut DNA fragments amplified by RT-PCR from either PHA blasts, HUT78, Jurkat or U937 cells into the cloning site of a pGEX-2T vector (Amersham Pharmacia Biotech). Recombinant DNA was checked by DNA sequencing after small-scale protein expression. Fusion proteins were isolated from DH5α bacteria following induction of expression with IPTG and purification by affinity binding to glutathione beads. The generation of WW domain GST fusion proteins has been previously described [23–26]. The GST-FE65 WW mutant protein (W69F, P72A) is unable to bind typical FE65-interacting sequences [23].

### 2.3. Precipitation and Western blotting

For immunoprecipitation or precipitation with fusion proteins, cells were washed once in phosphate-buffered saline and lysed in Triton X-100 (Sigma, Deisenhofen, Germany) or Nonidet P-40 (Fluka Chemie, Buchs, Switzerland) lysis buffer [1% (v/v) of detergent in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, with protease and phosphatase inhibitors aprotinin (10 µg/ml), leupeptin (10 µg/ml), 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate (all from Sigma), and 10 mM sodium fluoride (Fluka)]. Lysates remained on ice for 10 min before centrifugation at 4°C and 14 000 rpm for 10 min. Supernatants were transferred into fresh tubes for further analysis. For precipitation with fusion proteins or immunoprecipitation, supernatants were incubated for 2 h rotating at 4°C with 2–5 µg of the respective antibody or 20–50 µg of indicated GST fusion protein. 50 µl of a 50% slurry of protein A Sepharose CL4B, protein G Sepharose, or glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) were added directly to the samples. The beads were pelleted, washed thrice in cold lysis buffer, boiled in sample buffer containing β-mercaptoethanol and electrophoresed on SDS polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (Hybond C-Extra, Amersham Pharmacia Biotech). Protein loading and efficiency of transfer were monitored with Ponceau S (Sigma). The blots were blocked with 5% bovine serum albumin (Sigma) in Tris-buffered saline (TBS) for 1 h and proteins analyzed with the indicated primary (i.e. anti-CD95L mAb clone G247-4 from PharMingen) and horseradish peroxidase (HRP)-conjugated secondary antibodies (i.e. sheep anti-mouse IgG, Amersham Pharmacia Biotech) and ECL detection reagents. For analysis of WW domain binding to FasL, KFL-9 cells were lysed in RIPA100 buffer with inhibitors as described before [22] and also using the Complete® protease inhibitor cocktail (Roche, Mannheim, Germany). 0.5 mg of the protein extract was incubated with 20 µg of the indicated GST fusion protein and 20 µl of GSH beads in 0.3 ml IP buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween 20, 5% (v/v) glycerol and inhibitors) for 4 h at 4°C. Samples were washed with RIPA 100 without inhibitors and separated on an 11% SDS gel. After blocking (5% non-fat milk TBST (20 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20)) bound FasL

was detected by probing with 0.75 µg/ml anti-FasL G247-4 followed by anti-mouse HRP (Jackson ImmunoResearch, West Grove, PA, USA) and ECL visualization.

### 2.4. Peptide competition assay

Peptide competition assays were performed as described earlier [27]. Different 10mer peptides corresponding to overlapping stretches within the cytosolic portion of CD95L were purchased from Sigma/Genosys (Fig. 3A). All peptides were dissolved in water and used at concentrations specified in Section 3. For competition experiments, 10 µg of the respective fusion protein were incubated with 20 µl of glutathione beads and the indicated peptides (2 mM) for 2 h at 4°C with constant rotation. 50 µl of filtered cell lysates corresponding to 3–5 × 10<sup>6</sup> KFL-9 cells were added and incubation was prolonged for 10 min. The beads were then washed extensively and subjected to SDS-PAGE and Western blotting as described.

## 3. Results

The goal of the present study was to identify signaling proteins that define biochemical components of the reverse signal transduction through CD95L. With a focus on proline interaction domains, we screened for putative binding partners of the full-length human CD95L protein in transfectants and activated T cells.

### 3.1. CD95L is expressed as a protein doublet in transfectants

The KFL-9 cells used in this study constitutively express substantial levels of surface CD95L and low levels of CD95 [19]. The cells are resistant to CD95L and anti-CD95-mediated death and capable of inducing cell death in CD95-positive Jurkat cells via CD95L/CD95 interaction (not shown). To establish a protocol for a reproducible detection of CD95L by Western blotting, we lysed KFL-9 cells in Triton lysis buffer and subjected the lysate to immunoprecipitation with several anti-CD95L mAbs (Alf-2, generated in D.R.K.'s laboratory [19], NOK-1 and G247-4 from PharMingen, and anti-Fas ligand (FasL) clone 33, F37720 from Transduction Laboratories) or purified polyclonal rabbit antibodies (FasL (N20) sc-834 from Santa Cruz Biotechnology, and fas ligand (Ab-1), PC78 from Calbiochem). Immune complexes were separated by SDS-PAGE, and CD95L detected with mAb G247-4 (PharMingen). As shown in Fig. 1B, CD95L was immunoprecipitated with four of six of the used reagents as a distinct protein doublet of 37–42 kDa from KFL-9 cells. Of note, mAb 33 is specified by the manufacturer as applicable for precipitation of denatured protein only and pAb Ab-1 as not applicable for precipitation. For subsequent experiments, mAbs NOK-1 or ALF-2 were used as a positive control for precipitation of CD95L whereas mAb clone G247-4 was used for Western blot detection. The nature of the two different forms of CD95L reflected by the two protein bands is yet unsolved. A doublet was also seen in transiently transfected 293T cells (not shown) but not in activated T cells.

### 3.2. CD95L binds to different SH3 domains

We started to test the specificity and selectivity of SH3-mediated CD95L interactions by using fusion proteins that had been generated earlier in our laboratory [20,21]. Unexpectedly from the data published in [18], not only the SH3 domain of p59<sup>fyn(T)</sup> but also SH3 regions of p56<sup>lck</sup> and of the p85 subunit of PI 3-kinase and the Grb2 molecule fused to GST were capable of precipitating full-length CD95L from

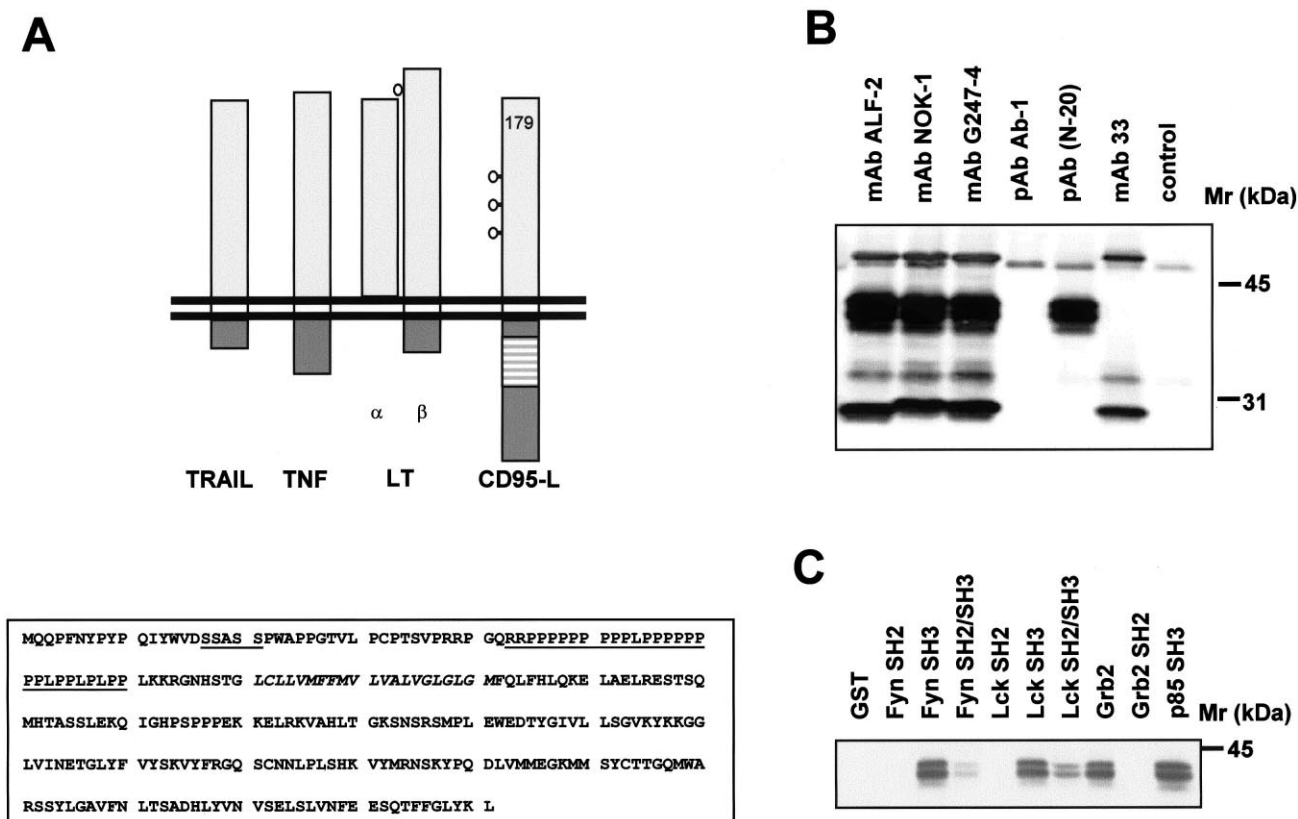


Fig. 1. Strategy to identify CD95L-interacting proteins. A: Schematic representation of TNF family death factors highlighting special features of CD95L. Membrane-bound CD95L has an 80 amino acid long cytosolic tail which is absolutely unique for TNF family members. Within the cytoplasmic region, a CKI consensus motif (SSASS) and a polyproline stretch (both underlined) have been suggested to be involved in CD95L transport and/or expression. B: Several commercially available monoclonal antibodies (mAb) or polyclonal antisera (pAb) were used to precipitate CD95L from KFL-9 transfectants. Immune complexes were precipitated with protein A Sepharose beads which also served as a control in the absence of antibodies. Anti-CD95L mAb: ALF-2, generated in D.R.K.'s laboratory, NOK-1 and G247-4 from PharMingen (San Diego, CA, USA) and Anti-Fas ligand (FasL) clone 33, F37720 from Transduction Laboratories (Lexington, KY, USA); purified polyclonal rabbit antibodies (pAb): fas ligand (Ab-1), PC78 from Calbiochem (San Diego, CA, USA) and Fas-L (N20) sc-834 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-CD95L mAb clone G-247.4 from PharMingen was used for Western blot detection. C: First indication for multiple SH3 domain binding to CD95L. In GST pull-down assays from KFL-9 lysates, CD95L precipitated with SH3 domains of Fyn, of Lck, and the p85 adapter subunit of PI 3-kinase and the full-length Grb2 protein fused to GST. Absolutely no binding was seen in GST controls or with SH2 domain containing fusion proteins.

KFL-9 transfectants (Fig. 1C) whereas GST or SH2 domain fusion proteins of the two Src-related kinases or Grb2 failed to do so. Encouraged by these initial findings, we performed pull-down assays with a large collection of SH3 domains of different enzymes and adapter proteins involved in multiple signal transduction pathways. To this end,  $1.5 \times 10^7$  KFL-9 cells per sample were lysed in 1% Triton lysis buffer and subjected to precipitation with the indicated fusion protein or GST and anti-CD95L mAb NOK-1 as controls. Proteins were electrophoresed, transferred to nitrocellulose and CD95L analyzed with mAb G247-4. Using SH3 fusion proteins of different origin, we confirmed CD95L binding to SH3 domains of p59<sup>fyn</sup>(T), p56<sup>lck</sup>, p85 and Grb2. Importantly, we identified a number of additional SH3 domains as potential CD95L binding partners (e.g. of Src-related kinases, Grb2-related adapter proteins (Grap and Gads), p47phox (=neutrophil cytosolic factor, NCF-1) and Nck (Fig. 2A,B)). Importantly, from the observed binding patterns we are able to deduce specificity and selectivity of the observed SH3 interactions. Thus, many of the tested fusion proteins did not

precipitate (or coprecipitate) any detectable CD95L. Also, and more importantly in functional terms, in most cases only one of two (Grb2 family) or two of three (Nck) SH3 domains of the respective proteins were able to interact with CD95L under these conditions.

### 3.3. CD95L/SH3 interaction from lysates of activated T lymphocytes

All SH3 interactions observed in pull-down assays with CD95L transfectants were verified in CD95L-expressing normal T cells. Peripheral blood mononuclear cells were stimulated with the T cell mitogen PHA for 3 days and expanded in rIL-2-containing medium. Around days 10–14, CD95L expression was induced with phorbol ester (TPA at 10 ng/ml) and calcium ionophore (ionomycin at 500 ng/ml) for 3–5 h before lysis of the cells. As shown in Fig. 2B, the pattern of SH3 domains precipitating CD95L from restimulated T cells (as a single protein band at 38 kDa) nearly corresponds to the one of KFL-9 cells. In our opinion some of the differences seen in the experiments in Fig. 2 may be due to experimental

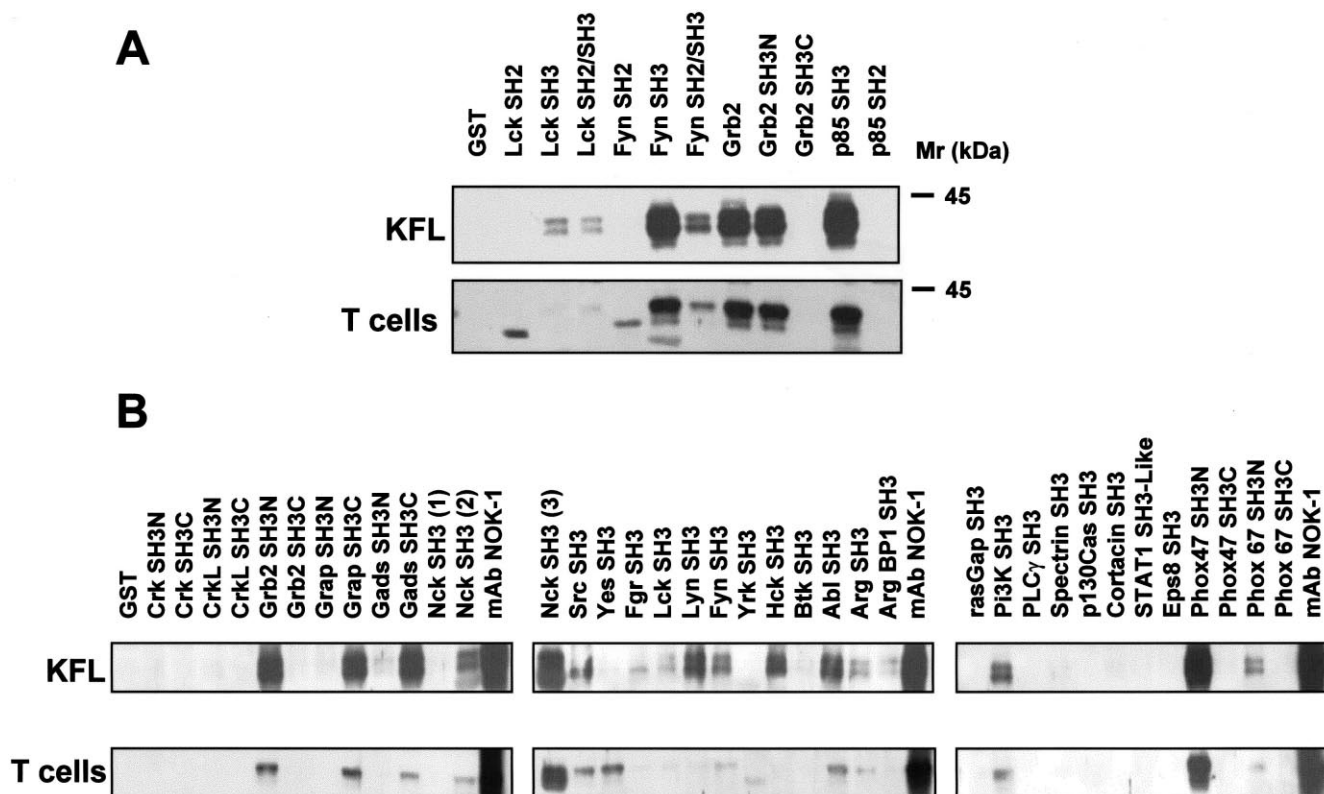


Fig. 2. Comparing precipitable CD95L from lysates of transfectants and activated T cells. A: GST-SH3 fusion proteins were used to precipitate CD95L from lysates of KFL-9 cells and phorbol ester- and calcium ionophore-stimulated PHA blasts (T cells). CD95L was precipitated as the usual doublet from transfectants and as a single band from T cells. Note: reducing the number of KFL-9 per ml of cell lysate revealed an obviously stronger association with Fyn SH3 domains as compared to Lck. This was also seen in various experiments with T cell blasts. B: Screening for CD95L-interacting SH3 domains. A large panel of different GST fusion proteins was used to identify other signaling proteins that may associate with CD95L via SH3-mediated interactions. Although the intensity of CD95L detected in precipitates from T cell blasts is much lower, the overall patterns were quite similar.

problems using primary T cells. For example, high CD95L expression was not observed in all PHA blasts tested. Also, for PHA blasts, a large number of cells (75–100×10<sup>6</sup>) per sample was necessary to get enough protein to visualize CD95L on a Western blot.

### 3.4. Characterization of putative CD95L/SH3 interaction sites

In order to define which part of the proline-rich region within CD95L mediates SH3 binding, we chose a peptide competition strategy [27]. Individual SH3 domains were pre-incubated for 2 h with the respective peptides before adding CD95L-containing KFL-9 lysates for 10 min. The proteins precipitated under these conditions were separated by SDS-PAGE, blotted and analyzed for the presence of CD95L with mAb G247-4. Not unexpectedly, peptide 4 (RRPPPPPPPP) containing two arginine residues in the context of eight prolines was most effective in competing off the SH3/CD95 association, e.g. with the N-terminal SH3 domain of Grb2, the SH3 domains of Fyn and PI 3-kinase or the C-terminal SH3 domains of Gads and Grap (Fig. 3B). This peptide contains a so-called type I SH3 recognition motif defined by the amino acid sequence RXXPPXXP with the critical N-terminal arginine (Fig. 3C). Of note, however, also other peptides (e.g. peptides 6 (PPLPPLPLPP) and 7 (PLPLPPLKKR)) reduced the level of CD95L precipitated from KFL-9 cells significantly and reproducibly.

### 3.5. CD95L also binds to a set of WW domains

WW domains are small protein modules characterized by two separated tryptophan (W) residues. They are found in a wide range of cytosolic or nuclear signaling molecules and bind proteins containing short linear peptide motifs that are proline-rich or contain at least one proline [28–32]. Thus we tested whether WW domains of different proteins were also capable of binding to the CD95L molecule. As shown in Fig. 4, WW domains of FE65 (wild-type but not mutated), FBP11 and dystrophin but not of Nedd4, YAP or Ess1 were able to precipitate CD95L from RIPA lysates of KFL-9 cells. Note that the strongest association was seen with FE65.

## 4. Discussion

Recent studies with mutant mice indicate that the CD95L, a 40-kDa transmembrane type II protein of the TNF family, serves not only as a death factor and ligand for CD95 but also as a costimulatory receptor and modulator of T cell activation *in vivo*. For CD4-positive cells, CD95L engagement was reported to inhibit TCR-driven cell cycle progression leading to increased rates of apoptotic CD4 cells [14–16]. Interestingly, a population-specific bias of CD95L action was suggested by several reports showing that under similar conditions, CD8-positive cells become activated to enter the cell cycle and to proliferate when CD95L is engaged together with

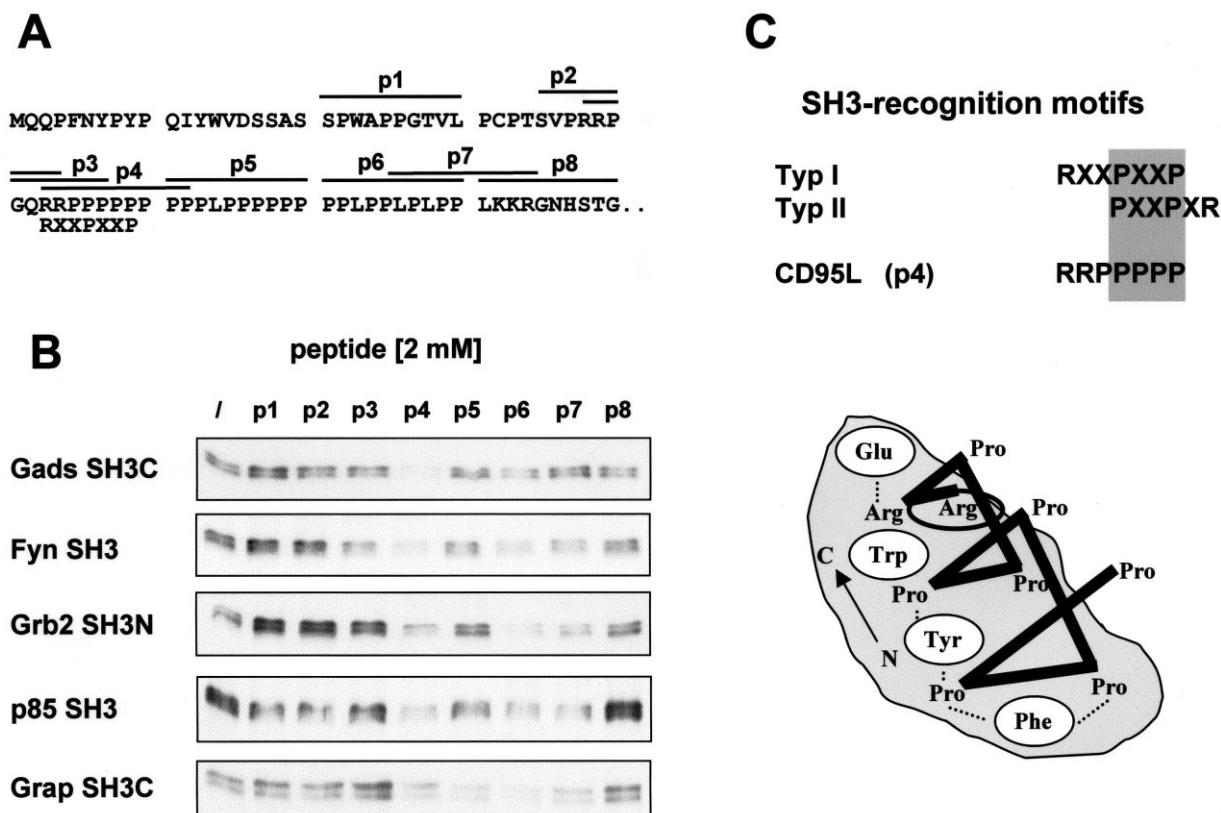


Fig. 3. Peptide competition to define CD95L/SH3 interaction sites. A: Location of peptides used for competition assays within the CD95L cytoplasmic tail. Peptides p1–p7 correspond to overlapping stretches of the proline-rich portion of CD95L and peptide p8 to the membrane-proximal 10 amino acids containing no proline. B: 10  $\mu$ g of the indicated GST fusion proteins were incubated with 20  $\mu$ l of glutathione beads and the respective peptides (2 mM) for 2 h at 4°C with constant rotation. 50  $\mu$ l of filtered cell lysates corresponding to  $3\text{--}5 \times 10^6$  KFL-9 cells were added and incubation was prolonged for 10 min. The beads were then washed extensively and subjected to SDS-PAGE and Western blotting and CD95L detection with mAb G247-4. Peptide 4 (RRPPPPPPPP) was most effective in competing off the SH3/CD95L association. C: Type I and type II SH3 interaction motifs (adapted from [54,55]). According to this definition, peptide p4 represents a typical type I SH3 binding motif.

the TCR [13–16]. The molecular basis for the reverse signaling of the death factor CD95L explaining the observed costimulatory function remained unclear.

So far, two lines of evidence have been reported that point to possible mechanisms of CD95L reverse signal transduction: (i) six of the 15 known members of the TNF family (CD27L, CD30L, CD40, CD137L, TNF $\alpha$ , CD95L) contain a so-called CKI consensus motif (-SXXS-). Only for membrane-bound TNF $\alpha$ , a role of CKI in phosphorylation of the cytoplasmic tail has been recently demonstrated and associated with reverse signaling [17], whereas the role of CKI in reverse signaling of other TNF family proteins including CD95L has not been proven. (ii) The CD95L cytoplasmic portion shows several other features unique among the protein family that make it likely to serve as a signal-transducing transmembrane protein. The length of the cytoplasmic region with 80 amino acids and the high degree of conservation amongst species by itself is an indication for function. Biochemically relevant, the membrane-proximal portion of the cytoplasmic tail contains a polyproline stretch indicative of conveying associations with proline-reactive interaction domains such as SH3 or WW domains [28,33]. In this context, it was reported earlier that peptides corresponding to the mouse CD95L polyproline region (amino acids 44–70) selectively interact with the SH3 domain of the T cell-specific ty-

rosine kinase p59<sup>fyn(T)</sup> but not with SH3 domains of p56<sup>lck</sup> and ras-GAP and only weakly with the two SH3 domains of Grb2 [18]. The authors suggested that Fyn may be involved in the regulation of CD95L transport processes or membrane expression. However, experimental evidence for the possible involvement of SH3-mediated interactions in regulating reverse costimulatory signals has not yet been reported.

In 1996, Hachiya and coworkers deposited several protein fragments in the NCBI database that they had 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 formin binding protein 11 (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 three SH3 domains), and FLAF3 represents a portion of the BAI1-associated protein 2 (BAP2)- $\beta$  (with a single SH3 domain). Again, a possible role of SH3 or WW domain-mediated interactions of these proteins in regulating reverse (costimulatory) signals had not been analyzed.

In the present report, we demonstrate that in addition to

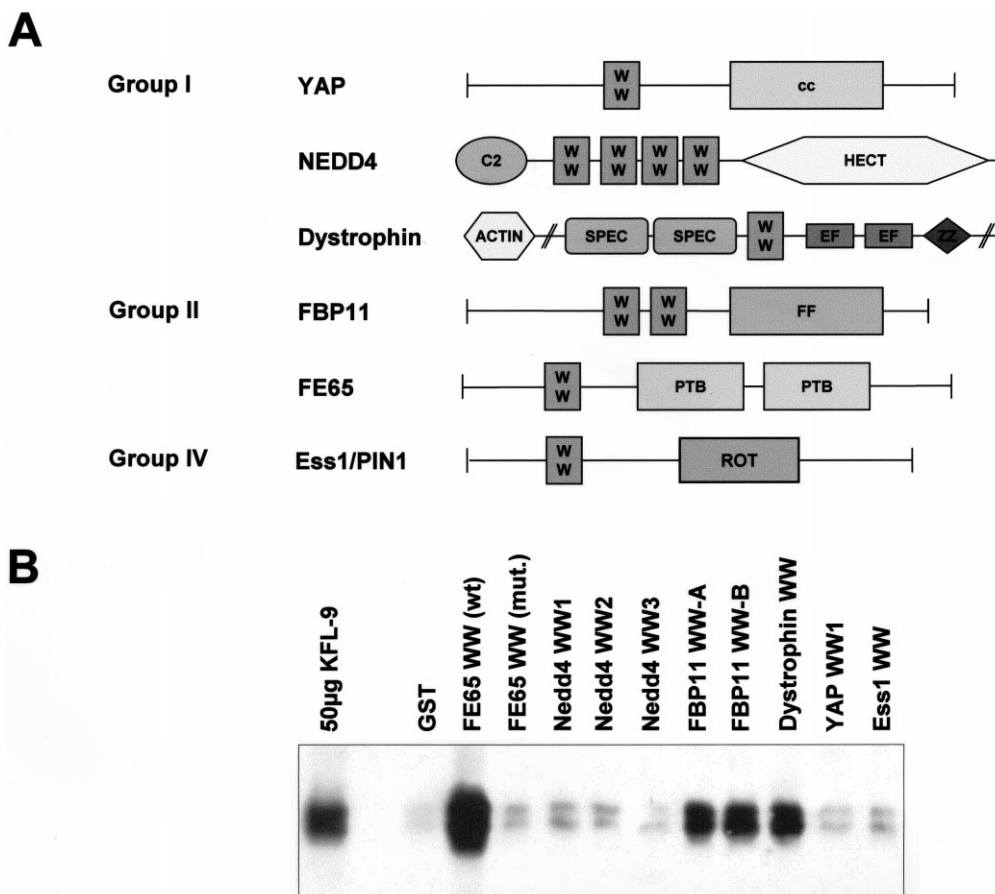


Fig. 4. WW domain binding to CD95L. A: Domain structure, group classification and putative binding specificity of proteins containing WW domains used in this study. Abbreviations for other domains are as follows: ROT, rotamase or isopropyl isomerase domain; cc, coiled-coil domain; C2, phospholipid binding domain; HECT, E3 ubiquitin ligase domain; ACTIN, actin binding domain shared with actinin and spectrin; SPEC, spectrin repeats; EF, EF hands potentially binding calcium; ZZ, domain composed of two zinc fingers; FF, novel motif that often accompanies WW domains (often contains two conserved Phe (F) residues); PTB, phosphotyrosine binding domain (adapted from [http://www.bork.embl-heidelberg.de/Modules/ww\\_module\\_str.html](http://www.bork.embl-heidelberg.de/Modules/ww_module_str.html)). B: 0.5 mg of the protein from post-nuclear RIPA extracts from KFL-9 transfectants was incubated with 20 µg of the indicated GST fusion protein and 20 µl of glutathione beads. The samples were separated on an 11% SDS gel. After blocking, FasL was detected by probing with mAb G247-4 and ECL visualization. 50 µg of KFL-9 lysate was loaded as a positive control for protein content, GST served as a negative control for precipitations with fusion proteins. FE65 WW domains were used as wild-type and mutated forms. Strong binding to CD95L was detected in the case of wild-type FE65 WW, intermediate association with both WW domains of FBP11 and dystrophin WW and only background with Nedd4, YAP or Ess1 WW domains.

Fyn SH3 domains, a number of other SH3 or WW modules are also able to effectively and specifically interact with the full-length CD95L molecule overexpressed in transfectants and inducibly expressed in stimulated human T lymphocytes. The identification of various Src-related kinases and different adapter proteins of the Grb2, PI 3-kinase, Nck and Phox families and the WW group 1 and group 2 domain proteins dystrophin, FE65 and FBP11 as putative CD95L-interacting molecules, opens new insights into the potential of CD95L to regulate and to modulate activation processes in T cells and in CD95L-expressing tumor cells. The role of Src-related kinases (e.g. Lck, Fyn and Yes expressed in T cells) [34]), PI 3-kinase [35], adapter proteins including Grb2, Gads [36–38], and Nck [39–40] in T cell activation has been extensively studied over recent years. All of these molecules mediate critical steps in cell activation and complex assembly associated with antigen receptor, adhesion molecule or costimulatory signals. The leukocyte NADPH oxidase subunit p47phox [41] seems to be expressed in granulocytes and B cells but not in T cells [42].

Interestingly, we were able to amplify the p47phox SH3 domains from RNA of Jurkat T cells and U937 histiocytic lymphoma cells. Nonetheless, a role for the p47 subunit of the NADPH oxidase in T cell activation has not been reported. Of note, the CD95L molecule seems to have a tendency to self-organize in a trimeric fashion, obviously a process that is mainly regulated by the extracellular portion of the molecule [43]. Thus, one could imagine that more than one signaling molecule may interact with the cytoplasmic tails of such trimers at the time. The role of WW domain interactions with CD95L also needs further elucidation. We know that WW domains are found in a wide range of cytosolic or nuclear signaling molecules and have different binding specificities with regard to proline content of the interaction motifs [28–32]. WW domains are for example involved in the regulation of transcription, RNA polymerase activity and cell cycle progression [32,44,45]. Recent results by several groups also suggest that binding of at least some of the WW domains is dependent on the phosphorylation state of serine or threo-

nine residues within the WW domain consensus binding sequence [32].

Another interesting phenomenon associated with CD95L upon TCR activation in the course of activation-induced cell death and during cytotoxicity mediated by cytolytic T cells is that CD95L surface expression as seen on CD4-positive T cells [27,46–48] and lysosomal expression and transport associated with cytotoxic (CD4+ or CD8+) T cells and NK cells [49–52] 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 [19,53]. 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. 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 antigen receptors on T and B cells (as suggested by the mentioned *in vivo* studies), or growth factor receptors on CD95L-positive tumor cells, respectively. The presented observations may also help to identify the biochemical basis for the functional sorting of CD95L during expression either on cell surfaces or in secretory lysosomes.

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