Interaction of peptides derived from the Fas ligand with the Fyn-SH3 domain

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Abstract **Interaction of the widely expressed Fas with its membrane-bound ligand (FasL) leads to rapid cell death via apoptosis. To avoid pathological tissue damage, the activity of FasL requires tight regulation. Here, we report that the Src homology 3 (SH3) domain of Fyn binds to the proline-rich cytoplasmic region of FasL. Binding of the SH3 domain occurs between amino acid residues 44–71 which contains several potential SH3 interaction sites. This binding is specific, as SH3 domains of Lck, Grb2 and ras-GAP bind only weakly or not at all. We suggest that FasL activity may be modulated by SH3 domains of the src-like Fyn kinase.**

Key words: Fyn; SH3 domain; Fas ligand; Apo-1; Apoptosis

1. Introduction

The Fas (Apo-1) system plays an important role in T cell homeostasis [1]. Mutant mice lacking functional FasL (*gld* mice) or Fas (*lpr* mice) develop lymphadenopathy and exhibit autoimmune disease symptomatology [2–4]. Together with perforin and granzymes, Fas contributes to the lytic activity of cytolytic T cells [5–8].

FasL is a 40-kDa type II membrane protein [4,9]. A salient feature of FasL is its proline-rich cytoplasmic sequence which is not observed in any other member of the TNF family (Fig. 1). Of the 28 residues proximal to the hydrophobic transmembrane region, 16 are highly conserved prolines. Short proline-rich motifs are known to interact with the src-homology region SH3 present in a variety of proteins important in cytoskeletal architecture or signal transduction [10]. Fyn and Lck are two SH3-containing members of the src protein kinase family that are associated with T cell receptor (TcR) and CD4/CD8, respectively [11]. In this study, we investigated the possible interaction between SH3 domains and the proline-rich cytoplasmic segment of FasL.

2. Materials and methods

2.1. Peptides

Peptides were synthesized on a Applied Biosystems 431A synthesizer using Fmoc chemistry. When required, peptides were coupled to CNBr-activated Sepharose beads (Pharmacia, Zürich, Switzerland).

2.2. Interaction of recombinant SH3-domains with FasL-derived peptides

Sepharose-bound peptide P7 (10 μl of a 50:50 slurry, 5 μg of immobilized peptide) was incubated with 3 μg (total input, L) of the various GST-fusion proteins (Santa Cruz Biotechnology, Santa Cruz, CA, and a gift from D. Cantrell, ICRF, London, UK) in a total volume of 50 μl of incubation buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4) and incubated for 2 h at 25°C. The beads were washed 3× in incubation buffer, resuspended in sample buffer and subjected to SDS-PAGE (10%) [12]. For inhibition studies, soluble peptides (500 μg) were included during the 2 h incubation time.

2.3. Interaction of cellular Fyn with FasL peptides

Precleared supernatants of lysed PMA/ionomycin-stimulated splenocytes were incubated overnight at 4°C with FasL proline-rich peptide P7 (or with P64, DAYLKVFFGGQEFR as a control) coupled to Sepharose beads. Precipitated proteins were separated by SDS-PAGE (1×10^7 cell equivalent per sample) and examined by immunoblot analysis using polyclonal antibodies against Fyn (Santa Cruz Biotechnology). In the inhibition experiments, the FasL P7 peptide (or control peptide P16, FAKLNCRLYRKANKSSKL) was included at a final concentration of 20 mg/ml.

2.4. Molecular model of FasL-Fyn SH3 domain

The experimental co-ordinates of SH3 domain of the Fyn proto-oncogene tyrosine kinase were obtained from the Brookhaven Protein Data Bank (entry 1SHF). The poly-proline type II helix co-ordinates were generated using knowledge-based protein modelling methods as implemented in the modelling tool ProMod [13]. The structure of the helix elements was deduced from the experimentally determined poly-proline peptide of cytochrome C-551 (residues 58–65, PDB entry: 1CCH). The elements were assembled in a long 'rod' and the relevant residues mutated to reflect the FasL sequence. Two copies of the Fyn SH3 domain were docked interactively to the poly-proline peptide as defined by the recent experimental elucidation of SH3-peptide complexes. Limited energy minimization with CHARMM (500 cycles of conjugate gradient minimization) were used to idealize the stereochemistry of the model and optimize the intermolecular contacts. The interaction between the peptide SH3-domain and the peptide was continuous and presented no cavities as evidenced by solvent-accessible surface overlap analysis.

3. Results and discussion

We assessed the ability of the proline-rich region of FasL to bind various SH3-containing proteins. Mouse FasL peptide (residues 44–71, see Fig. 1) was coupled to Sepharose beads and incubated with the GST-SH3 fusion proteins of Fyn, Lck, Grb2 and ras-GAP (Fig. 2A). The GST-Fyn-SH3 and GST-Fyn-SH3-SH2 fusion proteins strongly interacted with the FasL peptide, while weak binding was detected with the respective N- and C-terminal SH3 domains of Grb2. In contrast, no binding of Lck or ras-GAP was observed. In order to exclude that the absence of binding of Lck was due to a partial folding of the respective GST-Lck-SH3 fusion protein, a peptide corresponding to residues 1142–1157 (KGTDEVPVPPPVPPRR) of mouse Sos1 was analysed for its relative affinities to the SH3

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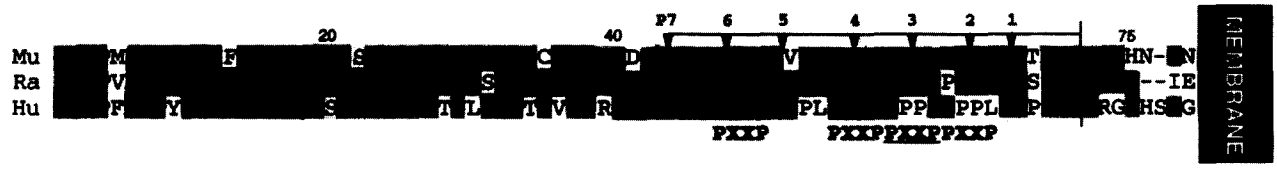

Fig. 1. Interaction of proline-rich sequences of FasL with SH3 domains. Alignment of the mouse, rat and human sequences from the cytoplasmic region of the FasL [22]. Peptides synthesized are indicated. P1 (residues 67–71 of mouse FasL), P2 (64–71), P3 (60–71), P4 (56–71), P5 (52–71), P6 (48–71), P7 (44–71). Four potential SH3-binding motifs (P-X-X-P) are indicated, the one shown in Fig. 4 is underlined.

domains. This peptide has been reported to interact with various SH3 domains including Lck and Fyn [14] and indeed, binding of the Sos1 proline-rich peptide to Lck and Fyn was detected (Fig. 2B).

To determine the structural motif mediating Fyn-binding, proline-rich peptides of distinct lengths (P1-P7, Fig. 1) were immobilized. Binding to Fyn-SH3 was observed with P3 which contains residues 60–71 of FasL (Fig. 3). P4 also interacted with Fyn-SH3, but interestingly, binding was not strengthened with peptides containing additional prolines (P5/P6). Maximal Fyn-SH3 precipitation was achieved with immobilized P7 suggesting that more than one recombinant Fyn-SH3 domain interacts with the immobilized peptides or that the conformation of SH3-binding motif was altered in the P7 peptide resulting in increased affinity.

Similar results were obtained when the soluble proline-rich FasL peptides were used as competitive inhibitors (Fig. 3). A 100-fold molar excess of FasL peptides partly or completely abolished FasL P7/GST-Fyn-SH3 interaction as long as the peptide included the PPPSQPLP sequence (P3-P4). In contrast, no inhibition was observed when the peptide ended with LPP (P2).

Structural and mutagenic studies have revealed that the core peptides which associate with SH3 domains consist of seven residues and contain the P-X-X-P motif [15]. The FasL contains several potential binding motifs [16,17], four of which are indicated in Fig. 1. SH3-binding peptides adopt left-handed type II helices [16]. Thus, a molecular model of the proline-rich segment (residues 44–71) of FasL was constructed [13], based on the experimentally elucidated 3-D structure of the poly-proline type II helix present in cytochrome C-551. The resulting helix contains 9 turns and is 75 Å long (Fig. 4). A gap of at least 30 Å remains between the FasL-bound Fyn-SH3 domain the fatty acid membrane anchor of Fyn. Consistent with our model, the SH3 domain of Fyn is preceded by 84 residues at the N-terminus. This would provide a sufficiently long structure capable of spanning the gap between membrane and SH3 domain.

We next investigated the interaction of cellular Fyn with the FasL-derived peptides. When the immobilized peptide P7 of FasL was incubated with extracts of activated splenocytes, Fyn was detected in immunoblots (Fig. 5), while no anti-Fyn-reactive band was seen when the cellular extracts were exposed to Sepharose beads to which an irrelevant peptide had been coupled. Moreover, Fyn-binding to FasL peptide P7 was inhibited by soluble P7 peptide while the control peptide was ineffective.

Considering the dramatic effect seen after systemic delivery of anti-Fas antibodies to mice [18], a tight regulation of FasL surface expression and activity is unquestionably important. In primary allogeneic cytolytic T cells, Fas-and perforin-mediated target cell death is highly polarized, sparing innocent bystander cells from killing [5]. Perforin is harbored in cytoplasmic gran-

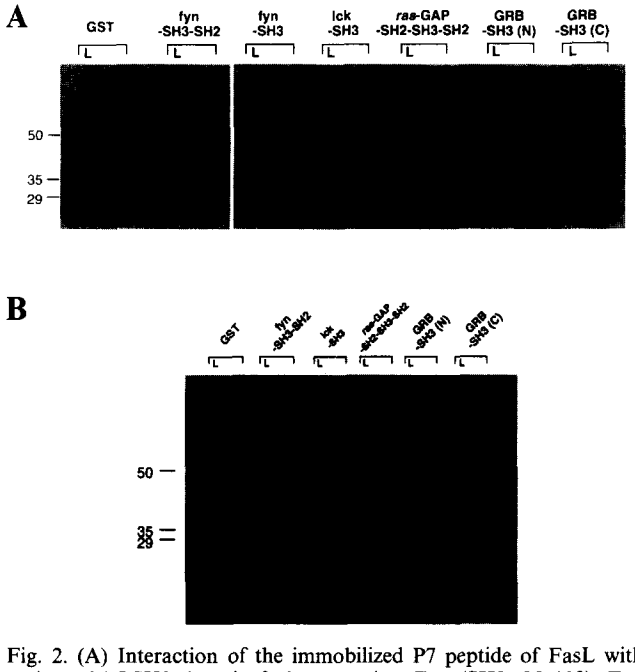

Fig. 2. (A) Interaction of the immobilized P7 peptide of FasL with various GST-SH3 domain fusion proteins. Fyn (SH3, 85–139), Fyn (SH3-SH2, 85–247), Lck (SH3, 54–120), ras-GAP (SH2-SH3-SH2, 171–448), grb-N (SH3, 1–68) and grb-C (SH3, 156–199). Negative control. GST non-fusion protein. L shows the total input of the respective fusion proteins. (B) As Fig. 2A, but the interaction of immobilized mouse Sos1 peptide (residues 1142–1157) with the indicated GST-SH3 fusion proteins was analysed.

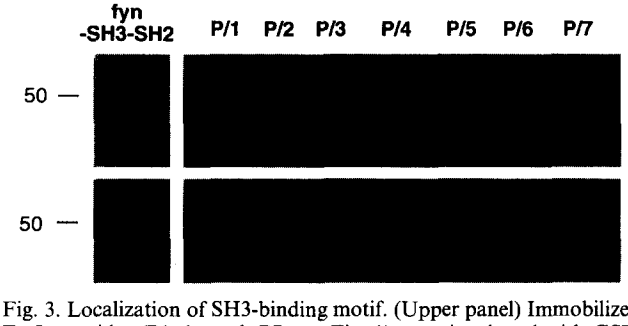

Fig. 3. Localization of SH3-binding motif. (Upper panel) Immobilized FasL peptides (P1 through P7, see Fig. 1) were incubated with GST-Fyn-SH3-SH2 and analysed by SDS-PAGE. (Lower panel) Inhibition of the interaction of immobilized FasL P7 with GST-Fyn-SH3-SH2 by soluble peptides P1 through P7. Peptides were added at a 100-fold excess relative to immobilized P7. The left-most band represents the interaction of the protein in the absence of any competitor peptide (lower panel) or the total input of protein (upper panel).

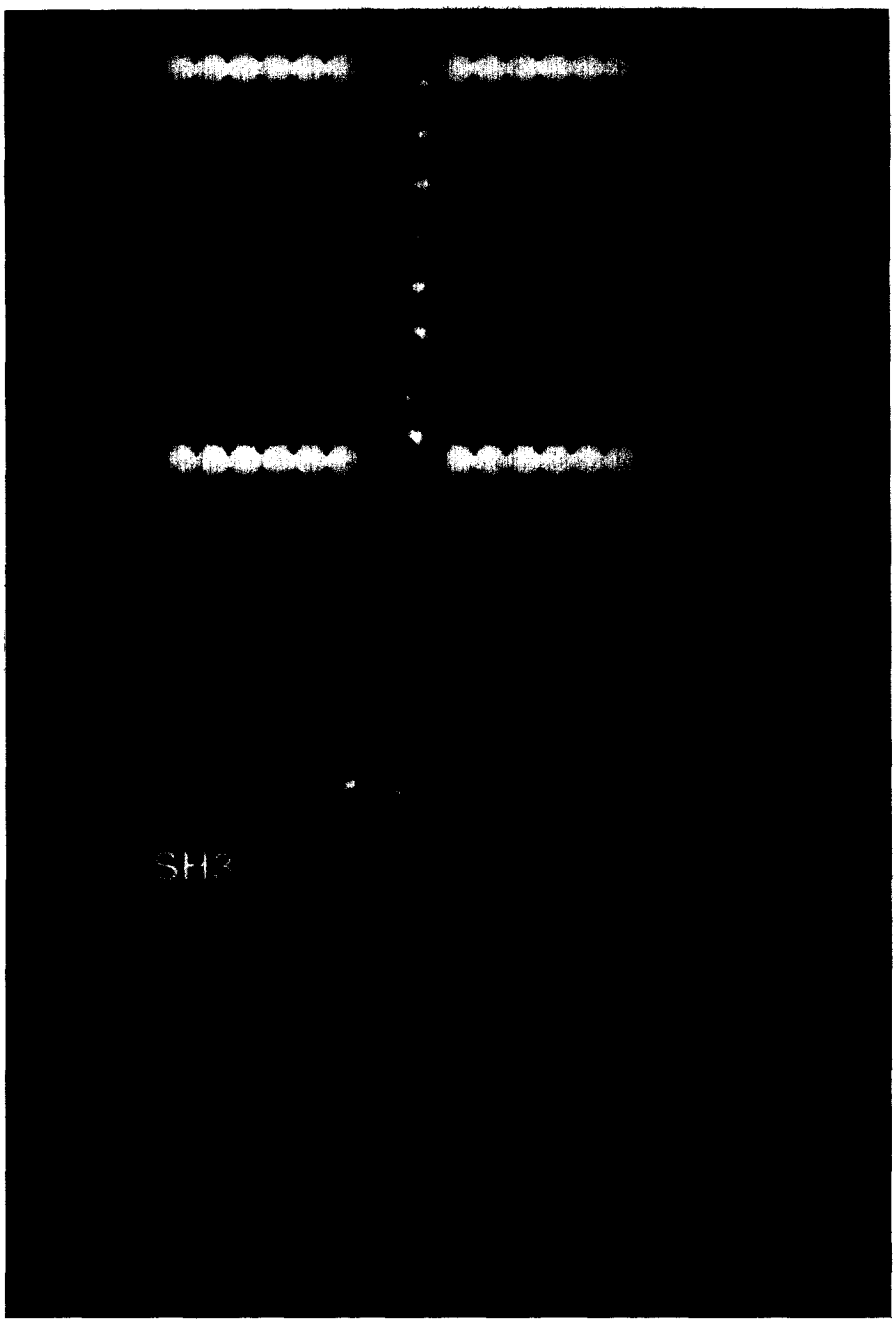

Fig. 4. Molecular model of the proline-rich cytoplasmic segment of mouse FasL interacting with a SH3 domain of Fyn. The SH3 interaction sequence shown binds in the forward (Class I) orientation [17], but their precise localization is putative. The SH3 domain shown binds to the PSQP (residues 58–61, see Fig. 1) motif of FasL (PXXP-motif). The proline-rich sequence of FasL is modeled in a poly-proline II helix conformation. The FasL peptides used in this study (P1-P7, see fig. 1) are indicated indicated. Proline residues are coloured in red.

ules which are guided by microtubules to release their contents only towards the site of target cell contact [19]. The functional importance of the FasL-Fyn interaction is unknown. However, one could envisage that this interaction may modulate the surface expression of newly synthesized FasL. Secretory vesicles that have the poly-proline-rich sequence of FasL cytoplasmatically exposed might be directly targeted to the cell surface via Fyn and/or Lck SH3 domains, both of which are known to cap at the site of TcR receptor/target cell interaction [20]. This implies that the resulting polarized vesicle-cell membrane fusion could engender local exposure of other vesicle-harbored proteins, including adhesion molecules and cytokines [19]. Alternatively, FasL may follow the regular non-polarized secretory pathway. If this occurs, subsequent redistribution and retention of FasL by Fyn or other proteins containing SH3 domains (our study does not exclude this possibility) could result in high local accumulation of the lytic ligand in the region around the activated TcR complex. Thus, SH3 domains may not only dictate the position of soluble signaling molecules within the cell, but also decide on the location of membrane proteins with respect to the docking site of an interacting cell. A recent report demonstrated that apical surface expression of

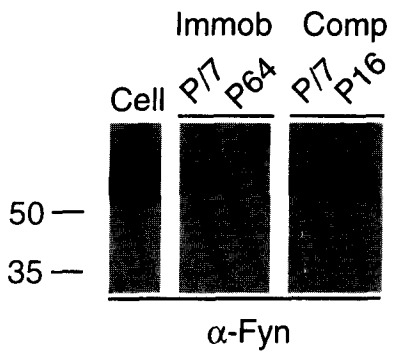

Fig. 5. Precipitation of Fyn from splenocytes with immobilized FasL proline-rich peptide (P/7). Precipitates were analysed by immunoblotting using the respective antibodies. Control precipitates with an irrelevant peptide (Immob, P64) were negative. Fyn interaction with immobilized P/7 was inhibited in the presence of soluble P7 peptide (Comp P/7), but not the P16 control peptide. Specificity of the anti-Fyn antibody was confirmed by Western blots of whole splenocyte extracts (Cell).

the amiloride-sensitive sodium channel is dependent on its proline-rich SH3-binding domain [21]. This may even point to a more general mechanism whereby SH3-domains control sorting and localization of membrane proteins in specialized or polarized cells. However, more experiments are required to support this hypothesis.

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References

[1] Krammer, P.H., Behrmann, I., Daniel, P., Dhein, J. and Debatin, K.M. (1994) Curr. Opin. Immunol. 6, 279–89.
[2] Watanabe, F.R., Brannan, C.I., Copeland, N.G., Jenkins, N.A. and Nagata, S. (1992) Nature 356, 314–7.
[3] Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T. and Nagata, S. (1994) Cell 76, 969–976.
[4] Nagata, S. and Golstein, P. (1995) Science 267, 1449–1456.
[5] Lowin, B., Hahne, M., Mattmann, C. and Tschopp, J. (1994) Nature 370, 650–2.
[6] Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H. and Golstein, P. (1994) Science 265, 528–30.
[7] Walsh, C.M. et al. (1994) Proc. Natl. Acad. Sci. USA 91, 10854–8.
[8] Kojima, H. et al. (1994) Immunity 1, 357–364.
[9] Suda, T., Takahashi, T., Golstein, P. and Nagata, S. (1993) Cell 75, 1169–78.
[10] Pawson, T. (1995) Nature 373, 573–580.
[11] Chan, A.C., Desai, D.M. and Weiss, A. (1994) Annu. Rev. Immunol. 12, 555–92.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Peitsch, M.C. and Tschopp, J. (1995) Mol. Immunol. (in press).
[14] Gout, I. et al. (1993) Cell 75, 25–36.
[15] Lim, W.A., Richards, F.M. and Fox, R.O. (1994) Nature 372, 375–379.
[16] Saraste, M. and Musacchio, A. (1994) Nature Struct. Biol. 1, 835–837.
[17] Feng, S.B., Chen, J.K., Yu, H.T., Simon, J.A. and Schreiber, S.L. (1994) Science 266, 1241–1247.
[18] Ogasawara, J. et al. (1993) Nature 364, 806–809.
[19] Kupfer, A. and Singer, S.J. (1989) Annu. Rev. Immunol. 7, 309–338.
[20] Gassmann, M., Amrein, K.E. and Burn, P. (1993) J. Receptor Res. 13, 711–24.
[21] Rotin, D., Bar, S.D., O'Brodovich, H., Merilainen, J., Lehto, V.P., Canessa, C.M., Rossier, B.C. and Downey, G.P. (1994) EMBO J. 13, 4440–50.
[22] Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda, T. and Nagata, S. (1994) Int. Immunol. 6, 1567–1574.