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Interaction of Fas(Apo-1/CD95) with proteins implicated in the ubiquitination pathway

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Abstract Fas(Apo-1/CD95), a receptor belonging to the tumor necrosis factor receptor family, induces apoptosis when triggered by Fas ligand. Upon its activation, the cytoplasmic domain of Fas binds several proteins which transmit the death signal. We used the yeast two-hybrid screen to isolate Fas-associated proteins. Here we report that the ubiquitin-conjugating enzyme UBC9 binds to Fas at the interface between the death domain and the membrane-proximal region of Fas. This interaction is also seen in vivo. UBC9 transiently expressed in HeLa cells bound to the co-expressed cytoplasmic segment of Fas. FAF1, a Fas-associated protein that potentiates apoptosis (Chu et al. (1996) Proc. Natl. Acad. Sci. USA 92, 11894–11898), was found to contain sequences similar to ubiquitin. These results suggest that proteins related to the ubiquitination pathway may modulate the Fas signaling pathway.

Key words: Apoptosis; Fas(Apo-1/CD95); Yeast two-hybrid; Ubiquitin

1. Introduction

Apoptosis, or programmed cell death, is essential for the homeostasis of multicellular organisms [1]. Apoptosis is most efficiently induced by Fas (Apo-1/CD95) [2,3]. Fas is a transmembrane receptor of the TNF family of receptor proteins which are characterized by a conserved cysteine-rich extracellular domain [4]. Upon binding of the natural ligand (FasL) or crosslinking with antibodies, cells expressing Fas undergo rapid apoptosis [5,6].

Despite its importance, the mechanisms underlying Fas-mediated apoptosis still remain unclear. Members of the growing family of cysteine proteases specific for aspartic acid (Caspases) appear to be implicated in the execution phase of cell death [7]. Signaling of apoptosis immediately following activation of Fas leads to activation of caspases via the Fas-interacting protein FADD/MORT1 [8,9] and subsequent activation of FLICE/MACH proteases [8,10].

In order to define additional candidate proteins implicated in Fas signaling, we performed a two-hybrid screen using the intracellular domain of Fas as bait. We screened a mouse embryo library and obtained several clones coding for two distinct proteins. One of these represents UBC9, a member of the E2 family of ubiquitin conjugating enzymes.

Ubiquitination of proteins plays a role as a signal for the intracellular degradation of proteins by proteasomes [11,12]. Briefly, in the ubiquitination reaction, ubiquitin is first activated by an activation enzyme, E1. It is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme (E2 enzyme). The E2 enzyme, either alone or together with a ubiquitin-protein ligase, E3, catalyzes the transfer of ubiquitin to a lysine residue of the target protein which is thereby marked for degradation by proteasomes. In yeast, UBC9 represents one out of at least ten different E2 enzymes. It mediates destruction of S- and M-phase cyclins and is essential for viability [13]. The mouse and human UBC9 homologues were recently cloned as proteins interacting with the Rad51 recombination protein [14].

We now show that UBC9 is present in the cytosol and nucleus of transfected cell lines and that it associates with the intracellular domain of the Fas receptor.

2. Material and methods

2.1. Cloning of Fas cytosolic domain fusion proteins

A DNA fragment coding for the cytosolic domain of murine Fas (Ser^{183}–Glu^{306}) was excised *Xba*I–*Pst*I from pKS(+) containing the entire murine Fas [15], and subcloned into PKS(+) (Stratagene). The cytosolic fragment of Fas in this vector from *Not*I (5′) (filled in with Klenow polymerase) to *Sal*I (3′) was cloned into the *Bam*HI (filled in) and *Sal*I sites of the vector PGBT9 (Clontech) thus creating a fusion protein of the Gal4-DNA binding domain and Fas (pGBT-Fas).

A series of fragments and deletion mutations of the Fas cytosolic domain were produced by PCR (using oligonucleotides that contain an added *Sma*I site at the 5′ end or an added *Bam*HI site at the 3′ end of the insert) and cloned in frame into the *Sma*I and *Bam*HI sites of the Gal4-DNA binding domain vector PGBT9 (Clontech). The following fragments were amplified: Arg^{166}–Glu^{306}, Arg^{166}–Asp^{288}, Leu^{200}–Leu^{289}, Ile^{207}–Leu^{289}, Leu^{289}–Glu^{306}. The Fas cytosolic domain lpr^{cg} ($I_{225}N$) (Arg^{166}–Glu^{306}) mutation was created by PCR using mutated oligonucleotides and equally cloned into pGBT9. A further truncated version of Fas was constructed by digestion with *Xho*I (Ala^{220}) and relegation into the *Sal*I site of the vector (Arg^{166}–Ala^{220}).

2.2. Two-hybrid screen

A mouse embryo cDNA library cloned into the *Not*I-site of the vector pVP16 [16] which codes for fusion proteins of the viral transcription activation domain VP16 was obtained from S. Hollenberg (Vollum Institute, Portland, Oregon). Yeast strain Y153 [17] was transformed with pGBT-Fas and the library plasmids and analyzed for interaction of the proteins [18]. Mating tests [19] were performed using yeast Y187 (MATα) and Y190 (MATa) transformed with the bait and library plasmids, respectively.

2.3. Transfection of mammalian cells

For expression in mammalian cells, the cDNAs corresponding to mouse UBC9 (R. Bernards, Amsterdam, Netherlands) or fragments thereof were cloned into pCR3 (Invitrogen) and tagged with either a flag or a myc epitope at the N-terminus [20]. The complete cytosolic domain of Fas (Arg^{166}–Glu^{306}) was tagged with a flag epitope at its N-terminus and cloned into pCR3 (Invitrogen). The FADD cDNA in the pcDNA3 vector (Invitrogen) was obtained from J.C. Martinou, Glaxo, Geneva.

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Plasmids were transiently transfected into HeLa or 293T cells (293 cells, ATCC CRL 1573, expressing the SV40-T antigen, obtained from M. Peter, Heidelberg) using the calcium-phosphate precipitation method [21]. Protein expression was controlled by separating cell extracts on a polyacrylamide gel and subsequent immunoblotting.

2.4. Immunofluorescence staining

Transfected HeLa cells were grown on sterile coverslips prior to immunofluorescence. Cells were then washed in PBS, fixed in 4% formaldehyde and permeabilized with 0.1% saponin in PBS. Transfected proteins were stained with monoclonal antibody to the flag epitope (M2, Kodak). After extensive washing, an antibody against mouse immunoglobulins conjugated to FITC (Dianova, Hamburg, Germany) was added. Slides were mounted using FluorSave Reagent (Calbiochem) and analyzed using a Zeiss Axiophot microscope.

2.5. Immunoprecipitation

293T cells were transfected with flag-tagged Fas cytosolic domain and myc-tagged UBC9 in a 25 cm^2 plate. Cells were lysed in 1 ml lysis buffer [22] and precipitated with a monoclonal antibody to the flag tag coupled to agarose (M2, Kodak). Co-precipitation of myc-tagged UBC9 was analyzed by Western blot using the monoclonal anti-myc antibody 9E10 (gift from R. Iggo, ISREC, Epalinges). Anti-FADD monoclonal antibodies were purchased from Transduction Laboratories, Lexington.

3. Results

3.1. Identification of UBC9 as a protein binding partner of Fas

We used the yeast two-hybrid system to screen for proteins that interact with the cytosolic domain of the Fas/Apo-1 receptor. As bait we fused the fragment of Fas from Ser^{183} to Glu^{306} to the Gal4 DNA binding domain. This plasmid was transformed into yeast strain Y153 along with a library of mouse embryo cDNA fused to the transcription activation domain VP16 [22]. Twenty-seven positive clones were obtained from 5×10^6 clones screened. Sequencing of the inserts revealed that four inserts corresponded to the mouse homologue of the *S. cerevisiae* ubiquitin-conjugating enzyme UBC9 [13,14]. Three of the clones were identical. All of the clones lacked the 2 C-terminal amino acids of full-length UBC9, and one clone additionally lacked 6 amino acids at the N-terminus. The full-length mouse UBC9 cDNA (obtained from R. Bernards) was subsequently cloned into the vectors for the two-hybrid system to confirm the results. UBC9 interacted specifically with Fas (Fig. 1), but not with the proteins lamin A, LDL receptor, or poly Ig receptor (not shown) used as control.

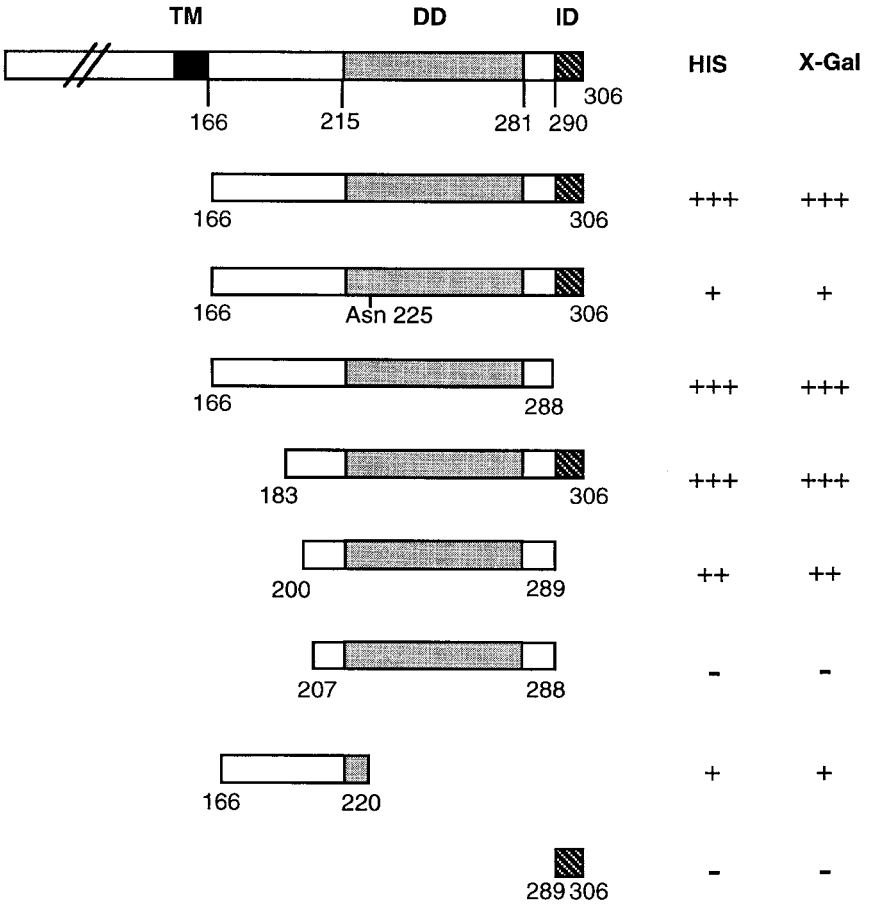

Fig. 1. Interaction of UBC9 and Fas in the yeast two-hybrid system. TM, transmembrane segment of Fas; DD, death domain; ID, inhibitory domain, binds to FAP-1. Several deletion mutations of the cytosolic domain of Fas were cloned as fusion proteins with the DNA binding domain of Gal4. Interaction of these with the VP16 fusion protein of UBC9 was tested in the two-hybrid system. Growth on histidine-lacking media and β-galactosidase activity is indicated. +++: strong reaction (large colonies, dark blue 213color), +: weak interaction (small colonies, faint blue color).

3.2. Localization of the Fas–UBC9 interaction site

In order to characterize the interactions of UBC9 and Fas more precisely, we constructed several truncated versions of the Fas cytosolic domain (Fig. 1). Both the complete UBC9 protein and the fragments obtained in the two-hybrid screen interacted very strongly with the entire cytosolic domain of Fas (Arg^{166}–Glu^{306}) and with the fragments which lacked either 17 amino acids adjacent to the membrane-interacting segment (Ser^{183}–Glu^{306}) or the C-terminal 18 amino acids (Arg^{166}–Asp^{288}) (Fig. 1). Neither of the UBC9 proteins interacted with the fragment of Fas corresponding to the death domain (Ile^{207}–Asp^{288}) alone or with the C-terminal 18 amino acids (Leu^{289}–Glu^{306}) known to interact with FAP-1 [23]. In contrast, weak but detectable interaction was found with fragments in which the death domain was flanked by additional 15 amino acids at its N-terminus (Leu^{200}–Leu^{289}) or when the fragment contained the complete membrane proximal part, but only 7 residues of the death domain (Arg^{166}–Ala^{220}). In addition, UBC9 interacted only weakly with the cytosolic domain of Fas which contained a mutation in the death domain corresponding to the *lpr*^{cg} [24] mutation in mice which renders Fas inactive [25]. From these experiments, we conclude that efficient binding of UBC9 to the cytosolic domain of Fas requires both a functional death domain and part of the membrane proximal segment.

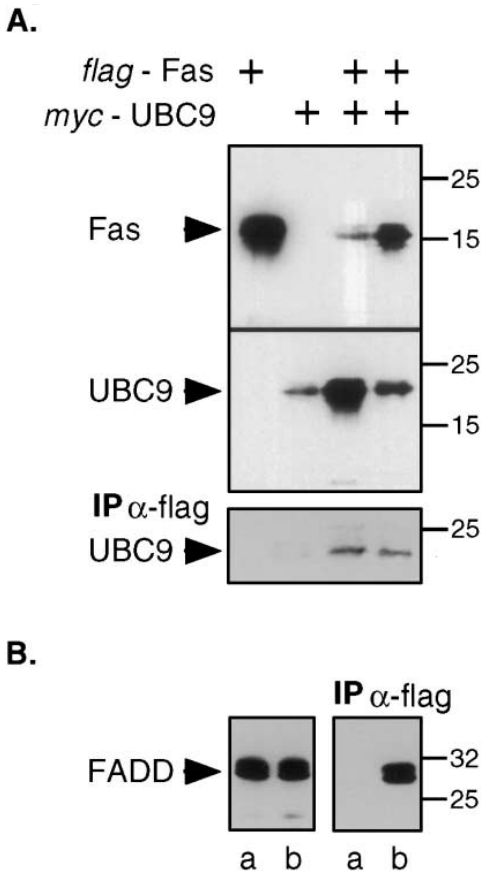

Fig. 2. Interaction of UBC9 and Fas in mammalian cells. (A) 293T cells were transfected with, from left to right, (a) flag-tagged Fas cytosolic domain (10 μg plasmid), (b) myc-tagged UBC9 (2 μg), (c) flag-tagged Fas cytosolic domain (2 μg plasmid) and myc-tagged UBC9 (8 μg), and (d) flag-tagged Fas cytosolic domain (8 μg plasmid) and myc-tagged UBC9 (2 μg). Cells were lysed and postnuclear supernatant analyzed by Western blot (top two panels) using Fas-detecting anti-flag and UBC9-detecting anti-myc antibodies, respectively. In the third panel, experiments are shown where Fas was first precipitated (IP) from lysates of transfected cells with anti-flag antibodies bound to agarose. Precipitates were subsequently analyzed by Western blot for the co-precipitation of myc-tagged UBC9. (B) Control experiment: interaction of FADD and Fas in mammalian cells. 293T cells were either transfected with FADD cDNA alone (a) or co-transfected with FADD and flag-tagged Fas cytosolic domain (b). The left panel shows a Western blot of lysates of transfected cells using an anti-FADD antibody. In the right panel (IP), Fas, when present, was first precipitated (IP) with anti-flag antibodies bound to agarose. Precipitates were subsequently analyzed by Western blot for the co-precipitation of FADD.

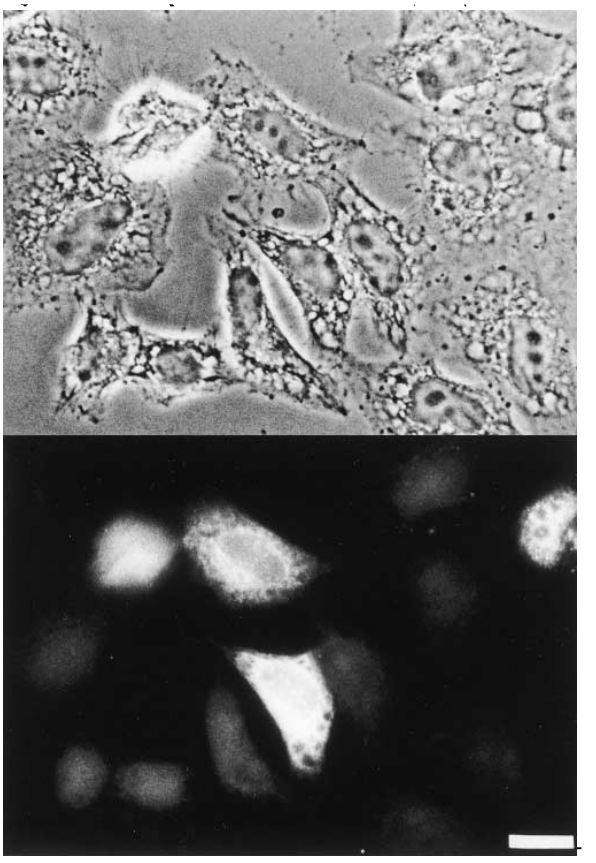

Fig. 3. Immunofluorescence of UBC9. HeLa cells were transfected with UBC9 tagged with a flag epitope at its N-terminus, fixed on a coverslip, and stained with anti-flag antibody (lower panel). The corresponding phase-contrast image is shown in the upper panel. The bar corresponds to a distance of 15 μm.

3.3. Interaction of UBC9 and Fas in vivo

To confirm that interaction of Fas with UBC9 occurs in vivo, 293T human embryo kidney cells were co-transfected with a plasmid coding for the cytosolic domain of Fas tagged with a flag epitope and a plasmid coding for mouse UBC9 tagged with a myc epitope. Cells were lysed and subjected to immunoprecipitation using an anti-flag epitope antibody. The immunoprecipitates were analyzed by Western blot using an antibody against the myc epitope. UBC9 was only precipitated if it was coexpressed with Fas (Fig. 2A). If the two proteins were expressed separately and the cell lysates were mixed before immunoprecipitation, no binding was observed (data not shown) indicating that Fas and UBC9 interaction is weak. As expected, the transiently expressed cytoplasmic segment of Fas strongly interacted with FADD/Mort1 (Fig. 2B).

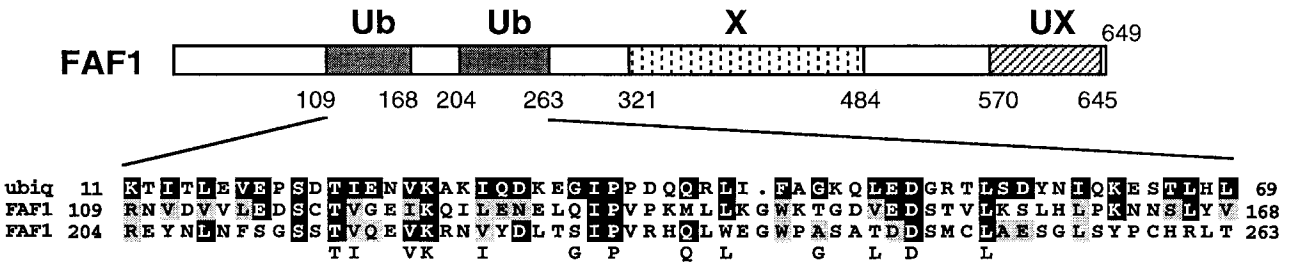

Fig. 4. Sequence homology of FAF1 with proteins involved in the ubiquitin pathway. For establishing the homology, the generalized profile method was used. Alignment of the two regions of FAF1 homologous to ubiquitin (ubiq) is shown. The most conserved amino acids in ubiquitin-like proteins are listed below the alignment. Black-boxed residues correspond to identical amino acids in at least two of the three sequences shown, shaded amino acids represent conserved residues. X = homology with *C. elegans* ORF C281.1; UX = homology with proteins involved in ubiquitination pathway. Accession numbers: mouse FAF1: gb: U39643, human ubiquitin: P02248 (76 aa), C28g1.1: gb: U41026.

3.4. UBC9 is present in the cytosol and the nucleus

Yeast UBC9 has been reported to be active in the nucleus of yeast [13]. Since our experiments indicated that UBC9 was interacting with the cytoplasmic domain of Fas, it was important to investigate whether UBC9 was also present in the cytoplasm of mammalian cells. The flag-tagged UBC9 expression construct was transiently expressed in HeLa cells and localization of the protein was analyzed by immunofluorescence. As shown in Fig. 3, UBC9 was expressed in the cytosol of transfected cells. A considerable portion was also found in the nucleus suggesting that the protein shuttles between the two cellular compartments. The same result was obtained with a myc-tagged version of UBC9 in COS cells (data not shown). This localization is consistent with the binding of UBC9 to the cytosolic domain of Fas.

4. Discussion

Using the two-hybrid screen, we have identified the ubiquitin-conjugating enzyme UBC9 as a Fas-interacting protein, increasing to date the number of proteins known to associate with the cytoplasmic segment of Fas to five. This finding is in agreement with recently published data by Wright et al. [26], who reported that Fas interacts with UBC-FAP (identical to UBC9). In addition to UBC9, two death domain containing proteins, i.e. FADD/MORT1 [8,9] and RIP [27], specifically interact with the death domain of Fas. Whereas the role of FADD/MORT1 is to physically link the FLICE/MACH proteases to Fas and thus initiate apoptosis [10,28], RIP's function in Fas-mediated cell death is less clear as it preferentially associates with TNFR1 via the death domain protein TRADD [29]. Fas also interacts with two proteins that lack death domains, i.e. FAF1 and FAP-1. FAF1, if overexpressed in mammalian cells, potentiates apoptosis [30], in contrast to the phosphatase FAP-1 which has been reported to act as inhibitor of cell death [31]. FAP-1 binds to the carboxyl-terminal 15 amino acids of Fas, a sequence known to represent a negative regulator of Fas-induced apoptosis [32]. The FAF1 binding region in Fas has not been mapped precisely. Similar to UBC9, FAF1 is unable to interact with the Fas lpr^{cg} mutation and no homology with any known sequence in the data banks was found. Using a generalized profile method [33], however, sequence similarity with several sequence motifs present in proteins of the ubiquitination pathway became apparent (Fig. 4). First, one repeated motif homologous to ubiquitin itself is present in the N-terminus of FAF1. Second, the C-terminus comprises a UX sequence motif present in proteins implicated in ubiquitin conjugation [34]. Finally, the middle region of FAF1 shows sequence similarity with a motif (X) found in the *C. elegans* ORF C28G1.1 where this (X) motif is found adjacent to a sequence homologous to UBC9.

The physiological relevance of the association of Fas intracellular domain with two proteins apparently implicated in the ubiquitination pathway remains to be elucidated. When FAF1 is overexpressed, the percentage of apoptotic cells increases through Fas signaling, suggesting that it is a positive regulator of apoptosis. We have observed no effect on overexpression of UBC9 in mammalian cells, probably reflecting the high endogenous levels already present in most mammalian systems [14]. There are several possible explanations for the UBC9/Fas interaction. First, UBC9 may be directly involved in ubiquitin-dependent degradation of Fas by proteasomes. Fas signaling leads to rapid apoptosis and one mechanism to control this potentially dangerous death pathway could be to limit the half-life of the receptor. Although nothing is known on the stability of Fas and possible ubiquitination, the related TNFR1 appears to be short-lived [35]. Interestingly, TNFR was shown to be ubiquitinated [36] and to bind a protein related to a proteasome subunit upstream of the death domain [35,37]. Second, Fas may target UBC9 to membrane sites where ubiquitination of neighboring proteins may trigger events required for apoptosis. Proteasome proteolytic activity is required for certain apoptotic pathways in neurons and thymocytes [38,39]. However, modification of proteins by ubiquitin can have consequences other than direct targeting to the 26S proteasome. Ubiquitination may act as a signal for receptor endocytosis to lysosomes as recently reported for a yeast plasma membrane receptor [40]. Moreover, the biosynthesis or activity of several receptor proteins has been shown to be regulated by ubiquitination, as in the case of the cystic fibrosis transmembrane regulator, the T-cell receptor, the platelet-derived growth factor receptor and an IκBα protein kinase [41]. Understanding the role of the ubiquitination pathway related proteins FAF1 and UBC9 in Fas signaling will certainly lead to a better understanding of how apoptotic signals are modulated.

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