The EMBO Journal vol.14 no.22 pp.5579–5588, 1995

Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor

Frank C.Kischkel, Stefan Hellbardt, Iris Behrmann[1], Matthias Germer[2], Michael Pawlita[3], Peter H.Krammer and Marcus E.Peter[4]

Tumor Immunology Program, Division of Immunogenetics and [3]Applied Tumor Virology Program, German Cancer Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
[1]Present address: Institute for Biochemistry, RWTH Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany
[2]Present address: Haemostasis Research Unit, Kerckhoff Clinic, Sprudelhof 11, D-61231 Bad Nauheim, Germany

[4]Corresponding author

F.C.Kischkel and S.Hellbardt contributed equally to this work

APO-1 (Fas/CD95), a member of the tumor necrosis factor receptor superfamily, induces apoptosis upon receptor oligomerization. In a search to identify intracellular signaling molecules coupling to oligomerized APO-1, several cytotoxicity-dependent APO-1-associated proteins (CAP) were immunoprecipitated from the apoptosis-sensitive human leukemic T cell line HUT78 and the lymphoblastoid B cell line SKW6.4. CAP1–3 (27–29 kDa) and CAP4 (55 kDa), instantly detectable after the crosslinking of APO-1, were associated only with aggregated (the signaling form of APO-1) and not with monomeric APO-1. CAP1 and CAP2 were identified as serine phosphorylated MORT1/FADD. The association of CAP1–4 with APO-1 was not observed with C-terminally truncated non-signaling APO-1. In addition, CAP1 and CAP2 did not associate with an APO-1 cytoplasmic tail carrying the lpr^{cg} amino acid replacement. Moreover, no APO-1–CAP association was found in the APO-1$^+$, anti-APO-1-resistant pre-B cell line Boe. Our data suggest that *in vivo* CAP1–4 are the APO-1 apoptosis-transducing molecules.

Keywords: APO-1 (Fas/CD95)/apoptosis/CAP1–4/MORT1/FADD/signaling

Introduction

APO-1 (Fas/CD95) is an apoptosis-inducing receptor expressed on a variety of cells including activated T and B cells (Trauth *et al.*, 1989; Yonehara *et al.*, 1989; Itoh *et al.*, 1991; Oehm *et al.*, 1992). APO-1 is involved in apoptosis within the immune system. It is a member of the tumor necrosis factor (TNF) receptor superfamily that includes the NGF receptor (Johnson *et al.*, 1986), CD27 (Camerini *et al.*, 1991), CD30 (Dürkop *et al.*, 1992), CD40 (Stamenkovic *et al.*, 1989), the MRC OX-40 antigen (Mallett *et al.*, 1990), two receptors for TNF called TNF-RI and TNF-RII (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990), 4-1BB (Kwon and Weissman, 1989) and SFV-T2, an open reading frame in Shope fibroma virus (Upton *et al.*, 1987). The members of this family are characterized by several cysteine-rich motifs of ~40 amino acids in the extracellular part of the molecules (for a review see Krammer *et al.*, 1994).

The expression of APO-1 is strongly reduced in lpr mutant mice (Davidson *et al.*, 1986; Mariani *et al.*, 1994) that develop autoimmunity and a lymphoproliferative disorder characterized by the accumulation of non-malignant CD4$^-$CD8$^-$B220$^+$ T lymphocytes. The discovery that a single-point mutation within the APO-1 cytoplasmic tail of the lpr^{cg} mutant mouse causes the same autoimmune disease strongly indicates that APO-1 itself transduces the apoptosis signal (Watanabe-Fukunaga *et al.*, 1992). The cytoplasmic region of human APO-1 (325 amino acids) consists of 145 amino acids, and does not contain any known consensus sequences for enzymatic functions or binding sites for any known signaling molecules. It has homology to TNF-RI, which has also been shown to transduce a cytotoxic signal (Tartaglia *et al.*, 1991). Itoh and Nagata (1993) defined an area of 65 amino acids (the death domain), present in both APO-1 and TNF-RI, as being responsible for promoting the death signal. The ability of the death domain to self-aggregate was shown to be responsible for the generation of a death signal (Boldin *et al.*, 1995a). Using the yeast two-hybrid system, two groups have recently isolated a protein, MORT1/FADD, that associates with the APO-1 death domain (Boldin *et al.*, 1995b; Chinnaiyan *et al.*, 1995). Another protein, called RIP, was cloned using the same strategy (Stanger *et al.*, 1995). Both proteins contain a sequence homologous to the APO-1 death domain. The overexpression of MORT1/FADD or RIP induces apoptosis in transfected cells (Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995). These experiments have suggested that the two proteins associate with APO-1 *in vivo* and mediate APO-1-dependent apoptosis. However, this has not been shown directly. Thus, because APO-1 signaling requires oligomerization (Dhein *et al.*, 1992), we sought to find proteins that physically associate *in vivo* with the activated receptor. We show that a set of proteins (cytotoxicity-dependent APO-1-associated proteins; CAP1–4), two of which are identical to MORT1/FADD, associate only with aggregated APO-1 containing a functional death domain, resulting in the formation of the death-inducing signaling complex (DISC). CAP1–4 are therefore prime candidates for death-transducing signaling molecules.

Results

Crosslinking of APO-1 results in rapid receptor aggregation

Induction of apoptosis through the APO-1 receptor requires receptor crosslinking (Dhein *et al.*, 1992). Crosslinking of

© Oxford University Press

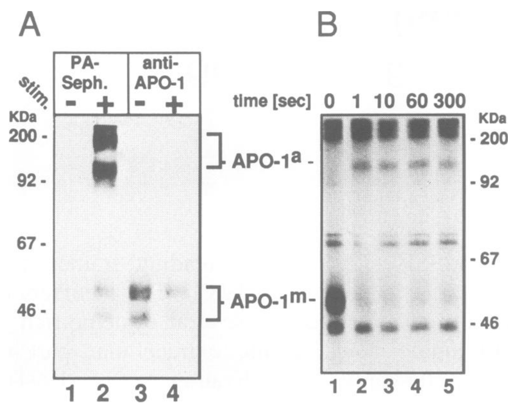

Fig. 1. Crosslinking of APO-1 results in receptor aggregation. (**A**) APO-1 Western blot using biotinylated anti-APO-1. HUT78 cells were incubated in the absence (–) or presence (+) of anti-APO-1 for 5 min. Cells were lysed in a 1% Brij-58-containing buffer. Solubilized APO-1 was immunoprecipitated with Protein A–Sepharose (lanes 1 and 2), followed by covalently coupled anti-APO-1 (lanes 3 and 4). Under non-reducing conditions, monomeric APO-1 in HUT78 cells sometimes appears as a double band, probably because of an inhomogeneity in the N-linked glycosylation. (**B**) Anti-APO-1 immunoprecipitates of ^{35}S-labeled K50 cells stimulated with anti-APO-1 for different periods of time and lysed in a 1% Triton X-100-containing buffer. Crosslinked APO-1 was then immunoprecipitated using Protein A–Sepharose and analyzed by SDS–PAGE. The migration positions of the monomeric (APO-1^{m}) and aggregated (APO-1^{a}) APO-1 are indicated.

surface APO-1 in HUT78 cells by the agonistic IgG3 anti-APO-1 mAb and immunoprecipitation with Protein A–Sepharose resulted in the detection of SDS-stable, high molecular weight APO-1 aggregates (Figure 1A, lane 2). Subsequently, non-crosslinked APO-1 was immunoprecipitated with anti-APO-1 and was found primarily in a monomeric form (Figure 1A, lane 4). Aggregate formation required anti-APO-1 binding because untreated cells predominantly contained monomeric APO-1 (Figure 1A, lane 3). A significant amount of APO-1 oligomers was detected when a Brij-58-containing lysis buffer was used that best preserved APO-1 aggregates. However, under these conditions APO-1 was not efficiently solubilized. Hence, in all subsequent APO-1 immunoprecipitation experiments, a Triton X-100-containing buffer was used that allowed for the efficient APO-1 extraction and detection of APO-1 aggregates. The formation of high molecular weight APO-1 species was very rapid and occurred instantly (1 s) after receptor crosslinking (Figure 1B). In APO-1-transfected BL-60 Burkitt's lymphoma cells (K50), a high molecular weight APO-1 species of ~110 kDa was detected after crosslinking with anti-APO-1 when the lysate was immunoprecipitated with Protein A–Sepharose (Figure 1B, lanes 2–5). In this immune complex, virtually no monomeric APO-1 was detectable (Figure 1B).

Crosslinked APO-1 associates specifically with CAP

To identify the signaling molecules interacting directly with activated oligomerized APO-1, metabolically labeled K50 cells were treated with IgG3 anti-APO-1. After lysis, crosslinked APO-1 was immunoprecipitated with Protein A–Sepharose beads (Figure 2). Only in the anti-APO-1-stimulated K50 cells were four CAPs detected (CAP1, 27 kDa, pI 5.2; CAP2, 28 kDa, pI 5.15; CAP3, 27 kDa, pI 5.05; and CAP4, 55 kDa, pI 5.02; Figure 2B). These

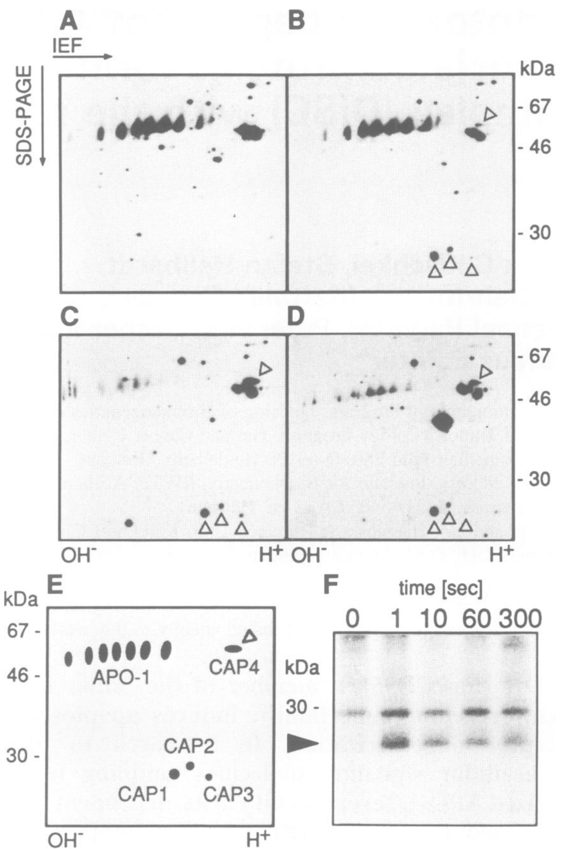

Fig. 2. Identification of CAPs. (A–D) Anti-APO-1 immunoprecipitates of ^{35}S-labeled cells. (**A**) Metabolically labeled K50 cells were first lysed in a Triton X-100-containing buffer and then treated with anti-APO-1. (B–D) Metabolically labeled K50 (**B**), HUT78 (**C**) or SKW6.4 (**D**) cells were stimulated with anti-APO-1 for 5 min and then lysed. APO-1 was immunoprecipitated in all cases with Protein A–Sepharose and analyzed on 2-D gels. CAP1–4 are labeled by arrowheads. As shown previously in malignant cells, APO-1 can hardly be metabolically labeled because of a very low turnover rate (Peter *et al.*, 1995). Despite high APO-1 expression (Table I), APO-1 spots in HUT78 and SKW6.4 cells were faint (C and D). In K50 cells, APO-1 is expressed under the control of the cytomegalovirus promoter. APO-1 spots were therefore more intense (A and B). (**E**) Schematic illustration of the migration positions of APO-1 and CAPs. (**F**) Kinetics of association of the small CAP. Anti-APO-1 immunoprecipitates of ^{35}S-labeled K50 cells treated with anti-APO-1 for the indicated time periods. Protein A–Sepharose was used for immunoprecipitation, and immunoprecipitates were analyzed by SDS–PAGE. The low molecular weight area of the gel is shown. The migration position of the small CAP is indicated by an arrowhead. The band at 30 kDa represents a background protein.

proteins were not found in immunoprecipitates from untreated K50 cells (Figure 2A). CAP1–4 were also present in stimulated HUT78, SKW6.4 (Figure 2C and D), the Burkitt's lymphoma cell line Raji and the leukemic T cell line H9, but not in unstimulated cells (data not shown). The association of CAP1, CAP2 and CAP3 was detected efficiently when cells were lysed in a buffer containing NP-40 or Triton X-100, but was hardly detectable when lysed in Brij-58 probably because of the inefficient solubilization of either APO-1 or CAPs (data not shown). The association of CAPs occurred as early as 1 s after anti-APO-1 stimulation (Figure 2F). These kinetics correlated with the formation of SDS-stable APO-1 species (Figure 1B). This indicates that the associa-

tion of CAPs is likely to mark the initiation of the APO-1 death signal. To test whether the occurrence of APO-1-associated proteins depended solely on the crosslinking of APO-1 with a cytotoxic anti-APO-1 mAb, the experiments were performed using a non-cytotoxic IgG2b anti-APO-1 isotype (Figure 3). Consistent with earlier results (Dhein *et al.*, 1992), only the IgG3 anti-APO-1 isotype was able to kill K50 cells (Figure 3A, column 4). The use of both the IgG3 and the IgG2b antibody isotype, but not an IgG3 control antibody (Figure 3B), resulted in the immunoprecipitation of equal amounts of APO-1 (Figure 3C and D). However, only the IgG3 isotype coimmunoprecipitated CAP (Figure 3C), suggesting that the detection of these proteins was associated with the induction of apoptosis whereas the immunoprecipitation of bound anti-APO-1 (IgG2b) did not detect any specifically associated proteins (Figure 3D). The association of CAPs was specific for APO-1 because they were not associated with other receptors such as the transferrin receptor (TfR; Figure 4B) or major histocompatibility complex class II in anti-APO-1 crosslinked SKW6.4 or K50 cells (data not shown).

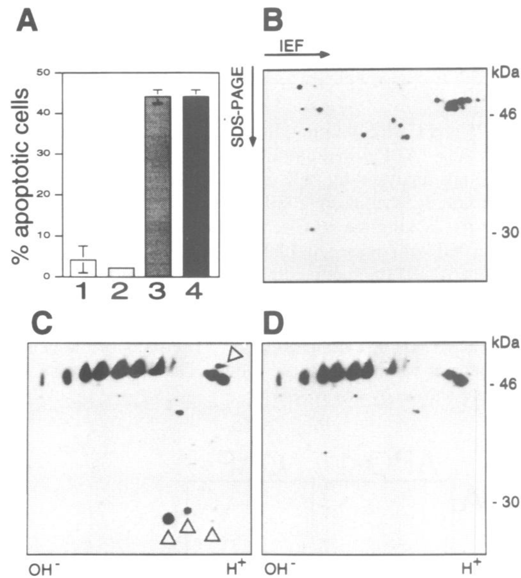

Fig. 3. Demonstration of APO-1–CAP association requires triggering by a cytotoxic anti-APO-1 mAb. (**A**) Apoptosis sensitivity assay with K50 cells incubated in medium (lane 1), with 2 µg/ml anti-APO-1 (IgG2b) (lane 2), with 2 µg/ml anti-APO-1 (IgG2b) + 10 µg/ml anti-mouse IgG (Fc-specific) (lane 3), or with 2 µg/ml anti-APO-1 (IgG3) (lane 4) for 20 h. The frequency of apoptotic cells was determined as described in Materials and methods. Experiments were performed in triplicate. The mean values and standard deviations are shown. (**B–D**) APO-1 immunoprecipitation from ^{35}S-labeled cells. Metabolically labeled K50 cells were first lysed. A non-binding IgG3 control mAb FII23c was added (B), or cells were first incubated with IgG3 anti-APO-1 (C) or IgG2b anti-APO-1 (D) and then lysed. In all cases, antibodies were immunoprecipitated with Protein A–Sepharose and immunoprecipitates were analyzed on 2-D gels. The parts of the gels covering APO-1 and the CAPs are shown. IgG2b anti-APO-1 crosslinked with anti-mouse IgG gave the same result as IgG3 anti-APO-1 (data not shown). The migration positions of the CAPs are labeled.

CAP association requires a functional APO-1 death domain

To test whether a functional death domain of APO-1 was required for CAP association, BL-60 cells were transfected with truncated APO-1 lacking the 57 C-terminal amino acids (APO-1Δ). Such a truncated APO-1 lacking about one-third of the death domain has been shown to be incapable of transducing a death signal (Itoh and Nagata, 1993). Despite high APO-1Δ expression (Figure 5A), these transfectants were even more resistant to apoptosis than the untransfected parental BL-60 cells which express low amounts of endogenous APO-1, indicating that the truncated receptor functions as a dominant negative mutant (Figure 5B). Transfectants were also found to be resistant when truncated receptors were expressed in about the same amount as endogenous APO-1 (data not shown). CAP1–4 were not associated with APO-1Δ when cells were crosslinked with IgG3 anti-APO-1 (Figure 5D). Metabolically labeled CAP1 and CAP2, but not CAP3 and CAP4 (data not shown), were also found to be associated with recombinant APO-1 cytoplasmic tail coupled to agarose beads (Figure 6C). CAP1 and CAP2 were not associated with agarose-coupled cytoplasmic tails with an lpr^{cg} amino acid substitution (Figure 6D) or with a 39 amino acid deletion (Figure 6E). Hence, CAP1–4 only associate with APO-1 containing a functional death domain.

CAPs associate exclusively with activated APO-1

To test which APO-1 species bound CAPs, HUT78 cells were treated with anti-APO-1 (Figure 7). Lysates were

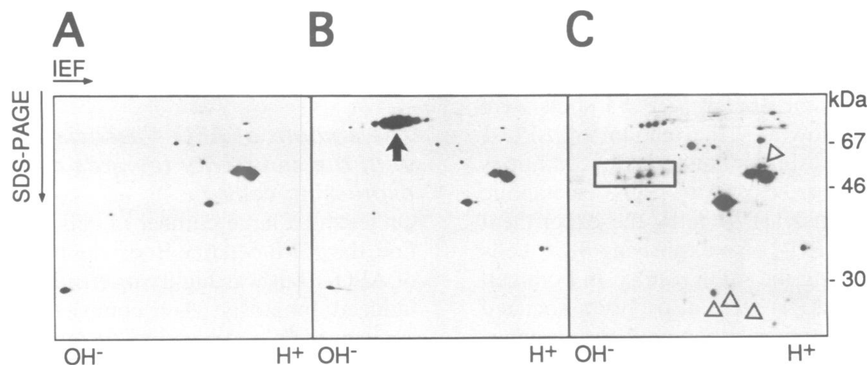

Fig. 4. The CAP–APO-1 association is specific. Metabolically labeled SKW6.4 cells were incubated with IgG3 anti-APO-1 for 5 min, lysed and subsequently immunoprecipitated with anti-IgG1 agarose beads (**A**), followed by an IgG1 anti-TfR mAb coupled to anti-IgG1 agarose beads (**B**) and Protein A–Sepharose (**C**). TfR is labeled by an arrow and APO-1 by a closed box. CAP1–4 are marked by open arrowheads.

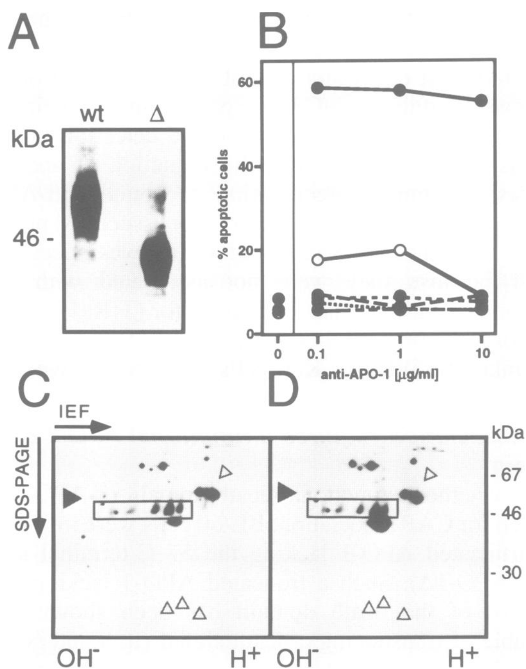

Fig. 5. CAP1–4 do not associate with truncated APO-1 that functions as a dominant negative mutant. (**A**) An analysis of surface biotinylated APO-1 (wt) and one representative APO-1Δ clone (Δ) by SDS–PAGE. (**B**) Apoptosis sensitivity assay of BL-60 (○—○), K50 (●—●) and four high APO-1Δ-expressing clones (K1.2, ●·····●; K2.2, ●– – – –●; K7.2, ●- - - -●; and K13.2, ●– – –●). Cells were incubated with increasing amounts of anti-APO-1 for 16 h. Apoptotic cells were determined as described in Materials and methods. The APO-1Δ clones expressed about the same amount or more APO-1Δ than the wild-type (wt) APO-1-expressing clone K50, as determined by immunoprecipitation and flow cytometry (data not shown). The decrease of apoptosis in the BL-60 cells at high anti-APO-1 concentrations is probably caused by the monovalent binding of the anti-APO-1 mAb to the weakly expressed APO-1. (**C** and **D**) APO-1 immunoprecipitation from metabolically labeled APO-1Δ cells incubated with anti-APO-1 after (unstimulated, C) or prior to (stimulated, D) lysis. Proteins were analyzed as described in the legend to Figure 2. Closed arrowhead, endogenous full-length APO-1; box, APO-1Δ; open arrowheads, migration positions of CAPs. Using the anti-MORT1 rabbit antibody (see Figure 8), the APO-1Δ-expressing cells were shown to express similar amounts of CAP1 and CAP2 as K50 cells (data not shown).

first immunoprecipitated with anti-APO-1 followed by an immunoprecipitation with protein A–Sepharose. Only crosslinked APO-1 was associated with CAPs (Figure 7B, right panel). Residual non-crosslinked APO-1 in the same lysates was not complexed with CAPs (Figure 7A, right panel). Because of inefficient labeling, APO-1 spots were weak in HUT78 cells. However, the amount of APO-1 immunoprecipitated was similar under all conditions (Figure 7, left panels, arrows). As APO-1 metabolic labeling was inefficient in HUT78 cells, the experiment was repeated with the APO-1-overexpressing K50 cells (Figure 7C and D), yielding the same results. In both cell lines, CAPs (including CAP4) were also only associated with crosslinked APO-1 when lysates were immunoprecipitated first with Protein A–Sepharose and then with anti-APO-1 beads (data not shown). These experiments suggest that heterotypically aggregated complexes exist next to monomeric APO-1 in the same cell.

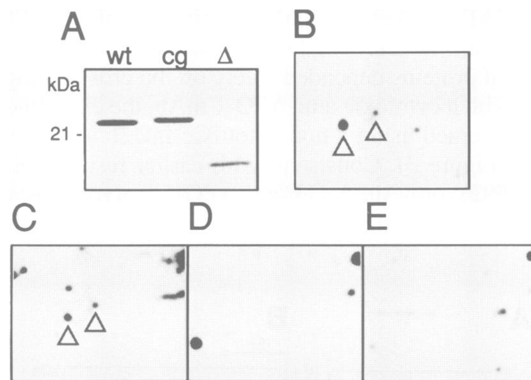

Fig. 6. CAP1 and CAP2 bind to wild-type but not to mutated APO-1 cytoplasmic tails. (**A**) Purified and Coomassie-stained APO-1 cytoplasmic tails analyzed by 15% SDS–PAGE. wt, wild-type cytoplasmic tail; cg, cytoplasmic tail carrying the lpr^{cg} mutation; Δ, cytoplasmic tail lacking the 39 C-terminal amino acids. (**B**) CAP1 and CAP2 associated with aggregated APO-1 in metabolically labeled SKW6.4 cells. (C–E) Immunoprecipitation of metabolically labeled SKW6.4 cells after lysis with a 1% NP-40-containing buffer with agarose beads coupled to recombinant cytoplasmic $tail^{wt}$ (**C**), cytoplasmic $tail^{cg}$ (**D**) or cytoplasmic $tail^{\Delta}$. The parts of the gels with the low molecular weight CAPs are shown. The positions of CAP1 and CAP2 are shown by arrowheads.

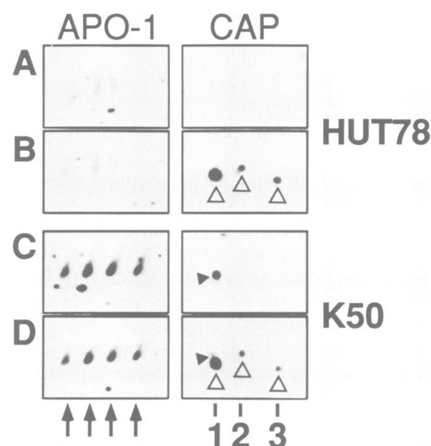

Fig. 7. CAPs associate only with crosslinked APO-1. Anti-APO-1 immunoprecipitates of metabolically labeled HUT78 (**A** and **B**) or K50 (**C** and **D**) cells incubated with anti-APO-1 for 5 min, lysed and immunoprecipitated with covalently coupled anti-APO-1 (A and C) followed by Protein A–Sepharose (B and D). The gel parts covering APO-1 (left panels) and CAP1–3 (right panels) are shown. Open arrowheads, CAP1–3. The migration positions of APO-1 are labeled by arrows. Closed arrowheads mark the position of the metabolically labeled IgG light chain of K50 cells which unspecifically binds to beads and migrates very close to the position of CAP1.

The amount of APO-1-associated CAPs correlates with the sensitivity towards apoptosis of APO-1^{hi}-expressing cells

On testing a large number of cell lines we found only one line, the pre-B cell line Boe, which expressed high amounts of APO-1 but was highly insensitive towards anti-APO-1-induced apoptosis when compared with other cells with similar APO-1 expression (data not shown). Cells were further selected for apoptosis resistance from wild-type Boe cells by continuous culture with IgG3 anti-APO-1 for 4 months. By now, Boe^R cells still expressed large amounts of APO-1 (Figure 8A and Table I) but were

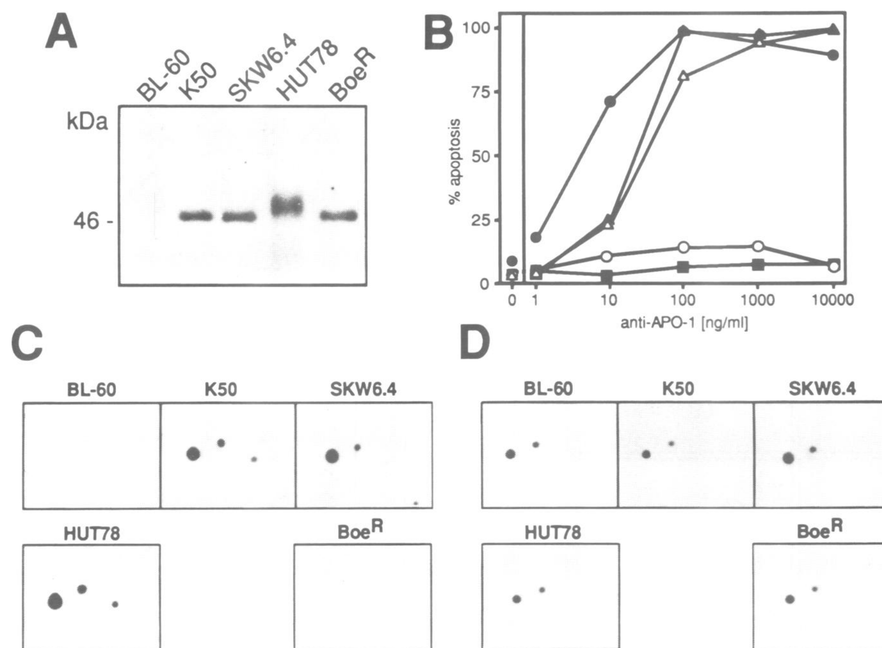

Fig. 8. CAP association with APO-1 correlates with sensitivity to APO-1-mediated apoptosis. (**A**) Western blot analysis of APO-1 in various cells. (**B**) Sensitivity to anti-APO-1-induced apoptosis of various cell lines. BL-60 (○); K50 (●); SKW6.4 (▲); HUT78 (△); Boe^R (■). (**C**) Anti-APO-1 immunoprecipitates from stimulated ^{35}S-labeled SKW6.4 cells. Shown is the area on 2-D gels of coimmunoprecipitating CAP1–3. (**D**) Anti-MORT1 immunoprecipitates from unstimulated ^{35}S-labeled cells under denaturing conditions. Shown is the same gel area as in (C). The specificity of the anti-MORT1 antibody was confirmed by blocking MORT1 immunoprecipitation in the presence of a 4-fold excess of MORT1 peptide (data not shown).

virtually resistant to anti-APO-1-induced apoptosis (Figure 8B). When tested for APO-1–CAP association, various cell lines showed a negative correlation between the association of CAP1–3 and the resistance of the cells (Figure 8C). CAP4 association followed the same pattern (data not shown). Using an affinity-purified rabbit anti-peptide antibody directed against the death domain of the recently cloned MORT1 protein (Boldin *et al.*, 1995b), CAP1 and CAP2 were identified as MORT1. This was demonstrated directly by a specific re-immunoprecipitation of APO-1-associated CAP1 and CAP2 with anti-MORT1 antibodies (data not shown); the published apparent molecular weight of MORT1 on SDS–PAGE (27/28 kDa) was very similar to that of CAP1 (28 kDa) and CAP2 (29 kDa). CAP3 was not found in any of the anti-MORT1 immunoprecipitates. CAP2 may represent a post-translationally modified form of MORT1. Despite differential apoptosis sensitivity, all cells tested expressed similar amounts of MORT1 (Figure 8D). Thus, the apoptosis resistance of Boe^R cells may be caused by an altered CAP association rather than by a lack of MORT1 expression.

CAP1 and CAP2 represent phosphorylated MORT1

To test whether the CAPs were phosphorylated, *in vivo* phosphorylation experiments were performed. When stimulated with anti-APO-1 on 2-D gels, two phosphorylated proteins were detected (Figure 9B) that comigrated with metabolically labeled CAP1 and CAP2, and were also immunoprecipitated with the anti-MORT1 antibody (Figure 8D). Under both conditions, CAP1 produced the more intense spot compared with CAP2. CAP1 and CAP2 were also found only with crosslinked APO-1 in an *in vitro* kinase assay. Here CAP2 was the more abundant species

Table I. Analysis of different cell lines for APO-1 surface expression

Cell lines	% Positive cells	
	control	anti-APO-1
BL-60	1.2 (4.0)	10.0 (22.5)
K50	2.5 (2.6)	99.2 (57.8)
SKW6.4	0.2 (3.3)	99.9 (57.8)
HUT78	0.3 (3.2)	99.9 (56.7)
Boe^R	2.4 (3.9)	95.0 (71.0)

Values in parenthesis are mean fluorescence intensity. A phycoerythrin-conjugated goat anti-mouse IgG antibody was used as second step reagent. Control cells were stained with the second step reagent only.

(Figure 9D), suggesting that CAP2 represents a higher phosphorylated form of CAP1. Only phosphorylated CAP2 was found when the APO-1 cytoplasmic tail^{wt} was used for immunoprecipitation but not when the lpr^{cg} mutant tail was employed (Figure 9F and E), again suggesting that CAP2 represents a more highly phosphorylated form of CAP1. The identity of CAP2 as MORT1 was additionally established by the re-immunoprecipitation of APO-1- and APO-1 cytoplasmic tail-associated CAP2 (data not shown). A phospho-amino acid analysis revealed that CAP1 (data not shown) and CAP2 (Figure 9G) are phosphorylated on serine residues.

Discussion

Many biological processes, such as cell growth, differentiation or apoptosis, are regulated in part by cell surface receptors. Their ligands (polypeptide growth factors or

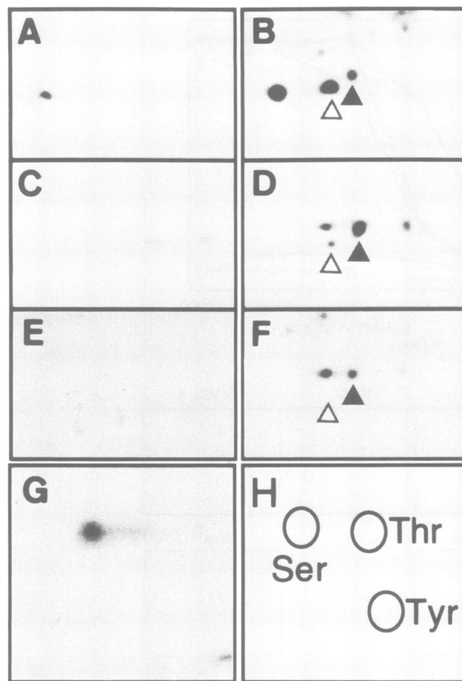

Fig. 9. CAP1 and CAP2 represent phosphorylated MORT1. (**A** and **B**) Protein A–Sepharose immunoprecipitates of *in vivo*-phosphorylated SKW6.4 cells. (**C** and **D**) *In vitro* kinase assay on APO-1 immunoprecipitated with protein A–Sepharose. SKW6.4 cells were first lysed and then treated with anti-APO-1 (A and C), or first stimulated with anti-APO-1 for 5 min and then lysed (B and D). (**E** and **F**) *In vitro* kinase assay on APO-1 cytoplasmic tail^{cg} (E) and APO-1 cytoplasmic tail^{wt} (F) immunoprecipitates from unstimulated SKW6.4 cells lysed in a 1% NP-40-containing buffer. (**G**) Phosphoamino acid analysis of *in vitro*-labeled CAP2. (**H**) The migration positions of phosphorylated amino acids by 2-D thin-layer electrophoresis.

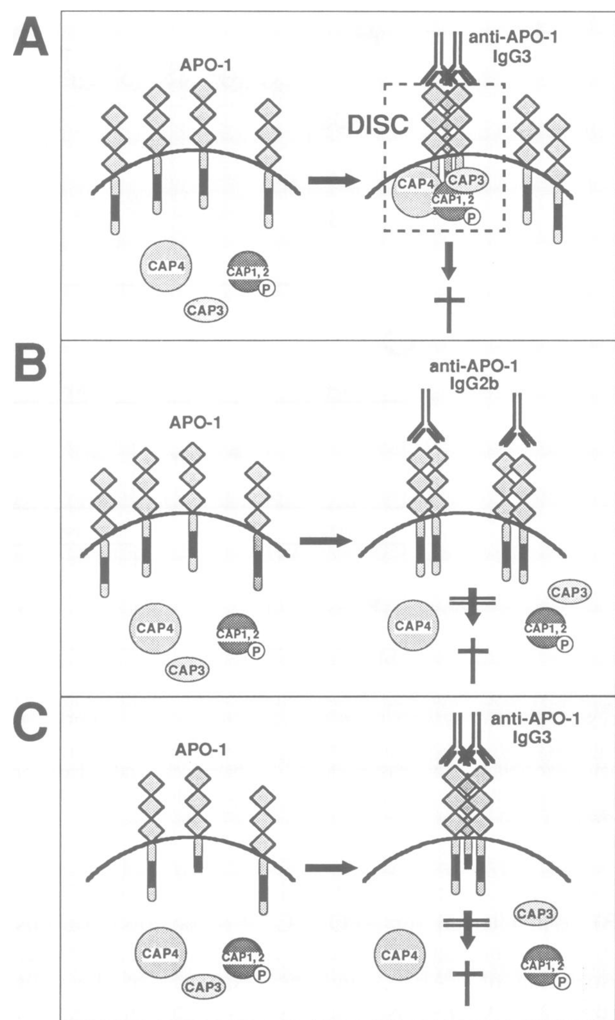

Fig. 10. The role of CAP1–4 in mediating anti-APO-1-induced apoptosis (model). (**A**) CAP1–4 associate only with IgG3 anti-APO-1-oligomerized but not with monomeric APO-1 forming the DISC. (**B**) APO-1 dimerization induced by the non-cytotoxic IgG2b anti-APO-1 mAb does not result in CAP association or cell death. (**C**) CAP1–4 do not associate with IgG3 anti-APO-1-induced oligomers containing APO-1Δ, preventing apoptosis.

cytokines) are unable to penetrate hydrophobic cell membranes. Therefore, the signal mediated by such molecules must be transduced into the cell by other mechanisms, which may include receptor dimerization or oligomerization (for a review see Heldin, 1995), e.g. TNFβ has been crystallized together with TNF-RI and shown to form a trimer (Banner *et al.*, 1993). It has been proposed that all receptors of the TNF-R family function as trimers (Banner *et al.*, 1993). Thus, as shown previously, APO-1 must also be oligomerized to signal apoptosis (Dhein *et al.*, 1992).

Here we show that a number of proteins (termed CAPs) associate with oligomerized but not monomeric APO-1 in an activation-dependent fashion as early as 1 s after receptor crosslinking and form a DISC (Figure 10A). CAPs do not associate with dimerized APO-1 or TfR (Figures 10B and 4B). Truncated APO-1 (APO-1Δ) lacking the 57 C-terminal amino acids was found to be signaling deficient, consistent with a previous report (Itoh and Nagata, 1993). In addition, it acted as a dominant negative inhibitor of APO-1-induced apoptosis, similar to the situation in TNF-RI signaling. Both APO-1 and TNF-RI contain a death domain (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993), and both receptors can induce apoptosis. TNF-RI signaling could be inhibited by transfecting cells with a truncated TNF-RI lacking the entire cytoplasmic region (Brakebusch *et al.*, 1992; Tartaglia and Goeddel, 1992). An activated complex of APO-1 and APO-1Δ did not associate with CAPs, indicating that a minimal number of APO-1 molecules with an intact death domain is required for efficient CAP association and DISC formation (Figure 10C). Taken together, our data indicate that CAP association is receptor specific and depends on functionally active APO-1. Since this association is also linked functionally to apoptosis, CAP1–4 are prime candidates for death-transducing molecules.

The early events of the APO-1 signaling pathway are controversial. A recent report has shown that the Ca^{2+} flux is important for APO-1 signaling (Oshimi and Miyazaki, 1995). We and others have not been able to confirm these data (Vignaux *et al.*, 1995; unpublished data). Tyrosine phosphorylation was also reported to be an early and requisite event in APO-1 signaling (Eischen *et al.*, 1994). However, our own experiments are not in line with these results (Schraven and Peter, 1995). Further downstream signaling events were shown to be involved in APO-1 signaling: (i) the activation of an acidic sphingomyelinase (Cifone *et al.*, 1993); (ii) the activation of p21^{ras} (Gulbins *et al.*, 1995); (iii) the involvement of a member of the

ICE protease family (Los *et al.*, 1995); (iv) the proteolytic processing of a member of the cdc2 kinase family (Lahti *et al.*, 1995); and (v) the translocation of the RNA binding protein TIAR from the nucleus to the cytoplasm (Taupin *et al.*, 1995).

Using the yeast two-hybrid system, three APO-1-associated proteins have been cloned recently that might be involved in APO-1 signaling. The first is FAP-1, a protein tyrosine phosphatase that associates with the 15 C-terminal amino acids of APO-1 (Sato *et al.*, 1995) and is probably not directly part of the APO-1 signaling cascade because it is not expressed in highly APO-1-sensitive cells (e.g. Jurkat T cells); it may also confer resistance to APO-1-mediated apoptosis. The other two are MORT1/FADD and RIP, which bind to the APO-1 death domain and contain a death domain themselves (Boldin *et al.*, 1995b; Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995).

CAP1 and CAP2 were both identified as MORT1/FADD using an affinity-purified anti-MORT1 rabbit antibody. CAP1 and CAP2 detected on 2-D gels from *in vivo*-phosphorylated and ^{35}S-labeled cells migrated to the same position as the anti-MORT1 immunoprecipitates. Both proteins could be specifically re-immunoprecipitated with the anti-MORT1 rabbit antibody. CAP1 and CAP2 most probably represent phosphorylated MORT1/FADD. CAP3 was not associated with activated APO-1 in *in vivo*-phosphorylated cells (Figure 9B). Therefore, it is unlikely that it represents another phosphorylated MORT1 species. No associated protein could be detected that corresponded to unphosphorylated MORT1. Thus, we conclude that only phosphorylated MORT1 associates with APO-1.

It is unlikely that the phosphorylation of MORT1 is the result of APO-1 aggregation for two reasons. (i) MORT1 associated instantly with APO-1 after receptor aggregation; this process was therefore too rapid for a protein kinase to be activated. (ii) CAP1 and CAP2 were also associated with the APO-1 cytoplasmic tail in cells which had not been activated. Hence, phosphorylated MORT1 was already present in non-activated cells.

In all cells tested, CAP1–4 were only found associated with oligomerized APO-1. Monomeric APO-1 from the same cell lysates was not complexed with the CAPs (Figure 10A). This situation seems to be similar to that with the T cell receptor (TCR)–CD3 complex. In addition, TCR aggregation is necessary for efficient signaling (Ratcliffe *et al.*, 1992). However, TCR signaling molecules such as $p56^{lck}$ or $p59^{fyn}$ are constitutively associated with the receptor and become activated upon receptor stimulation (Samelson *et al.*, 1990). Other signaling molecules, such as ZAP70 or phosphatidylinositol-3′ kinase, only associate upon receptor phosphorylation (for a review see Chan *et al.*, 1994). A similar situation is found for B cell receptor immunoglobulin and its association with the $p53/56^{lyn}$ and $p59^{fyn}$ kinases (Pleiman *et al.*, 1994). We have not seen APO-1 phosphorylation upon activation. Therefore, the association of CAPs was probably caused by receptor aggregation only. The association of signaling molecules with oligomerized receptors without a phosphorylation step has rarely been observed. The $p72^{syk}$ kinase associates with the aggregated high-affinity IgE receptor (Fcε RI; Benhamou *et al.*, 1993; Chacko *et al.*, 1994). In some reports, JAK1 and JAK2 kinases have been shown to associate with the interleukin 4 receptor and the growth hormone receptor, respectively, in an activation-dependent manner (Argetsinger *et al.*, 1993; Yin *et al.*, 1994). The oligomerization-dependent association of CAPs with APO-1 might uniquely facilitate apoptosis dependent on the quantity of the APO-1 ligand. The strength of the death signal might depend on the quantity of APO-1 and CAPs drawn into the DISC.

Boldin *et al.* (1995a) have found recently that death domains self-aggregate when transfected into HeLa cells, thereby signaling death. Therefore to prevent the death signal, APO-1 death domains must be kept apart. We have shown recently that APO-1 sensitivity can be modulated by surface sialylation (Peter *et al.*, 1995). The negative charges resulting from APO-1 sialylation could function as a repellent to separate APO-1 molecules and their death domains. In addition, intracellular protecting molecules constitutively binding to the APO-1 death domains might prevent self-aggregation and cell death. These molecules might be replaced by CAPs upon receptor oligomerization. However, no proteins were detected that were specifically associated with monomeric APO-1 and were not present in oligomerized APO-1 complexes. Alternatively, APO-1 could also assume a ligand-induced conformation which allows CAPs to bind. Two pieces of data seem to conflict with this model.

(i) MORT1/FADD was cloned originally using the yeast two-hybrid system and associating with monomeric APO-1 (Boldin *et al.*, 1995b; Chinnaiyan *et al.*, 1995). The binding of MORT1/FADD could be explained by a lower but sufficient binding affinity of MORT1/FADD to the APO-1 cytoplasmic tail in yeast cells than to crosslinked APO-1 in mammalian cells under physiological conditions.

(ii) The binding of CAP1 and CAP2 to 'non-activated' APO-1 cytoplasmic tail. APO-1 cytoplasmic tail had a high tendency to self-aggregate, possibly because of the lack of an extracellular domain with its negatively charged sialyl groups. On overloaded gels, SDS-stable oligomeric forms of APO-1 cytoplasmic tail could be detected (data not shown). This might explain why CAP1 and CAP2 also associated with APO-1 cytoplasmic tail^{wt} in lysates from non-activated cells.

The fact that MORT1/FADD and RIP associate with APO-1 suggested a biological importance for these molecules (Boldin *et al.*, 1995b; Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995). The N-terminus of FADD was shown to be both essential and sufficient to induce apoptosis in transfected cells (Chinnaiyan *et al.*, 1995), and RIP contains a putative N-terminal kinase domain (Stanger *et al.*, 1995). This qualifies both proteins as effector molecules. However, none of the reports established receptor association *in vivo*. This leaves two ways of action for these death domain-containing proteins (Cleveland and Ihle, 1995): (i) APO-1 aggregation activates receptor-associated signaling molecules (these molecules might be released and might function as effectors of cell death); or (ii) APO-1 aggregation might facilitate the recruitment of such molecules to the DISC.

Our data suggest that receptor oligomerization results in the instant recruitment of the death domain protein MORT1/FADD (CAP1/2) to the APO-1 receptor. Two other proteins (CAP3 and CAP4) acted in a similar fashion. We are now testing whether CAP4 (55 kDa) could be

proteolytically processed RIP (74 kDa; Stanger *et al.*, 1995).

The discovery of proteins that only associate with aggregated APO-1 provides the main step towards the identification of the DISC and the apoptosis mechanism. Further downstream, effector molecules can now be identified that transduce the death signal to the nucleus.

Materials and methods

Cells

The monocytic cell line U937, the leukemic T cell line HUT78, the B lymphoblastoid cell line SKW6.4, the pre-B cell line Boe, the group I Burkitt's lymphoma BL-60 cell line, its full-length APO-1 transfectants K4 and K50 (Oehm *et al.*, 1992), and the APO-1Δ-expressing clones K1.2, K2.2, K7.2 and K13.2 (see below) were maintained in RPMI 1640 (Whittaker, Walkersville, MD), Gibco Biocult (Eggenheim, Germany), 10 mM HEPES, pH 7.3, 10% fetal calf serum (FCS; Conco, Wiesbaden, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) in 5% CO_2. Boe^R, a variant of Boe and resistant towards anti-APO-1-induced apoptosis, was generated by continuous culture in medium containing anti-APO-1 (IgG3, κ, 1 μg/ml) for >4 months. Next, the cells were cultured further in medium without anti-APO-1.

Antibodies

The murine anti-APO-1 mAbs (IgG3 and IgG2b) recognized an epitope on the extracellular part of APO-1 (Trauth *et al.*, 1989; Dhein *et al.*, 1992). The TfR-specific mAb PA-1 (IgG1) was a generous gift from Dr G.Moldenhauer (Van Endert and Moldenhauer, 1992). FII23c is a non-binding murine mAb (IgG3). The rabbit anti-mouse IgG Fc-specific antibody was obtained from Dianova (Hamburg, Germany). A peptide corresponding to amino acids 372–390 with an N-terminal attached cysteine residue (CEDRYPRNLTERVRESLRIW) of the MORT1 primary structure (Boldin *et al.*, 1995b) was synthesized on an Applied Biosystems peptide synthesizer (Weiterstadt, Germany). A polyclonal rabbit serum was generated, and affinity purification of the antibody was performed as described previously (Peter *et al.*, 1995).

Escherichia coli expression of histidine-tagged APO-1 cytoplasmic tails

The cDNA corresponding to the cytoplasmic part of APO-1 was obtained by PCR from the full-length APO-1 cDNA (Oehm *et al.*, 1992). The primers used were 5′-GCCGAATCCAAGAGAAAGGAAGTACAG (sense primer) and 3′-CAGGGATCCCTAGACCAAGCTTTGGAT (antisense primer). Into both primers a *Bam*HI site was introduced to allow for cloning into the expression vector pQE-8 (Qiagen, Hilden, Germany) carrying the sequence for six consecutive histidine residues in front of its multiple cloning site. A mutant cytoplasmic tail carrying a Val238→Asn238 mutation, a point mutation leading to the signaling defect in lpr^{cg} mice, was made by site-directed mutagenesis using a two-step protocol (Higuchi *et al.*, 1988) and employing two additional oligonucleotides corresponding to positions 747–717 in the APO-1 cDNA: CGAAAGAATGGT**AAC**AATGAAGCC and GGCTTCATT**GT**-**T**ACCATTCTTTCG (altered bases are in bold). A deletion mutant was obtained by *Bam*HI and *Hin*dIII digestion. Blunt ends were generated by incubation with mung bean nuclease (Pharmacia) and subsequent ligation, leading to a mutated APO-1 cytoplasmic tail lacking the 39 C-terminal amino acids. All cytoplasmic tail constructs were sequenced in both directions. The expression of the cytoplasmic tail construct and its mutants was obtained by incubation of the K12-derived *E.coli* strain SG13009 (pREP4; Qiagen) with 2 mM isopropylthiogalactoside for 4 h according to the manufacturer's instructions. After lysis of the bacteria, as described previously (Wittinghofer *et al.*, 1983), the pellet was frozen in lysis buffer (0.01 M Tris–HCl, 0.1 M NaH_2PO_4, pH 8.0) containing 250 mM guanidine–HCl. Proteins were precipitated from the supernatant after thawing using the Ni^{2+}–NTA–agarose system, washed extensively with buffer (1 M NaCl, 10% glycerol, 50 mM NaH_2PO_4, pH 6.0, 1% Triton X-100, 25 mM imidazole) and stored in PBS/0.1% NaN_3 at 4°C.

Generation of stable BL-60 transfectants expressing an APO-1 deletion mutant

A cDNA lacking the 57 C-terminal amino acids of APO-1 was generated by PCR. Oligonucleotides contained either *Xho*I or *Xba*I restriction sites and plasmid pKEX-APO-1 (Oehm *et al.*, 1992) serving as a template were used. The amplimers were digested with the restriction enzymes *Xho*I and *Xba*I, purified by gel electrophoresis and cloned into the pKEX2XR vector (Rittner *et al.*, 1991). The correct sequence was verified by double-strand DNA sequencing. BL-60 cells were transfected, as described earlier (Oehm *et al.*, 1992).

Metabolic labeling, surface biotinylation, immunoprecipitation and PAGE

For ^{35}S-labeling, 3×10^7 cells were washed twice with PBS and incubated in 15 ml RPMI without cysteine and methionine (Gibco) for 1 h at 37°C. 0.5 mCi ^{35}S-Translabel (ICN, Meckenheim, Germany) were added and the cells were incubated at 37°C for 20 h. For stimulation, cells were then incubated with 2 μg/ml anti-APO-1, as specified in the figure legends, transferred to dry ice/isopropanol, washed with PBS and lysed in 1 ml of lysis buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and small peptide inhibitors, as described (Peter *et al.*, 1995), containing either 1% NP-40 (Serva, Heidelberg, Germany) or 1% Triton X-100 (Serva) and 10% glycerol for 15 min on ice. In unstimulated controls, 10 μg anti-APO-1 were added after cell lysis. Post-nuclear supernatants were added to 10 μg anti-APO-1 covalently coupled to 30 μl CNBr-activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden), 10 μg of the isotype-matched control mAb FII23c or 10 μg anti-APO-1 coupled to 30 μl Protein A–Sepharose (Pharmacia), and rotated for 1–3 h at 4°C. Using the same conditions, the TfR was immunoprecipitated as a control using anti-IgG1 agarose beads (Sigma, Deisenhofen, Germany). For the immunoprecipitation of MORT1, 10^7 metabolically labeled cells were lysed in 100 μl 1% NP-40-containing lysis buffer. Post-nuclear supernatant was boiled for 2 min in the presence of 0.5% SDS. After the addition of nine volumes of lysis buffer without SDS, MORT1 was immunoprecipitated using 20 μg anti-MORT1 antibody coupled to 30 μl Protein A–Sepharose. In some experiments, His-tagged APO-1 cytoplasmic tails coupled to Ni-NTA beads were used for the immunoprecipitation of unstimulated cells. All beads were washed five times with lysis buffer. Immune complexes were subjected to IEF/12% SDS–PAGE analysis, as described previously (Huber and Peter, 1994), or boiled in a standard reducing sample buffer for 3 min and separated on a 12% SDS–polyacrylamide gel. The biotinylation of surface proteins from 10^7 cells was performed as described previously (Peter *et al.*, 1995). Surface biotinylated proteins were immunoprecipitated using covalently coupled anti-APO-1 and analyzed by 10% SDS–PAGE, as described above.

Immune complex kinase assays, *in vivo* phosphorylation and phospho-amino acid analysis

A total of 10^7 cells were incubated with 10 μg anti-APO-1 in medium ($2–3\times10^5$ cells/ml) at 37°C. Cells were washed twice with Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.4, 130 mM NaCl), lysed in 500 μl lysis buffer and immunoprecipitated, as described previously (Peter *et al.*, 1995). Immune complexes were incubated in 40 μl of assay buffer [75 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1% Triton X-100, 100 nM [γ-^{32}P]ATP (ICN, specific activity 3000 Ci/mmol)] for 20 min at 25°C. Reactions were stopped by washing the beads once with lysis buffer. Labeled complexes were subjected to a 2-D gel analysis. For *in vivo* phosphorylation, 3×10^7 cells were washed twice with TBS and incubated in 30 ml Dulbecco's modified Eagle's medium without HPO_4^{2-} (Gibco) for 1 h at 37°C. For labeling, 0.5 mCi [^{32}P]orthophosphate (NEN, specific activity 9000 Ci/mmol) were added and cells were incubated at 37°C for 1 h. For stimulation, cells were then incubated with 2 μg/ml anti-APO-1, as specified in the figure legends, and washed twice with ice-cold PBS containing 10 mM NaF and 10 mM Na_3VO_4. Lysis, immunoprecipitation and IEF/12% SDS–PAGE analysis were performed as described above. Phospho-amino acid analysis was performed as described previously (Schraven *et al.*, 1991).

Detection of monomeric or aggregated APO-1 by Western blotting

For Western blot detection, APO-1 was immunoprecipitated from 10^7 cells treated with 2 μg/ml anti-APO-1 (IgG3) for 5 min at 37°C or untreated cells with 10 μg anti-APO-1 covalently coupled to CNBr-activated Sepharose CL-4B or 30 μl Protein A–Sepharose, as described above. Immune complexes were separated by 10% SDS–PAGE, transferred to Hybond nitrocellulose membrane (Amersham, Braunschweig, Germany), blocked with 5% dry milk in PBS for at least 1 h, washed with PBS/0.02% Tween-20 (PBS/Tween) and incubated with biotinylated anti-APO-1 (1:5000), as described previously (Peter *et al.*, 1995). The blot was washed with PBS/Tween and developed with streptavidin–biotinylated horseradish peroxidase complex (1:3000; Amersham). After

washing with PBS/Tween, the blots were developed with the chemiluminescence method ECL following the manufacturer's protocol (Amersham).

Cell stimulation and cytotoxicity assay

A total of 5×10^5 cells were incubated in 48-well plates (Costar, Cambridge, MA) with anti-APO-1 (IgG3) (10^6 cells/ml) for 18 h at 37°C. The quantification of DNA fragmentation as a specific measure of apoptosis was carried out essentially as described elsewhere (Peter *et al.*, 1995).

Immunofluorescence

For indirect immunofluorescence, 10^6 cells were washed and resuspended in 100 µl of affinity-purified antibody diluted in PBS (10 µg/ml), 5% FCS and 0.1% NaN_3. Cells were incubated for 30 min on ice, washed and resuspended in 100 µl of 1:200 diluted phycoerythrin-conjugated goat anti-mouse IgG (Dianova), incubated for 30 min on ice, washed again and analyzed on a FACScan (Becton-Dickinson, Heidelberg, Germany).

Acknowledgements

We are grateful to W.Müller, M.Oppenländer and U.Silberzahn for expert technical assistance. This work was supported by grants from the Bundesministerium für Forschung und Technologie, Bonn, and the Tumor Center Heidelberg/Mannheim, Germany.

References

Argetsinger,L.S., Campbell,G.S., Yang,X., Witthuhn,B.A., Silvennoinen,O., Ihle,J.N. and Carter-Su,C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell*, **74**, 237–244.

Banner,D.W., D'Arcy,A., Janes,W., Gentz,R., Schoenfeld,H.J., Broger,C., Loetscher,H. and Lesslauer,W. (1993) Crystal structure of the soluble human 55 kd TNF receptor–human TNF β complex: implications for TNF receptor activation. *Cell*, **73**, 431–445.

Benhamou,M., Ryba,N.J., Kihara,H., Nishikata,H. and Siraganian,R.P. (1993) Protein-tyrosine kinase $p72^{syk}$ in high affinity IgE receptor signaling. Identification as a component of pp72 and association with the receptor gamma chain after receptor aggregation. *J. Biol. Chem.*, **268**, 23318–23324.

Boldin,M.P., Mett,I.L., Varfolomeev,E.E., Chumakov,I., Shemer-Avni,Y., Camonis,J.H. and Wallach,D. (1995a) Self-association of the 'death domains' of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *J. Biol. Chem.*, **270**, 387–391.

Boldin,M.P., Varfolomeev,E.E., Pancer,Z., Mett,I.L., Camonis,J.H. and Wallach,D. (1995b) A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.*, **270**, 7795–7798.

Brakebusch,C., Nophar,Y., Kemper,O., Engelmann,H. and Wallach,D. (1992) Cytoplasmic truncation of the p55 tumour necrosis factor (TNF) receptor abolishes signalling, but not induced shedding of the receptor. *EMBO J.*, **11**, 943–950.

Camerini,D., Walz,G., Loenen,W.A.M., Borst,J. and Seed,B. (1991) The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. *J. Immunol.*, **147**, 3165–3169.

Chacko,G.W., Duchemin,A.M., Coggeshall,K.M., Osborne,J.M., Brandt,J.T. and Anderson,C.L. (1994) Clustering of the platelet Fc gamma receptor induces noncovalent association with the tyrosine kinase $p72^{syk}$. *J. Biol. Chem.*, **269**, 32435–32440.

Chan,A.C., Desai,D.M. and Weiss,A. (1994) The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu. Rev. Immunol.*, **12**, 555–592.

Chinnaiyan,A.M., O'Rourke,K., Tewari,M. and Dixit,V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 505–512.

Cifone,M.G., De Maria,R., Roncaioli,P., Rippo,M.R., Azuma,M., Lanier,L.L., Santoni,A. and Testi,R. (1993) Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. *J. Exp. Med.*, **177**, 1547–1552.

Cleveland,J.L. and Ihle,J.N (1995) Contenders in FasL/TNF death signalling. *Cell*, **81**, 479–482.

Davidson,W.F., Dumont,F.J., Bedigian,H.G., Fowlkes,B.J. and Morse,H.C.,III (1986) Phenotypic, functional, and molecular genetic comparisons of the abnormal lymphoid cells of C3H-lpr/lpr and C3H-gld/gld mice. *J. Immunol.*, **136**, 4075–4084.

Dhein,J., Daniel,P.T., Trauth,B.C., Oehm,A., Möller,P. and Krammer,P.H. (1992) Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. *J. Immunol.*, **149**, 3166–3176.

Dürkop,H., Latza,U., Hummel,M., Eitelbach,F., Seed,B. and Stein,H. (1992) Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's disease. *Cell*, **68**, 421–427.

Eischen,C.M., Dick,C.J. and Leibson,P.J. (1994) Tyrosine kinase activation provides an early and requisite signal for Fas-induced apoptosis. *J. Immunol.*, **153**, 1947–1954.

Gulbins,E. *et al.* (1995) Fas-induced apoptosis is mediated via a ceramide-initiated Ras signalling pathway. *Immunity*, **2**, 341–351.

Heldin,C.-H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell*, **80**, 213–223.

Higuchi,R., Krummel,B. and Saiki,R.K. (1988) A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acid Res.*, **16**, 7351–7367.

Huber,L.A. and Peter,M.E. (1994) Mapping small GTP-binding proteins on high-resolution two-dimensional gels by a combination of GTP binding and labeling with *in situ* periodate-oxidized GTP. *Electrophoresis*, **15**, 283–288.

Itoh,N. and Nagata,S. (1993) A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J. Biol. Chem.*, **268**, 10932–10937.

Itoh,N., Yonehara,S., Ishii,A., Yonehara,M., Mizushima,S., Sameshima,M., Hase,A., Seto,Y. and Nagata,S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66**, 233–243.

Johnson,D., Lanahan,A., Buck,C.R., Sehgal,A., Morgan,C., Mercer,E., Bothwell,M. and Chao,M. (1986) Expression and structure of the human NGF receptor. *Cell*, **47**, 545–554.

Krammer,P.H. *et al.* (1994) The role of APO-1-mediated apoptosis in the immune system. *Immunol. Rev.*, **142**, 175–191.

Kwon,B.S. and Weissman,S.M. (1989) cDNA sequences of two inducible T-cell genes. *Proc. Natl Acad. Sci. USA*, **86**, 1963–1967.

Lahti,J.M., Xiang,J., Heath,L.S., Campana,D. and Kidd,V.J. (1995) PITSLRE protein kinase activity is associated with apoptosis. *Mol. Cell. Biol.*, **15**, 1–11.

Loetscher,H., Pan,Y.-C., Lahm,H.-W., Gentz,R., Brockhaus,M., Tabuchi,H. and Lesslauer,W. (1990) Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell*, **61**, 351–359.

Los,M. *et al.* (1995) Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. *Nature*, **375**, 81–83.

Mallett,S., Fossum,S. and Barclay,A.N. (1990) Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes—a molecule related to nerve growth factor receptor. *EMBO J.*, **9**, 1063–1068.

Mariani,S.M., Matiba,B., Armandola,E.A. and Krammer,P.H. (1994) The APO-1/Fas (CD95) receptor is expressed in homozygous MRL/lpr mice. *Eur. J. Immunol.*, **24**, 3119–3123.

Oehm,A. *et al.* (1992) Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J. Biol. Chem.*, **267**, 10709–10715.

Oshimi,Y. and Miyazaki,S. (1995) Fas antigen-mediated DNA fragmentation and apoptotic morphologic changes are regulated by an elevated cytosolic Ca^{2+} level. *J. Immunol.*, **154**, 599–609.

Peter,M.E., Hellbardt,S., Schwartz-Albiez,R., Westendorp,M.O., Walczak,H., Moldenhauer,G., Grell,M. and Krammer,P.H. (1995) Cell surface sialylation plays a role in modulating sensitivity towards APO-1-mediated apoptotic cell death. *Cell Death Differ.*, **2**, 163–171.

Pleiman,C.M., Abrams,C., Gauen,L.T., Bedzyk,W., Jongstra,J., Shaw,A.S. and Cambier,J.C. (1994) Distinct $p53/56^{lyn}$ and $p59^{fyn}$ domains associate with nonphosphorylated and phosphorylated Igα. *Proc. Natl Acad. Sci USA*, **91**, 4268–4272.

Ratcliffe,M.J., Coggeshall,K.M., Newell,M.K. and Julius,M.H. (1992) T cell receptor aggregation, but not dimerization, induces increased cytosolic calcium concentrations and reveals a lack of stable association between CD4 and the T cell receptor. *J. Immunol.*, **148**, 1643–1651.

Rittner,K., Stöppler,H., Pawlita,M. and Sczakiel,G. (1991) Versatile eukaryotic vectors for strong and constitutive transient and stable gene expression. *Methods Mol. Cell. Biol.*, **2**, 176–181.

Samelson,L.E., Phillips,A.F., Luong,E.T. and Klausner,R.D (1990)

Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc. Natl Acad. Sci. USA*, **87**, 4358–4362.

Sato,T., Irie,S., Kitada,S. and Reed,J.C. (1995) FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science*, **268**, 411–415.

Schall,T.J. *et al.* (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell*, **61**, 361–370.

Schraven,B. and Peter,M.E. (1995) APO-1(CD95)-mediated apoptosis in Jurkat cells does not involve src kinases or CD45. *FEBS Lett.*, **368**, 491–494.

Schraven,B., Kirchgessner,H., Gaber,B., Samstag,Y. and Meuer,S.C. (1991) A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase CD45, the protein tyrosine kinase $p56^{lck}$ and pp32, a possible common substrate. *Eur. J. Immunol.*, **21**, 2469–2477.

Smith,C.A., Davis,T., Anderson,D., Solam,L., Beckmann,M.P., Jerzy,R., Dower,S.K., Cosman,D. and Goodwin,R.G. (1990) A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science*, **248**, 1019–1023.

Stamenkovic,I., Clark,E.A. and Seed,B. (1989) A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO J.*, **8**, 1403–1410.

Stanger,B.Z., Leder,P., Lee,T.-H., Kim,E. and Seed,B. (1995) RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell*, **81**, 513–523.

Tartaglia,L.A. and Goeddel,D.V. (1992) Tumor necrosis factor receptor signaling. A dominant negative mutation suppresses the activation of the 55-kDa tumor necrosis factor receptor. *J. Biol. Chem.*, **267**, 4304–4307.

Tartaglia,L.A., Weber,R.F., Figari,I.S., Reynolds,C., Palladino,M.A.,Jr and Goeddel,D.V. (1991) The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc. Natl Acad. Sci. USA*, **88**, 9292–9296.

Tartaglia,L.A., Ayres,T.M., Wong,G.H. and Goeddel,D.V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell*, **74**, 845–853.

Taupin,J.-L., Tian,Q., Kedersha,N., Robertson,M. and Anderson,P. (1995) The RNA-binding protein TIAR is translocated from the nucleus to the cytoplasm during Fas-mediated apoptotic cell death. *Proc. Natl Acad. Sci. USA*, **92**, 1629–1633.

Trauth,B.C., Klas,C., Peters,A.M.J., Matzku,S., Möller,P., Falk,W., Debatin,K.-M. and Krammer,P.H. (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science*, **245**, 301–305.

Upton,C., DeLange,A.M. and McFadden,G. (1987) Tumorigenic poxviruses: genomic organization and DNA sequence of the telomeric region of the Shope fibroma virus genome. *Virology*, **160**, 20–30.

Van Endert,P.M. and Moldenhauer,G. (1992) Inhibitory and stimulatory signaling via immunoglobulin receptors: dichotomous responses elicited in clonal B cell populations. *Eur. J. Immunol.*, **22**, 1229–1235.

Vignaux,F., Vivier,E., Malissen,B., Depraetere,V., Nagata,S. and Golstein,P. (1995) TCR/CD3 coupling to Fas-based cytotoxicity. *J. Exp. Med.*, **181**, 781–786.

Watanabe-Fukunaga,R., Brannan,C.I., Copeland,N.G., Jenkins,N.A. and Nagata,S. (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*, **356**, 314–317.

Wittinghofer,A., Guariguata,R. and Leberman,R. (1983) Bacterial elongation factor Ts: isolation and reactivity with elongation factor Tu. *J. Bacteriol.*, **153**, 1266–1271.

Yin,T., Tsang,M.L. and Yang,Y.C. (1994) JAK1 kinase forms complexes with interleukin-4 receptor and 4PS/insulin receptor substrate-1-like protein and is activated by interleukin-4 and interleukin-9 in T lymphocytes. *J. Biol. Chem.*, **269**, 26614–26617.

Yonehara,S., Ishii,A. and Yonehara,M. (1989) A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.*, **169**, 1747–1756.

Received on June 26, 1995; revised on August 4, 1995