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Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal

Maria Grazia Cifone[1], Paola Roncaioli[1], Ruggero De Maria[2], Grazia Camarda[2], Angela Santoni[3], Giovina Ruberti[4] and Roberto Testi[2,5]

[1]Department of Experimental Medicine, University of L'Aquila, 67100 L'Aquila, [2]Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', via di Tor Vergata 135, 100133 Rome, [3]Department of Experimental Medicine and Pathology, University of Rome 'La Sapienza', 00161 Rome and [4]Department of Immunobiology, Institute of Cell Biology, CNR, 00137 Rome, Italy

[5]Corresponding author

The early signals generated following cross-linking of Fas/APO-1, a transmembrane receptor whose engagement by ligand results in apoptosis induction, were investigated in human HuT78 lymphoma cells. Fas/APO-1 cross-linking by mAbs resulted in membrane sphingomyelin hydrolysis and ceramide generation by the action of both neutral and acidic sphingomyelinases. Activation of a phosphatidylcholine-specific phospholipase C (PC-PLC) was also detected which appeared to be a requirement for subsequent acidic sphingomyelinase (aSMase) activation, since PC-PLC inhibitor D609 blocked Fas/APO-1-induced aSMase activation, but not Fas/APO-1-induced neutral sphingomyelinase (nSMase) activation. Fas/APO-1 cross-linking resulted also in ERK-2 activation and in phospholipase A2 (PLA2) induction, independently of the PC-PLC/aSMase pathway. Evidence for the existence of a pathway directly involved in apoptosis was obtained by selecting HuT78 mutant clones spontaneously expressing a newly identified death domain-defective Fas/APO-1 splice isoform which blocks Fas/APO-1 apoptotic signalling in a dominant negative fashion. Fas/APO-1 cross-linking in these clones fails to activate PC-PLC and aSMase, while nSMase, ERK-2 and PLA2 activities are induced. These results strongly suggest that a PC-PLC/aSMase pathway contributes directly to the propagation of Fas/APO-1-generated apoptotic signal in lymphoid cells.

Keywords: apoptosis/aSMase/Fas/APO-1/PC-PLC

Introduction

Triggering of apoptotic cell death by surface receptor–ligand interactions is emerging as a central mechanism of tissue homeostasis and remodelling. Two distinct and widely expressed cell membrane receptors are responsible for initiating an intracellular apoptotic programme in mammalian cells, the tumour necrosis factor receptor p55 (TNF-R1) and the Fas/APO-1 receptor (Heller and Krönke, 1994; Krammer *et al.*, 1994; Nagata and Golstein, 1995). TNF-R1 and Fas/APO-1 belong to a family of receptors, which includes the low-affinity nerve growth factor receptor, CD40, CD30, CD27, OX40, tumour necrosis factor receptor p75, involved in mediating a variety of cell growth modulation or cell differentiation effects. While all members of the family typically display two or more cysteine-rich subdomains in the extracellular portion, TNF-R1 and Fas/APO-1 share also a region of limited homology close to the intracellular C-terminus, which is essential for the ability of the receptors to generate the apoptotic signal and which has been named 'death domain' (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). This region might be involved in binding putative cytosolic effectors, also expressing death domain-like regions, recently identified by yeast two-hybrid system searches (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995; Hsu *et al.*, 1995; Stanger *et al.*, 1995). Mutations or deletions within the receptor death domains, in fact, abrogate the death signal which follows TNF-R1 or Fas/APO-1 cross-linking (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). Moreover, the *lpr*^{cg} mouse, in which Fas/APO-1 is ineffective in transducing a death signal, carries a dominant point mutation within the Fas/APO-1 death domain (Watanabe-Fukunaga *et al.*, 1992).

Engagement of TNF-R1 or Fas/APO-1, however, may result also in the generation of non-apoptotic signals. TNF-R1, in fact, can mediate effects as diverse as antiviral activity, growth factor or adhesion receptor expression and cellular proliferation in different systems (Heller and Krönke, 1994), and Fas/APO-1 has been reported to generate co-stimulatory signals in lymphocytes (Alderson *et al.*, 1993). Efforts aimed at the identification of the intracellular mediators of this pleiotropy of effects have revealed that multiple signalling pathways can be initiated at the TNF-R1. A sequential phosphatidylcholine-specific phospholipase C/acidic sphingomyelinase (PC-PLC/aSMase) activation with ceramide generation can trigger the activation of a protease which degrades the nuclear transcription factor κB inhibitory subunit (IκB-α) (Machleidt *et al.*, 1994), eventually resulting in NFκB translocation to the nucleus and activation of its cognate DNA binding activity (Lowenthal *et al.*, 1989; Schütze *et al.*, 1992; Yang *et al.*, 1993). This pathway, potentially involved in multiple gene activation, requires the integrity of the very distal portion of the cytoplasmic domain of TNF-R1 (Wiegmann *et al.*, 1994). By contrast, a functionally and topologically distinct, neutral sphingomyelinase (nSMase)-mediated pathway would originate from receptor domains which are in close association with the cellular membrane (Dressler *et al.*, 1992; Wiegmann *et al.*, 1994), where ceramide production from sphingomyelin hydrolysis would result in the activation of several proline-directed

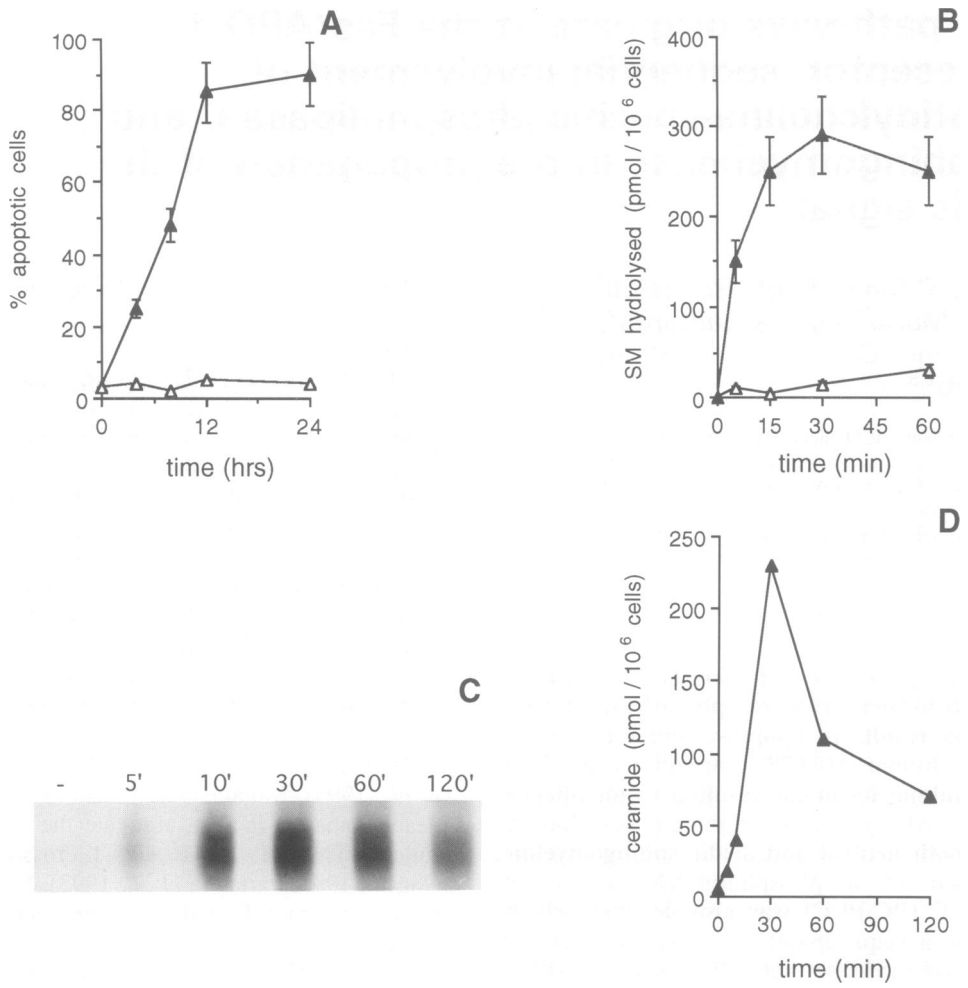

Fig. 1. (**A**) Time-course of apoptosis induction in HuT78 cells after Fas/APO-1 cross-linking. Cells were incubated in the presence of control IgM mAb (△) or 200 ng/ml anti-Fas mAb (▲), then nuclei processed and analysed for hypodiploid DNA content at the times indicated. Means and SD from six experiments are shown. (**B**) Time-course of *in vivo* sphingomyelin hydrolysis. HuT78 cells were labelled for 48 h with [*N*-methyl-^{14}C]choline, then treated as above. Hydrolysed SM was quantitated at the indicated times. Means and SD from three experiments are shown. (**C**) Time-course of ceramide accumulation. HuT78 cells were incubated in the presence of 200 ng/ml anti-Fas mAb, then lipid extracts at the indicated times and subjected to DAG-kinase assays. One representative out of five experiments is shown. (**D**) Quantitation data from the experiment shown in (C).

protein kinases, including members of the MAP kinase family (Raines *et al.*, 1993; Vietor *et al.*, 1993), a ceramide-activated protein kinase (Mathias *et al.*, 1991; Joseph *et al.*, 1993) and the stress-activated protein kinase JNK-1 (Kyriakis *et al.*, 1994). MAP kinases might then be responsible for cytosolic phospholipase A2 (PLA2) phosphorylation and activation, which also is observed following TNF-R1 engagement (Wiegmann *et al.*, 1994). In addition to the two ceramide-mediated pathways, a nitric oxide synthase (NOS) is activated by signals which require the integrity of both regions located upstream of the death domain and the death domain itself (Tartaglia *et al.*, 1993). Finally, a protein phosphatase 2A (Dobrowsky *et al.*, 1993; Fishbein *et al.*, 1993) has been shown to be activated by ceramides, and it is likely to play an important role in TNF-R1-mediated down-regulation of c-myc expression (Wolff *et al.*, 1994). Although a great deal of information has been gathered about TNF-R1 signalling, the intracellular pathway responsible for TNF-induced apoptosis has not been definitely identified. As exogenous ceramides directly trigger DNA fragmentation and apoptotic cell death in myeloid U937 cells (Obeid *et al.*, 1993; Jarvis *et al.*, 1994), a sphingomyelinase-dependent pathway is highly likely to be responsible for TNF-R1-mediated apoptosis, yet it is not clear which one of the two different sphingomyelinases is involved.

Unlike the case of TNF-R1, relatively little is known concerning the early biochemical changes involved in Fas/APO-1 signalling. Tyrosine kinase activity can be elicited following Fas/APO-1 cross-linking (Eischen *et al.*, 1994) and a tyrosine phosphatase might negatively regulate the apoptotic signal (Sato *et al.*, 1995). We have shown that Fas/APO-1-generated apoptotic signals activate an acidic sphingomyelinase in U937 myeloid cells, with ceramide accumulation (Cifone *et al.*, 1994). A recent report has suggested that ras can act as a downstream target for ceramides in Fas/APO-1 signalling (Gulbins *et al.*, 1995). Here we show that multiple phospholipid hydrolysis contributes to Fas/APO-1 signalling. Our attempts to identify among different effectors which one belongs to the apoptotic pathway, point toward the sequential PC-PLC/aSMase activation as a key step for the propagation of the death signal.

Results

Fas/APO-1 cross-linking induces sphingomyelin breakdown

Among several lymphoid tumour cell lines, a subline of the human T cell lymphoma HuT78 was selected for its high sensitivity to apoptotic Fas/APO-1 signalling. Approximately 80% of the cells undergo apoptosis within 12 h following Fas/APO-1 cross-linking, as detected by propidium iodide DNA staining and FACS analysis (Figure 1A). To investigate Fas/APO-1-induced sphingomyelin breakdown, HuT78 cells were labelled with [*N*-methyl-^{14}C]choline for 48 h, then stimulated with anti-Fas/APO-1 mAb. TLC analysis of labelled phospholipids revealed significant sphingomyelin hydrolysis, which was maximal at ~30 min after Fas/APO-1 cross-linking (Figure 1B). This was accompanied by ceramide accumulation, as detected by *in vitro* diacylglycerol kinase assay and TLC analysis of reaction products (Figure 1C and D).

Both neutral and acidic sphingomyelinases contribute to Fas/APO-1-induced sphingomyelin breakdown

Sphingomyelinases represent a widely distributed and heterogeneous family of type C phospholipases (Spence, 1993). Two classes of cell-associated sphingomyelinases can be defined based on *in vitro* pH optima, acidic sphingomyelinases (aSMase), which require pH 4.5–5.5, and neutral sphingomyelinases (nSMase), which prefer pH 7.0–7.5. While aSMase retain specific intracellular topology and functional requirements, as they are found associated with the lysosomal compartment and require diacylglycerol (DAG) for activation (Kolesnick *et al.*, 1987; Quintern *et al.*, 1987), nSMase are found either associated with the outer cell membrane (Rao and Spence, 1976) or within the cytosol (Okazaki *et al.*, 1994) and do not require DAG for activation but may require Mg^{2+}. aSMase, moreover, consistent with their subcellular localization *in vivo*, are particularly resistant to the action of proteases and phosphatases *in vitro*, unlike nSMase (Wiegmann *et al.*, 1994). We decided to investigate the individual contribution of each SMase to the sphingomyelin hydrolysis which followed Fas/APO-1 cross-linking in HuT78 cells. Cellular extracts from HuT78 cells stimulated for 15 min with anti-Fas mAb were reacted with radiolabelled SM vesicles to detect nSMase activation (Figure 2A), or aSMase activation (Figure 2B). Radiolabelled SM hydrolysis was assessed by TLC analysis. Residual SMase activity in the presence of protease and phosphatase inhibitors, β-glycerophosphate, ATP and Mg^{2+} at pH 5.5 or in the absence of protease and phosphatase inhibitors, β-glycerophosphate, ATP and Mg^{2+} at pH 7.4, was also assayed for specificity. Data presented in Figure 2 show that both nSMase and aSMase activity could be detected in extracts from Fas/APO-1-stimulated HuT78 cells, and displayed specific pH requirements. TNF, which is known to activate both SMases in U937 cells (Wiegmann *et al.*, 1994), also induced both nSMase and aSMase activation in HuT78 cells. Importantly, 30 min pretreatment of HuT78 cells with the phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor D609, prevented Fas/APO-1-induced SM hydrolysis by aSMase (Figure 2B), but not by nSMase (Figure 2A). This suggested that

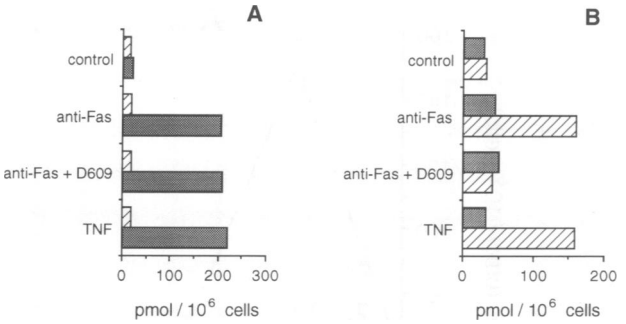

Fig. 2. (**A**) *In vitro* neutral sphingomyelinase activity. Cell extracts from HuT78 cells stimulated for 15 min with control IgM mAb, 200 ng/ml anti-Fas mAb, some after 30 min preincubation with 50 μg/ml D609, or stimulated with 100 ng/ml TNF, were reacted at pH 7.4 with labelled SM vesicles, in the presence (filled bars) or absence (hatched bars) of protease and phosphatase inhibitors, β-glycerophosphate, ATP and Mg^{2+}. Hydrolysed SM was quantitated and expressed as pmol/10^6 cells. (**B**) *In vitro* acidic sphingomyelinase activity. Cell extracts from HuT78 cells stimulated for 15 min with control IgM mAb, 200 ng/ml anti-Fas mAb, some after 30 min preincubation with 50 μg/ml D609, or stimulated with 100 ng/ml TNF, were reacted at pH 5.5 with labelled SM vesicles, in the presence (filled bars) or absence (hatched bars) of protease and phosphatase inhibitors and Mg^{2+}. Hydrolysed SM was quantitated and expressed as pmol/10^6 cells.

aSMase activation required an upstream PC-PLC activation *in vivo*. Taken together, these data indicate that Fas/APO-1 cross-linking induces sphingomyelin hydrolysis and ceramide production in HuT78 cells by the action of both a neutral and an acidic sphingomyelinase.

PC-PLC is activated following Fas/APO-1 cross-linking

The above results strongly suggested that Fas/APO-1-induced aSMase required previous activation of a PC-PLC. To address directly the possibility of Fas/APO-1 inducing PC-PLC activation, the release of DAG *in vivo* was evaluated following Fas/APO-1 cross-linking. HuT78 cells were loaded with [^{14}C]palmitoyl-lysoPC to label PC pools, then stimulated through Fas/APO-1 and released [^{14}C]DAG detected after TLC analysis. As shown in Figure 3A, cross-linking of Fas/APO-1 resulted in significant DAG release, which could be blocked by 30 min pretreatment with the PC-PLC inhibitor D609. Moreover, direct *in vitro* measurement of enzymatic activity using radiolabelled PC vesicles and TLC analysis of reaction products revealed the presence of PC-specific PLC activity in Fas/APO-1-stimulated HuT78 cell extracts, which again could be totally blocked by pretreatment with D609 (Figure 3B). These data indicate that, following Fas/APO-1 cross-linking, DAG is released from cellular PC pools through a PC-PLC. As D609 blocked Fas/APO-1-induced aSMase activation (Figure 2B), it is highly likely that PC-PLC and aSMase activation are two sequentially related steps of the same pathway.

Fas/APO-1 cross-linking activates a PLA2

Three different type C phospholipases were therefore found to contribute to Fas/APO-1 signalling. Systematic search for evidence of activation of other phospholipases indicated that cross-linking of Fas/APO-1 does not result in PI-PLC or PLD activation (data not shown), but it is

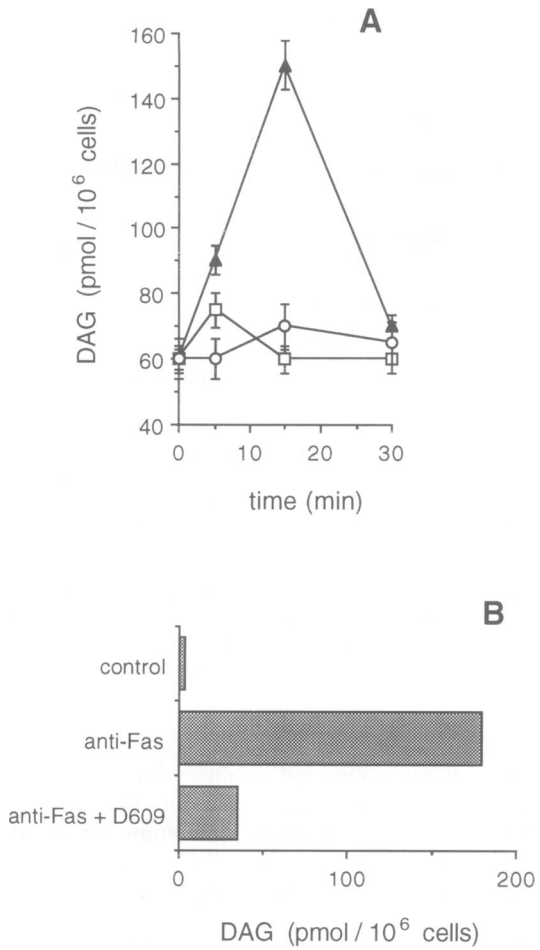

Fig. 3. (**A**) Time-course of *in vivo* PC-PLC activity. HuT78 cells labelled with [^{14}C]palmitoyl-lyso-PC were treated with control IgM mAb (○) or 200 ng/ml anti-Fas mAb (▲), without or after 30 min preincubation with 50 μg/ml D609 (□), then DAG production analysed at the indicated times. Means and SD from three experiments are shown. (**B**) PC-PLC activity *in vitro*. Cell extracts from HuT78 cells treated for 15 min with control IgM mAb, 200 ng/ml anti-Fas mAb, some after 30 min preincubation with 50 μg/ml D609, were reacted with radiolabelled PC vesicles, then DAG released quantitated by TLC.

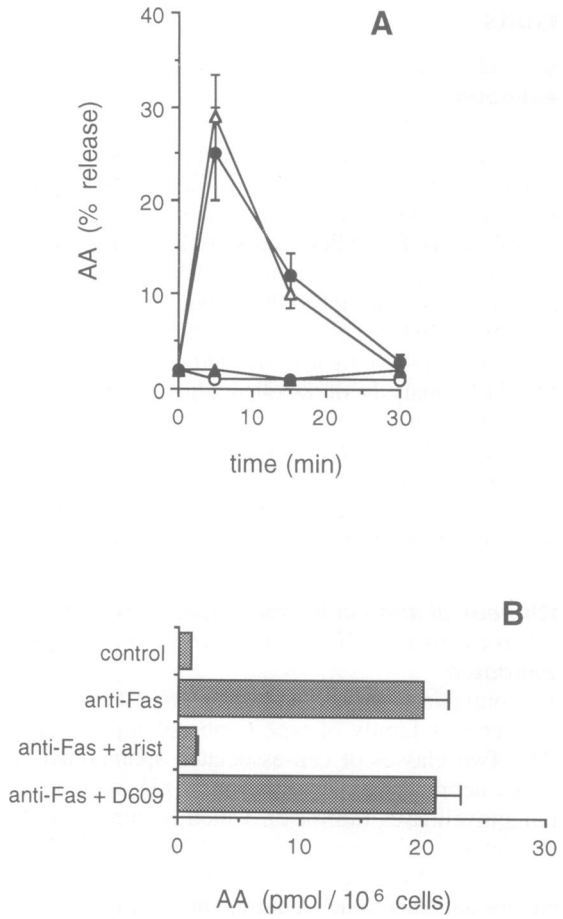

Fig. 4. (**A**) Time-course of AA release *in vivo*. HuT78 cells labelled with [^{3}H]AA were treated with control IgM mAb (○) or 200 ng/ml anti-Fas mAb (●), without or after 30 min preincubation with 50 μg/ml D609 (△), or 40 μM aristolochic acid (▲). Radioactive species were detected by liquid scintillation. Means and SD from four experiments are shown. (**B**) *In vitro* PLA2 assay. Cell extracts from HuT78 cells treated for 5 min with control mAb or 200 ng/ml anti-Fas mAb, without or after 30 min pretreatment with 40 μM aristolochic acid or 50 μg/ml D609, were reacted with radiolabelled PC vesicles. Released AA was analysed and quantitated by TLC. Means and SD from two experiments are shown.

able to activate a PLA2. HuT78 cells were loaded with [^{3}H]arachidonic acid (AA), then stimulated through Fas/APO-1 and ^{3}H-species released in the supernatant were evaluated as a measure of *in vivo* AA release. Figure 4A shows that Fas/APO-1 cross-linking induced AA release, and this could be blocked by 30 min pretreatment with PLA2 inhibitor aristolochic acid but not by PC-PLC inhibitor D609. Direct *in vitro* measurement of enzymatic activity using choline-radiolabelled PC vesicles indicated presence of PLA2 activity in Fas/APO-1-stimulated HuT78 cell extracts, as revealed by TLC detection of labelled AA (Figure 4B). Again, AA generation could be blocked by PLA2 inhibitor aristolochic acid but not by PC-PLC inhibitor D609, indicating that Fas/APO-1-induced PLA2 activation is not dependent from PC-PLC.

Fas/APO-1 cross-linking activates extracellular receptor kinase type 2 (ERK-2)

Phosphorylation by ERK-2 is known to be responsible for cytosolic PLA2 activation (Clark *et al.*, 1991; Lin *et al.*, 1993). We therefore investigated the possible activation of ERK-2 following Fas/APO-1 cross-linking. SDS–PAGE analysis and Western blot of HuT78 cell lysates indicated that cross-linking of Fas/APO-1 could induce a retardation shift of a fraction of ERK-2 in stimulated cells, which peaked at 15 min after stimulation (Figure 5A). Immunoprecipitation of ERK-2 and *in vitro* kinase assays on myelin basic protein substrate demonstrated kinase activation in Fas/APO-1 stimulated cells, peaking at 15 min after stimulation (Figure 5B). Moreover, C2-ceramide was able to activate ERK-2, as detected by *in vitro* kinase assays (Figure 5C), suggesting that ERK-2 activation may follow sphingomyelin breakdown after Fas/APO-1 cross-linking.

Signalling through Fas/APO-1 in Fas/APO-1-resistant cell clones

Thus, a variety of signalling pathways are triggered by Fas/APO-1 cross-linking, with potentially different functional relevance. To start to address the question of which one of these pathways is responsible for channelling the death signal, a number of Fas/APO-1-resistant cellular

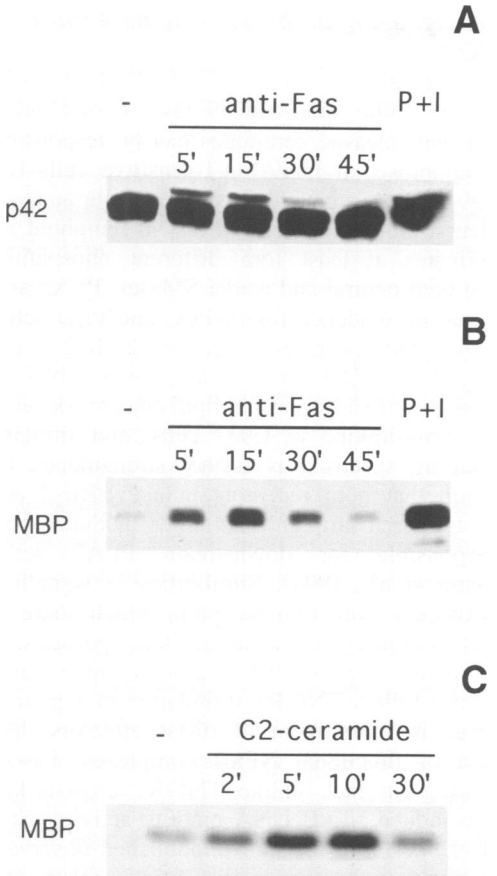

Fig. 5. (**A**) Time-course of ERK-2 activation. HuT78 cells were left untreated, stimulated with 1 μg/ml anti-Fas mAb for the indicated times, or with PMA plus ionomycin, then cell lysates analysed using SDS–PAGE, probed with anti MAP kinase R2 antiserum and visualized by ECL. One experiment out of several which gave similar results is shown. (**B**) Time-course of ERK-2 activity after Fas/APO-1 cross-linking. HuT78 cells were left untreated, stimulated with 1 μg/ml anti-Fas mAb for the indicated times, or with PMA plus ionomycin, then anti-ERK-2 immunoprecipitates reacted with myelin basic protein (MBP) in an *in vitro* kinase assay. One experiment out of several which gave similar results is shown. (**C**) Time-course of ERK-2 activity after C2-ceramide treatment. HuT78 cells were left untreated, stimulated with 50 μM C2-ceramide for the indicated times, then anti-ERK-2 immunoprecipitates reacted with myelin basic protein (MBP) in an *in vitro* kinase assay. One experiment out of two which gave similar results is shown.

clones were generated. HuT78 cells were grown in the presence of anti-Fas/APO-1 antibody, thus allowing selection for spontaneously occurring Fas/APO-1 resistant cells. A number of clones were obtained by limiting dilution cloning of surviving cells, and clones with surface Fas/APO-1 expression comparable with wild-type HuT78 cells were further investigated. Cross-linking of Fas/APO-1 was unable to trigger apoptosis in these cells, and the Fas/APO-1-resistant phenotype was stable over many generations. Importantly, exogenous ceramide was fully able to trigger apoptosis in these cells, indicating that downstream relevant targets of ceramide-mediated apoptotic pathway were functionally preserved (Figure 6). A search for Fas/APO-1 structural alterations as potentially responsible for the Fas/APO-1-resistant phenotype in these HuT78 clones revealed the presence, along with the normal receptor, of a spliced variant, called FasExo8Del, which

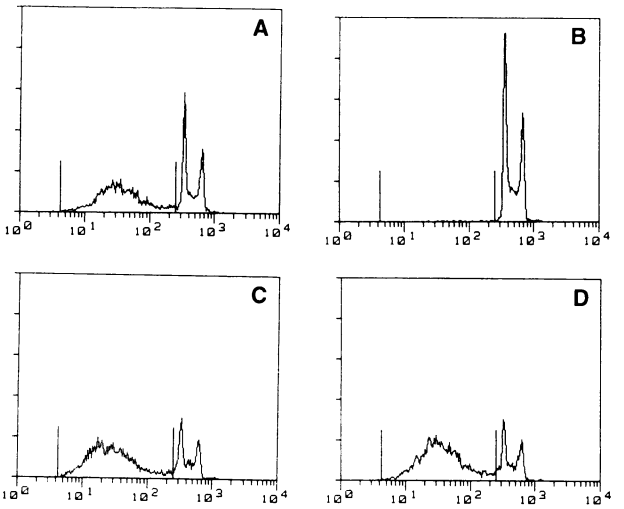

Fig. 6. FACS analysis of propidium iodide-labelled nuclei from HuT78 cells (**A**) or Fas-resistant clone HuT78-B1 (**B**) treated for 12 h with 200 ng/ml anti-Fas mAb, and HuT78 cells (**C**) or Fas-resistant clone HuT78-B1 (**D**) treated for 12 h with 50 μM C2-ceramide.

lacks the death domain, and affects Fas/APO-1-mediated death signal generation in a dominant negative fashion (I.Cascino, G.Papoff, R.De Maria, R.Testi and G.Ruberti, submitted). The different signal transduction pathways known to be triggered in Fas/APO-1-sensitive HuT78 cells were therefore investigated in Fas/APO-1-resistant cells expressing the death domain defective Fas/APO-1 isoform, and data from one representative clone out of five which gave identical results, are shown. In HuT78-B1 cells Fas/APO-1 cross-linking was able to activate nSMase, ERK-2 and PLA2, but not PC-PLC and aSMase (Figure 7). PC-PLC and aSMase, however, are expressed and functional in HuT78-B1 cells, as they could be activated by TNF.

Discussion

In an effort to define the molecular events which channel the apoptotic signal from the cell surface to distal intracellular targets, we investigated the early biochemical changes which follow cross-linking of Fas/APO-1, a widely distributed cell surface receptor able to trigger apoptosis upon ligand engagement (Nagata and Golstein, 1995). Fas/APO-1-induced cell death is a crucial event in the physiology of the immune system, being responsible for limiting clonal expansion and accumulation of lymphocytes following antigen challenge in lymphoid organs (Krammer *et al.*, 1994). Peripheral lymphocytes become susceptible to Fas/APO-1 cross-linking upon prolonged activation and proliferation (Klas *et al.*, 1993), and are eventually killed by direct interaction with Fas/APO-1-ligand expressing cells (Vignaux and Golstein, 1994) or autocrine suicide (Brunner *et al.*, 1995; Dhein *et al.*, 1995). Mice which are deficient in either Fas/APO-1 or Fas/APO-1-ligand expression, in fact, abnormally accumulate lymphocytes in peripheral lymphoid organs (Nagata and Suda, 1995). Perhaps more importantly, Fas/APO-1 might be involved in the immunologic clearance of transformed cells, as cytotoxic T lymphocytes can use Fas/APO-1-ligand to kill Fas/APO-1-expressing target cells (Kägi *et al.*, 1994), either virally infected cells or tumour cells.

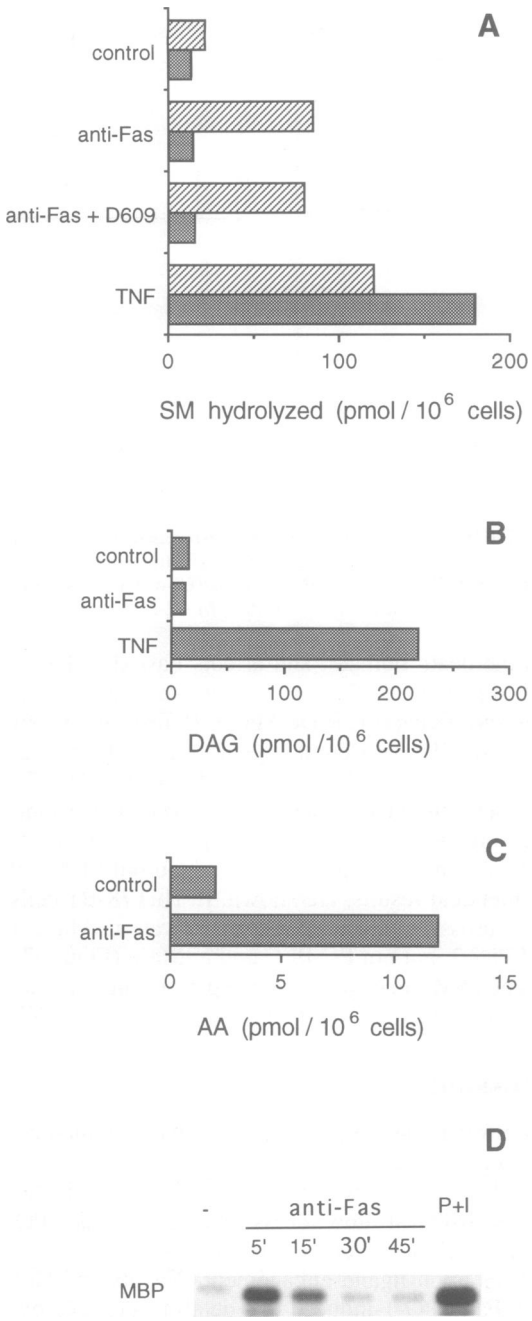

Fig. 7. (**A**) *In vitro* SMase assays. Cell extracts from HuT78-B1 cells treated with control IgM, 200 ng/ml anti-Fas mAb without or after preincubation with 50 μg/ml D609, or stimulated with 100 ng/ml TNF, were assayed for nSMase (hatched bars) and aSMase (filled bars) activities as described above. Five clones were independently tested and gave similar results. (**B**) *In vitro* PC-PLC assay. Cell extracts from HuT78-B1 cells treated with control IgM, 200 ng/ml anti-Fas mAb or 100 ng/ml TNF, were reacted with radiolabelled PC vesicles, then DAG released quantitated by TLC. Three clones were independently tested and gave similar results. (**C**) PLA2 activity. Cell extracts from HuT78-B1 cells treated with control IgM or 200 ng/ml anti-Fas mAb were reacted with radiolabelled PC vesicles. Released AA was analysed and quantitated by TLC. Two clones were independently tested and gave similar results. (**D**) Time-course of ERK-2 activity after Fas/APO-1 cross-linking. HuT78-B1 cells were left untreated, stimulated with 1 μg/ml anti-Fas mAb for the indicated times, or with PMA plus ionomycin, then anti-ERK-2 immunoprecipitates reacted with myelin basic protein (MBP) in an *in vitro* kinase assay. Two clones were independently tested and gave similar results.

Multiple signalling pathways originate form Fas/APO-1

We have recently shown that Fas/APO-1 cross-linking activates an acidic sphingomyelinase in myeloid U937 cells and that released ceramides can be responsible for cellular apoptosis in Fas/APO-1-sensitive cells (Cifone *et al.*, 1994). We systematically extended our analysis and found that stimulation of Fas/APO-1 in lymphoid HuT78 cells activates at least four different phospholipases, including both neutral and acidic SMases, PLA2 and PC-PLC, while no evidence for PI-PLC and PLD activation was found. Moreover, evidence for ERK-2 activation following Fas/APO-1 cross-linking was obtained. A similar pattern of early phospholipid changes occurs upon TNF-R1 cross-linking in U937 cells, and similarity in the signalling spectrum is further strengthened by the observation that both receptors induce ERK-2 activity, possibly triggered through a ceramide-mediated pathway and responsible for downstream PLA2 activation (Wiegmann *et al.*, 1994). Similarities in signalling are not unexpected from two receptors which share partial structural homology in the intracellular portions. These data, however, suggest that a more complex array of signals must follow TNF-R1 stimulation to explain major differences in recruitment of distal effectors, like the activation of functional NFκB complexes. Fas/APO-1 cross-linking, in fact, unlike TNF-R1 cross-linking, is unable to induce NFκB DNA binding activity (Schulze-Osthoff *et al.*, 1994, and our unpublished observations). This is likely to be responsible for a variety of gene expression events which accompany cellular responses to TNF and which can modulate the outcome and/or the kinetics of the cytotoxic signal (Clement and Stamenkovic, 1994). By contrast, Fas/APO-1-generated apoptotic signals do not require, and are not affected by, new gene expression (Schulze-Osthoff *et al.*, 1994; Wong and Goeddel, 1994). Likewise, signals which target mitochondrial respiratory enzymes are likely generated from TNF-R1, but not from Fas/APO-1, as several lines of evidence support the involvement of reactive oxygen intermediates in TNF-R1, but not Fas/APO-1, -mediated cell death (Schulze-Osthoff *et al.*, 1993, 1994; Jacobson and Raff, 1995).

Apoptotic and non-apoptotic signals from Fas/APO-1

Whereas it was conceivable that important similarities in signalling with the closely related TNF-R1 were detected, it was not clear which one of the different signals generated from the Fas/APO-1 receptor would eventually contribute to the apoptotic outcome. Our approach to this problem was to select HuT78 cellular clones which express a mutant receptor unable to couple with downstream effectors relevant to the progression of the death signal. Analysis of phospholipid changes following Fas/APO-1 cross-linking in these clones revealed that the different phospholipase activities belong to at least two independent pathways. A series of events, which appear insufficient to induce apoptosis, is triggered in Fas/APO-1-resistant clones following Fas/APO-1 cross-linking and include the activation of nSMase and PLA2. Moreover, the MAP kinase ERK-2 is activated following Fas/APO-1 cross-linking in resistant clones, providing a possible link between nSMase and PLA2 activations. Ceramides, in

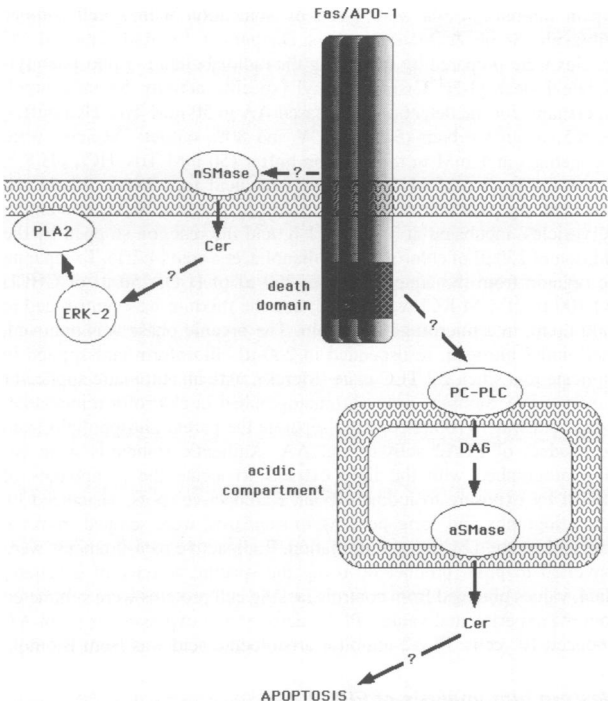

Fig. 8. Model for Fas/APO-1 signalling consistent with the data presented in this paper. nSMase, neutral sphingomyelinase; ERK2, extracellular receptor kinase 2; PLA2, phospholipase A2; PC-PLC, phosphatidylcholine-specific phospholipase C; DAG, diacylglycerol; aSMase, acidic sphingomyelinase; Cer, ceramide.

fact, activate ERK-2, and PLA2 is in turn activated by ERK-2 (Clark *et al.*, 1991; Lin *et al.*, 1993).

Another series of events, by contrast, seems required to allow the progression of the apoptotic signal triggered by Fas/APO-1 cross-linking, i.e. the sequential activation of PC-PLC and aSMase, as highlighted in our Fas/APO-1-resistant clones. As trimerization is expected to be crucial for Fas/APO-1 signalling, expression of the death domain-defective receptor isoform in Fas/APO-1-resistant HuT78 clones is likely to result in a dominant negative effect on death signalling. In fact, assembling of trimers which include death domain-defective monomers, may render the receptor unable to couple with relevant death domain-binding effectors (Stanger *et al.*, 1995), or unable to 'displace' death domain-binding effectors, as recently proposed (Chinnaiyan *et al.*, 1995). This results in the inability to activate both PC-PLC and aSMase, although these enzymes are present and functional. Moreover, these clones have a fully operative apoptotic machinery, including the relevant target for ceramides and subsequent downstream effectors. Since a ceramide-mediated pathway is sufficient to trigger apoptosis (Obeid *et al.*, 1993; Cifone *et al.*, 1994; Gulbins *et al.*, 1995), these data clearly support the involvement of aSMase, rather than nSMase, in generating the relevant ceramide (Figure 8). Further studies are needed to understand the general biochemical setting which allows aSMase-generated ceramide to mediate recruitment of distal apoptotic effectors. Such milieu could be contributed to and regulated by diffusible mediators derived from the nSMase pathway, or by additional signal transducers activated after Fas/APO-1 cross-linking, as well as by the differentiation status of each particular cell type (De Maria *et al.*, 1996).

Biological implications

Our observations point toward the sequential action of two phospholipases, PC-PLC and aSMase, as an important step for the progression of the Fas/APO-1-generated apoptotic pathway. This has potential relevance for both the understanding of intracellular apoptotic signal propagation and for pharmacological targeting of apoptotic effectors. It is noteworthy that cellular compartmentalization of diffusible messenger generation is crucial for determining functional outcomes. Generation of ceramides by nSMase-mediated sphingomyelin hydrolysis, in fact, does not result in activation of effectors able to propagate a death signal. As nSMase are likely located in close proximity to the cellular membrane, juxtamembrane-released ceramides either do not encounter effectors relevant to cell death or they are rapidly inactivated. On the contrary, ceramides released within lysosomes by aSMases should be postulated to interact with still unidentified effectors responsible for death signal propagation. Compartmentalization of the relevant downstream effector is a likely determinant, as we were unable to find structural differences, in terms of length or saturation of the acyl chain or of the sphingoid base, between ceramides released after Fas/APO-1 cross-linking in sensitive and resistant clones (data not shown). Defining subcellular compartments and relevant transducers of the apoptotic signal has obvious implications in directing procedures aimed at the pharmacological correction of accelerated apoptosis, such as in overt acquired immunodeficiency syndrome. Moreover, as tumour- or virus-transformed cells are likely to develop strategies to block Fas/APO-1-generated apoptotic signals, unravelling key steps of the Fas/APO-1 apoptotic pathway, should indicate possible targets for these strategies and provide suggestions on how to counteract them.

Materials and methods

Cells and reagents

The human T cell lymphoma HuT78 cells were grown in RPMI supplemented with 10% fetal calf serum, 1 mM glutamine and antibiotics. Fas-resistant clones were obtained by growing HuT78 cells in the presence of anti-Fas mAb, selecting surviving cells and cloning by limiting dilution. Clones were selected which expressed surface Fas/APO-1 receptor at levels comparable with wild-type cells, and which could be induced to undergo apoptosis by C2-ceramide (50 μM) exposure. The Fas/APO-1-resistant clones used in this study, along with the normal Fas/APO-1 transcript, constitutively expressed a Fas/APO-1 mRNA variant resulting from a defective splicing event occurring between exons seven and eight (I.Cascino, G.Papoff, R.De Maria, R.Testi and G.Ruberti, submitted). The resulting receptor lacked the intracytoplasmic region coded for by exons eight and nine, including the 'death domain'. Anti-Fas mAb (C-11) and TNF were from UBI (Lake Placid, NY). C2-ceramide (*N*-acetyl-D-sphingosine) was from Biomol (Plymouth Meeting, PA).

In vivo sphingomyelinase assay

Cells were labelled for 48 h with [*N*-methyl-^{14}C]choline (Amersham, Bucks, UK; 1 mCi/ml, specific activity 56.4 mCi/mmol) and then serum starved for 4 h in medium supplemented with 2% BSA. Aliquots of 1×10^7 cells were suspended in 1 ml of PBS and treated for the indicated times with control mAb or with anti-Fas mAb (200 ng/ml). Stimulation was stopped by immersion of samples in methanol/dry ice (–70°C) for 10 s followed by centrifugation at 4°C in a microfuge. Cell pellets were resuspended in ice-cold methanol:chloroform:water (2.5:1.25:1). Lipids were extracted, dried under nitrogen, resuspended in 200 μl chloroform and applied to a silica gel TLC plate (Merck, Darmstadt, Germany) with an automatic applicator (Linomat IV, Camag, Muttenz, Switzerland). Samples containing equal amounts of radioactivity were loaded. The amount of labelled PC, which remained constant when labelled at

equilibrium, was used as internal control to normalize for equal amounts of loaded material. Lipids were separated by TLC using a solvent system containing chloroform:methanol:acetic acid:water (100:60:20:5). LysoPC, PC and bovine brain sphingomyelin (Sigma) were used as standards and visualized in iodine vapour. The radioactive spots were visualized by autoradiography, scraped from the plate and counted by liquid scintillation. Radioactive measurements were converted to pmol product by using the specific activity of substrate. SMase activity was expressed as pmol SM hydrolysed/10^6 cells.

In vitro nSMase and aSMase analysis

Aliquots of 1×10^7 cells were suspended in 1 ml of RPMI medium and treated for the indicated times with control mAb or with anti-Fas mAb (200 ng/ml). Stimulation was stopped by immersion of samples in methanol/dry ice (–70°C) for 10 s followed by centrifugation at 4°C in a microfuge. To measure neutral SMase, pellets were dissolved in a buffer containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 30 mM *p*-nitrophenylphosphate, 10 mM β-glycerophosphate, 750 mM ATP, 1 μM PMSF, 10 μM leupeptin, 10 μM pepstatin (Sigma) and 0.2% Triton X-100. After incubation for 5 min at 4°C, cells were sonicated and the protein concentration measured with the Bradford protein assay kit (Bio-Rad Laboratories, Richmond, VA). Proteins (50–100 μg) were incubated for 2 h at 37°C in a buffer (50 μl final volume) containing 20 mM HEPES, 1 mM $MgCl_2$, pH 7.4 and 2.25 μl of [*N*-methyl-^{14}C]SM (0.2 μCi/ml, specific activity 56.6 mCi/mmol, Amersham). To measure acidic SMase, after treatment, the cells were washed, and the pellet was resuspended in 200 μl of 0.2% Triton X-100 and incubated for 15 min at 4°C. The cells were sonicated and the protein concentration assayed. 50–100 μg of protein were incubated for 2 h at 37°C in a buffer (50 μl final volume) containing 250 mM sodium acetate, 1 mM EDTA, pH 5.0, and 2.25 μl of [*N*-methyl-^{14}C]SM. The reaction was stopped by the addition of 250 μl of chloroform:methanol:acetic acid (4:2:1). Phospholipids were extracted, analysed on TLC plates and SM hydrolysis quantitated by autoradiography and liquid scintillation. SMase activation was expressed as pmol SM hydrolysed/10^6 cells.

Ceramide mass measurement (diacylglycerol kinase assay)

After stimulation, lipids were extracted and then incubated with *Escherichia coli* diacylglycerol kinase (Preiss *et al.*, 1986). Ceramide phosphate was then isolated by TLC using chloroform:methanol:acetic acid (65:15:5) as solvent. Authentic ceramide-1-phosphate was identified by autoradiography at R_f 0.25. Quantitative results for ceramide production are expressed as pmol ceramide-1-phosphate/10^6 cells.

PC-PLC activity

PC-PLC activity was determined either *in vivo* by monitoring [^{14}C]DAG production of cells prelabelled with [^{14}C]palmitoyl-lyso-PC, which allows rather selective labelling of PC pools, or *in vitro* by its ability to hydrolyse [^{14}C]PC vesicles to generate DAG. Lipids were separated by TLC using a solvent system containing petroleum ether:diethyl ether:acetic acid (70:30:1). DAG, lyso-PC and PC were used as standards and visualized in iodine vapour. The radioactive spots were visualized by autoradiography, scraped from the plate and counted by liquid scintillation. Radioactive measurements were converted to pmol product by using the specific activity of substrate. Blank values obtained from controls lacking cell proteins were subtracted from the experimental values. PC-PLC activity was expressed as pmol DAG/10^6 cells. PC-PLC inhibitor D609 was from Kamiya, Thousand Oaks, CA.

AA release

Cells (5×10^6) suspended in serum-free RPMI 1640 were incubated for 12 h with [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]AA (Amersham, 1 μCi/ml; 230 Ci/mmol). Labelled cells were then washed twice and resuspended in fresh medium containing 0.01% fatty acid-free BSA and treated as indicated. AA release assay was carried out in a shaking incubator bath at 37°C, and the reaction stopped by centrifugation at 8000 *g* for 1 min. The ^{3}H content of the supernatant was estimated in a β-counter.

PLA2 assay

Cells were treated for the indicated times with control mAb or with anti-Fas mAb (200 ng/ml), in the presence or absence of inhibitors. Stimulation was stopped by immersion of samples in methanol/dry ice (–70°C) for 10 s followed by centrifugation at 4°C in a microfuge. The pellets were then resuspended in 50 mM Tris–HCl buffer, pH 8.5, containing 10 μM PMSF, 100 μM bacitracin, 1 mM benzamidine, 1 μM aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 5 μg/ml soybean trypsin inhibitor. Cells were lysed by sonication with a cell sonifier (Vibracell, Sonic & Materials Inc., Danbury, CT). Radiolabelled PC vesicles were prepared by sonicating the radiolabelled L-3-phosphatidylcholine-1-stearoyl-2(^{14}C)arachidonoyl (specific activity 56 mCi/mmol, Amersham) for the detection of released AA in 50 mM Tris–HCl buffer, pH 8.5, in an ice bath (5 min, 5 W and 80% output). Vesicles were resuspended at 1 mM in the reaction buffer (50 mM Tris–HCl, pH 8.5, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.01% fatty acid free-BSA). Whole-cell lysate (60 μg proteins) was added to 250 μl reaction buffer containing the vesicles, incubated at 37°C for 1 h, and the reaction stopped by the addition of 250 μl of chloroform:methanol:acetic acid (4:2:1). To separate the organic from the aqueous phase, 250 μl of H_2O, 250 μl of $CHCl_3$ and 100 μl of 4 M KCl were added, and the mixture was centrifuged at 4000 r.p.m. in a microfuge for 5 min. The organic phase was removed, dried under nitrogen, resuspended in 200 μl chloroform and applied in duplicate to a silica gel TLC plate (Merck), with an automatic applicator (Linomat IV). Samples were chromatographed in chloroform:methanol:acetic acid:water (100:60:16:8) to separate the parent phospholipid from the product of PLA2 activity, i.e. AA. Authentic standards were co-chromatographed with the lipid extracts to locate the compounds of interest by exposure to iodine vapour. Radioactive spots, visualized by autoradiography and corresponding to standards, were scraped from the plate and counted by liquid scintillation. Radioactive measurements were converted to pmol product by using the specific activity of substrate. Blank values obtained from controls lacking cell proteins were subtracted from the experimental values. PLA2 activity was expressed as pmol AA produced/10^6 cells. PLA2 inhibitor aristolochic acid was from Biomol.

Western blot analysis of ERK-2

After starvation in FCS 0.1%, 1×10^6 cells were stimulated with anti-Fas (1 μg/ml in PBS) at 37°C. At the end of incubations, cells were spun at 4°C and pellets were resuspended in 50 μl of lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 0.5% NP-40). All subsequent manipulations were at 4°C. Lysates were cleared of nuclei and detergent insoluble material by centrifuging 10 min at 14 000 r.p.m., then assayed for total cell protein content (Bio-Rad) and equal amounts of proteins (typically 50 μg) were resolved by 12.5% SDS–PAGE using a Protean II apparatus (Bio-Rad) and transferred to nitrocellulose in a Bio-Rad transblot apparatus at 400 mA for 90 min. Blots were washed once in PBS–0.05% Tween-20 (PBS-T) and blocked in 5% non-fat dry milk in PBS-T for 60 min. After three washes in PBS-T, blots were incubated for 2 h with anti-MAP kinase R2 antiserum (UBI) (1 mg/ml in PBS-T). p42 ERK-2 was detected using an anti-rabbit HRP secondary antibody and the ECL detection system (Amersham).

Immunocomplex kinase assay

For kinase assay, 1×10^7 cells were stimulated with anti-Fas and lysed in 500 μl of lysis buffer. Lysates were incubated with anti-ERK-2 antibody (C-14, Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to protein A–Sepharose beads for 90 min at 4°C. Immunoprecipitates were washed three times with lysis buffer, once with kinase buffer (30 mM Tris–HCl, pH 8, 1 mM $MnCl_2$, 10 mM $MgCl_2$) and resuspended in 25 μl of kinase buffer containing 10 μM ATP, 8 μCi [γ-^{32}P]ATP and 7.5 μg MBP. *In vitro* kinase assay was performed at 30°C for 30 min. The reaction was stopped by adding 25 μl sample buffer 2× and boiling samples for 5 min. The mixture was electrophoresed using 12.5% SDS–PAGE, blotted to nitrocellulose and autoradiographed. To confirm the equal loading, blots were probed with anti-ERK antiserum and developed by ECL.

DNA labelling and evaluation of apoptotic cells

Cells were recovered and washed in PBS, and processed for apoptotic cell detection as previously shown (Cifone *et al.*, 1994). Briefly, cells were resuspended in hypotonic fluorochrome solution [propidium iodide 50 μg/ml (Sigma) in 0.1% sodium citrate plus 0.1% Triton X-100], kept 4–8 h at 4°C in the dark, and analysed by a FACScan cytofluorimeter. Percentage of apoptotic cells was determined by evaluating hypodiploid nuclei.

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