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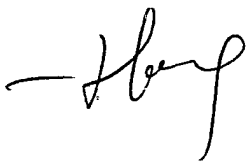

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PROGRESS REPORT (DAMD 17-99-1-9096)

BARC: A NOVEL APOPTOSIS REGULATOR

BAR is a multi-domain protein. A screen for cDNAs encoding suppressors of Bax-induced cell death was performed in the yeast *S. cerevisiae*, resulting in a cDNA containing an open reading frame (ORF) encoding a novel human protein of 450 amino acids (~52 kDa), which we assigned the acronym BAR, for Bi-functional Apoptosis Regulator (Accession number bankit 283835). The predicted BAR protein contains several protein domains, including: (1) an N-terminal zinc-binding RING domain at residues 24-86; (2) a SAM domain at amino acids 180-254; (3) a DED-like domain at 273-345, and (4) a C-terminal hydrophobic transmembrane (TM) domain at 400-428 (Figure 1A, B). The first two assignments have high statistical significance, with PSI-BLAST e. values of e^{-14} and e^{-28} respectively, indicating high confidence of the prediction. The DED-like domain is not recognized by any of the standard sequence analysis programs, and instead was identified by detailed comparison to known DED proteins, as described below. The DED-like domain of BAR was most homologous to the first DED domain of pro-caspases-10, sharing 21% amino acid sequence identity (39% similarity), respectively, with these proteins (Figure 1C). The BAR DED domain contains 14 of the 21 residues previously recognized to be conserved among DED-family domains based on sequence alignments (28), and 16 of the 19 hydrophobic residues known to be conserved among DED-family domains based on structural considerations (29). A consensus secondary-structure prediction based on PHD (30) and nearest neighbor (31) algorithms suggested the presence of six α–helices, consistent with the known structure of other DED-family proteins (29). The SAM domain of BAR shares greatest homology with the human Ephb2 SAM domain (17% identity), and is predicted by FFAS to adopt a four α–helix bundle structure typical of SAM domains (32), with excellent conservation of the signature residues found in other members of this domain family (Figure 1D).

BAR is a membrane-associated protein that suppresses Bax-induced cell death. Expression of full-length BAR protein in yeast rescued cells from Bax-induced death (Figure 2A), despite continued production of the Bax protein (not shown). Deletion of the RING domain from BAR did not interfere with its protective effect on Bax-induced cell death. In contrast, removal of the C-terminal TM domain completely abolished the ability of BAR to rescue against Bax, suggesting that membrane localization of BAR is important for its function as a Bax antagonist. Immunoblot analysis confirmed production of the BAR (ΔTM) protein at levels comparable to full-length BAR (not shown).

To investigate the effects of BAR on Bax-induced cell death in mammalian cells, plasmids encoding wild-type (wt) or mutant versions of BAR were co-transfected with a Bax-encoding plasmid into 293T cells, which contain low endogenous levels of BAR. Cells were recovered 24 hr later and analyzed for percentage apoptosis by staining with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (18). Both BAR and BAR(ΔR) suppressed Bax-induced apoptosis (Figure 2B), without interfering with Bax protein production (Figure 2C). In contrast, the BAR(ΔTM) protein was ineffective at suppressing Bax-induced apoptosis, even though the protein was produced at levels similar to full-length BAR in 293T cells. We conclude therefore that BAR suppresses Bax-induced cell death in both yeast and mammalian cells, requiring the TM but not RING domain for its Bax antagonistic function.

The BAR(ΔR) protein consistently accumulated to higher levels in cells compared to full-length BAR (Figure 2C, D), though no difference in plasmid-derived BAR and BAR(ΔR) mRNAs was observed (not shown). Similar to several other RING-containing proteins which are subject to proteosome-dependent degradation (33-35), the 26S proteosome protease inhibitor MG-132 markedly increased accumulation of BAR protein (Figure 2D). Thus, steady-state levels of the BAR protein may be controlled by proteosome-dependent degradation, mediated by the N-terminal RING domain of this protein, explaining the

greater suppression of apoptosis by BAR(ΔR) compared to BAR. Though deletion of the RING domain from BAR was helpful for enhancing accumulation of this protein in 293 cells, high-levels of full-length BAR were found endogenously in some tumor cell lines such as MCF7 breast cancer cells (Figure 2E), LOXIMVI and UACC-257 melanoma, and IGROV1 ovarian cancer cell lines (not shown).

The presence of a candidate TM domain in BAR suggested it could be a membrane-associated protein. Indeed, BAR protein was not extractable from cellular membrane preparations using alkaline (pH 11.5) solution, consistent with an integral membrane protein (not shown). Subcellular fractionation experiments (Figure 2F) revealed that BAR resides predominantly in the heavy-membrane (contains mitochondria) and nuclear fractions (presumably representing association with the nuclear envelope; see below), with little BAR present in cytosol or low-density membrane factions. Reprobing the same blots with antibodies to mitochondrial (COX-IV), cytosolic (caspase-3), and nuclear (Poly-ADP Ribosyl Polymerase; PARP) proteins validated the fractionation procedure (not shown). BAR also co-fractionated with Bcl-2 (Figure 2F). When examined by immunohistochemistry using normal human tissue-sections and monospecific anti-BAR antisera, BAR was present in an organellar pattern within the cytosol of most cells (Figure 2G and data not shown). Preimmune serum and anti-BAR antiserum preadsorbed with recombinant BAR protein resulted in negligible immunostaining, confirming the specificity of these results (not presented). Microscopy examination of cells expressing GFP-tagged BAR confirmed these findings (not shown). BAR mRNA and protein were widely expressed in human tissues, as determined by Northern and immunoblot analyses (unpublished observations).

BAR modulates Bax-induced apoptosis through a SAM domain-dependent mechanism. To explore how BAR might modulate Bax-induced cell death pathways, we examined whether the BAR protein can associate with members of the Bcl-2 family. For these experiments, Myc-epitope-tagged or untagged BAR or BAR(ΔR) proteins were co-expressed by transient transfection in 293T cells together with Bcl-2, Bcl-X_L, Bak, or Bax. Co-immunoprecipitation assays determined that BAR can specifically associate with anti-apoptotic Bcl-2 and Bcl-X_L proteins (Figure 3A), but not with Bax or Bak (not shown). The efficiency with which Bcl-2 co-immunoprecipitated with BAR was comparable to Bax (Figure 3A), under the conditions of these assays.

The region within BAR required for its interaction with Bcl-2 was mapped, using a series of N- and C-terminal truncation mutants and internal fragments of BAR for in vitro protein binding assays (Figure 3B). Fragments of BAR which lacked RING, DED, or TM retained Bcl-2-binding ability, indicating that none of these domains is necessary. In contrast, all fragments of BAR which retained the SAM domain bound Bcl-2 in vitro, while all fragments lacking this domain failed to bind Bcl-2. The dependence of the SAM domain for interactions with Bcl-2 was also confirmed by yeast two-hybrid assays (Figure 3C).

We next explored the relevance of the SAM domain of BAR for suppression of Bax-induced cell death in yeast and for Bax-induced caspase-activation and apoptosis in human cells. When co-expressed in yeast with Bax, the full-length BAR protein suppressed Bax-induced cell death and permitted growth on selective medium, whereas the BAR (ΔSAM) protein failed to rescue yeast from Bax (Figure 3D). Immunoblot analysis confirmed production of the BAR and BAR (ΔSAM) proteins at comparable levels in yeast (not shown). For mammalian cell experiments, 293T (Figure 3) cells were co-transfected with a Bax-producing plasmid together with plasmids encoding either BAR or BAR (ΔSAM) proteins. Cells were recovered 1 day later and either cell lysates were prepared for assaying caspase activity using the fluorigenic substrate Asp-Glu-Val-Glu-aminofluorocoumarin (DEVD-AFC) (Figure 3E) or the percentage of apoptotic cells was quantified based on DAPI-staining (Figure 3F). Over-expression of Bax induced caspase activation and apoptosis, which were both markedly suppressed by co-expression of full-length BAR but not by BAR (ΔSAM) (Figure 3E, F). Immunoblot analysis confirmed that neither BAR nor BAR(ΔSAM) interfered with Bax protein production and demonstrated that the BAR and BAR (ΔSAM) proteins were

produced at roughly equivalent levels in these transfected cells (Figure 3F and data not presented). Taken together, the results demonstrate that the SAM domain of BAR is required both for its interactions with Bcl-2 and its ability to suppress Bax-induced cell death.

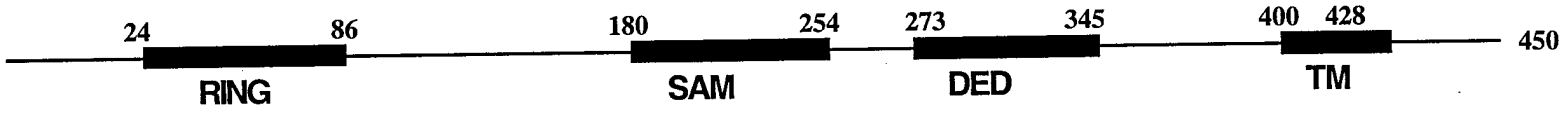
BAR binds DED-containing caspases and suppresses Fas-induced apoptosis. The presence of a DED-like domain in the BAR protein suggested that it might interact with other DED-containing proteins. Indeed, BAR and BAR(ΔR) specifically interacted with pro-caspase-8 and –10 in co-immunoprecipitation assays using lysates from transfected 293T cells (Figure 4A). In contrast, BAR and BAR(ΔR) did not co-immunoprecipitate with Fadd (Figure 4A), even though interactions of Fadd with pro-caspases-8 and 10 were detectable under the same experimental conditions (not shown). Thus, BAR associates with some but not all DED-family proteins. Mutagenesis studies confirmed a role for the DED domains of BAR, pro-caspase-8 and -10 for mediating their interactions (Figure 4B, C).

Because caspase-8 is essential for Fas-induced apoptosis (36, 37), we sought evidence that BAR could modulate this apoptotic pathway. For these experiments, 293 (Figure 4) or HT1080 (not shown) cells were transfected with plasmids encoding Fas in combination with either a control plasmid or plasmids producing the BAR, BAR(ΔR) and BAR(ΔDED) proteins. Caspase activity and apoptosis were then assayed after 1 day. 293 and HT1080 cells were chosen for these studies because over-expression of Fas triggers apoptosis in these cells through a Bcl-2-independent mechanism (20, 38), thus avoiding any contributions that BAR might make with respect to modulation of Bcl-2 family proteins. As shown, Fas stimulated activation of DEVD-cleaving caspases and triggered apoptosis (Figure 4D, E), both of which were partially blocked by co-expression of BAR or BAR(ΔR). In contrast, BAR(ΔDED) and BAR (ΔR/ΔDED), which failed to bind pro-caspase-8, were ineffective at blocking Fas-induced activation of caspases and apoptosis (Figure 4D, E). Thus, BAR requires the DED domain to interact with pro-caspase-8 and to suppress Fas-induced apoptosis. Immunoblot analysis also revealed correlations with Fas-induced processing of pro-caspase-8, with BAR reducing the amount of processed pro-caspase-8 produced as a result of Fas over-expression (Figure 4F).

BAR can mediate association of Bcl-2 and pro-caspase-8. Recognizing that BAR is capable of interacting with both Bcl-2 and pro-caspase-8, we determined whether BAR bridges these two proteins together in a complex. Accordingly, Bcl-2 and Flag-pro-caspase-8 were co-expressed in 293T cells by transient transfection, in the presence or absence of co-transfected BAR. Co-immunoprecipitation assays revealed that Bcl-2 was readily detected in caspase-8-containing immune-complexes when using lysates from cells over-expressing BAR but not in lysates of control transfected 293T cells (Figure 4G), which contain relatively little endogenous BAR protein. Co-expression of Bax prevented BAR-mediated co-immunopreciptation of Bcl-2 and pro-caspase-8 (not shown), suggesting that Bcl-2 cannot simultaneously bind Bax and BAR. We therefore conclude that BAR can bridge Bcl-2 and pro-caspase-8, at least when over-expressed, thus bringing together members of two important families of proteins involved in apoptosis regulation.

Appendix 1-Figures

A



B

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WASLYRERFLSERVNGRLLLTLTEEEFSKTPYTIENSSHRRAILMELERVKALGVKPPQNLWEYKAVNPGRSLFLLY**ALKSSPRLSLLYLYLFDYTDT**
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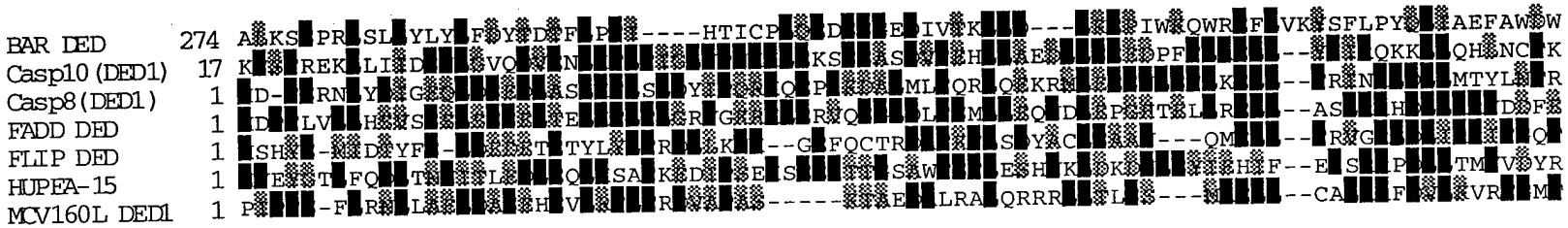
C



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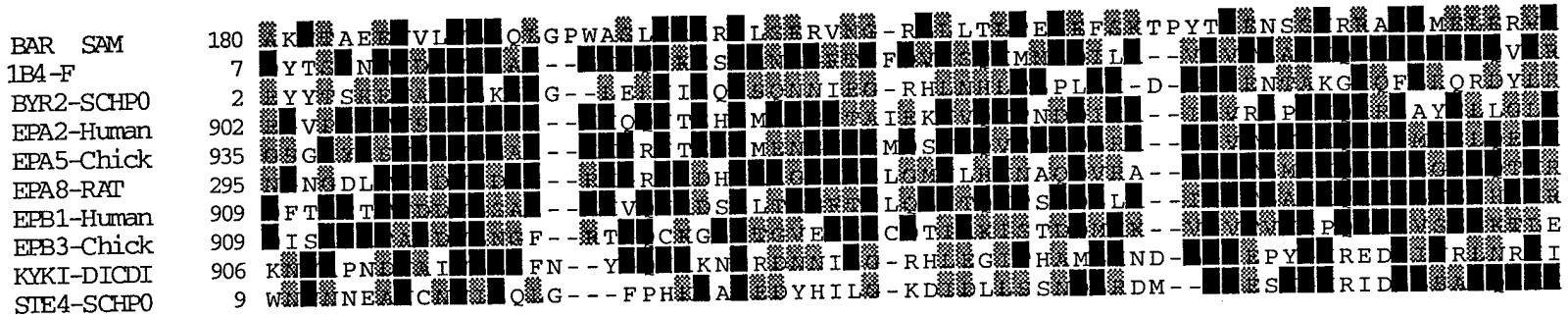

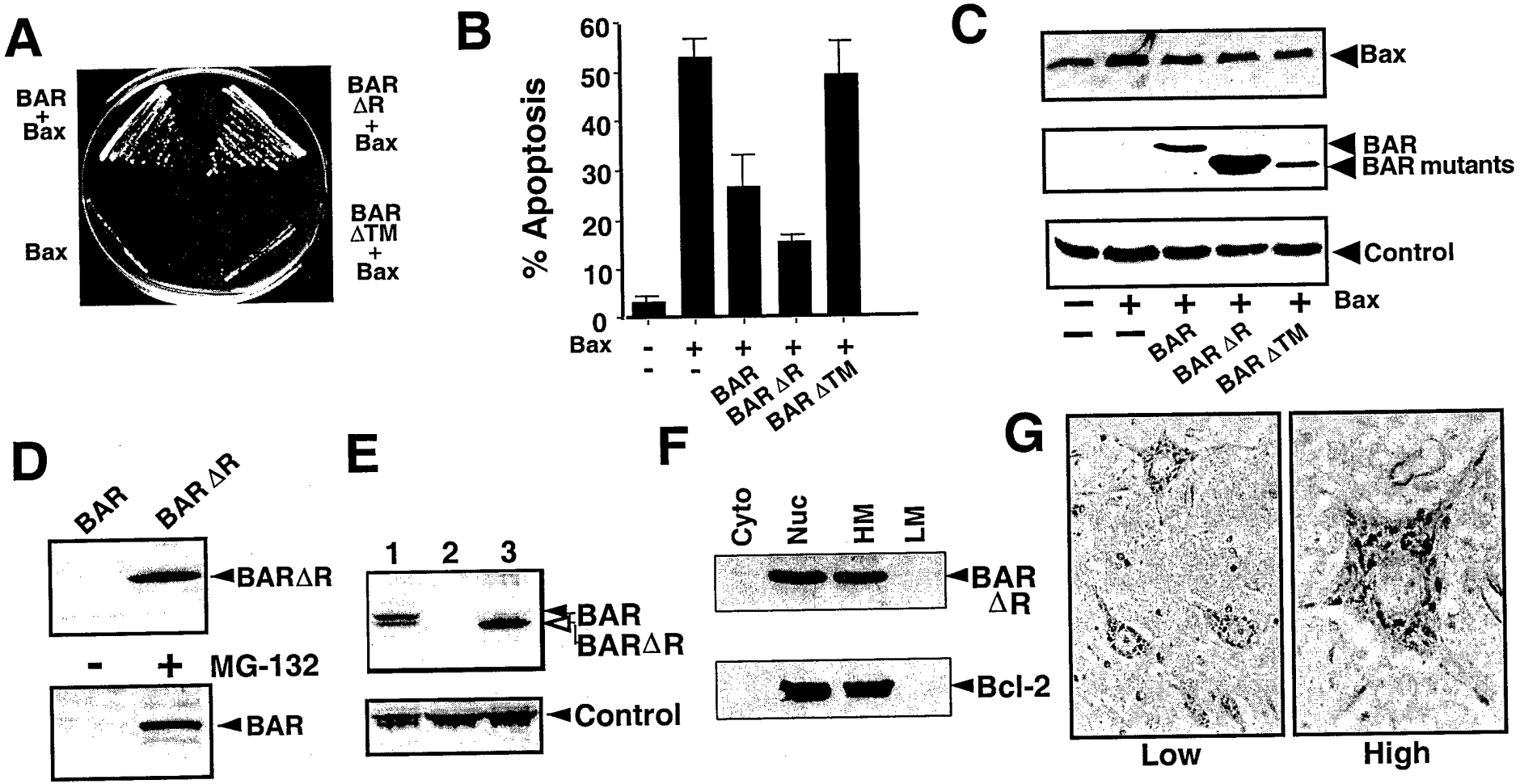
A
BAR + Bax
BAR ΔR + Bax
Bax
BAR ΔTM + Bax
B
% Apoptosis
60
50
40
30
20
10
0
Bax - + + + +
- - BAR BAR ΔR BAR ΔTM
C
Bax
BAR
BAR mutants
Control
Bax
- + + + +
- - BAR BAR ΔR BAR ΔTM
D
BAR
BAR ΔR
BARΔR
- + MG-132
BAR
E
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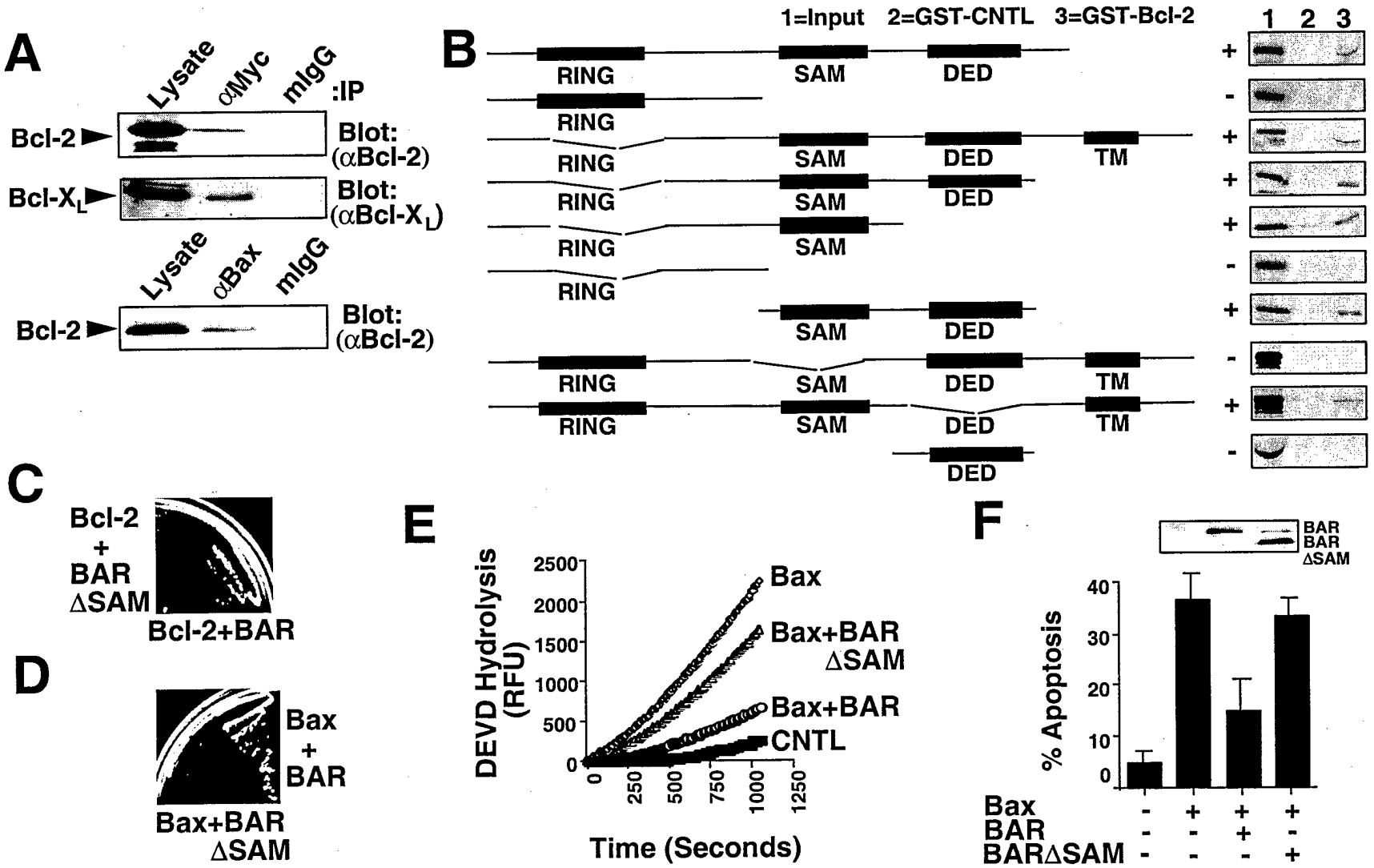
BAR
BARΔR
Control
F
Cyto
Nuc
HM
LM
BAR ΔR
Bcl-2
G
Low
High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Lysate
αMyc
mIgG
:IP
Bcl-2
Blot: (αBcl-2)
Bcl-X$_L$
Blot: (αBcl-X$_L$)
Lysate
αBax
mIgG
Bcl-2
Blot: (αBcl-2)
B
1=Input
2=GST-CNTL
3=GST-Bcl-2
1 2 3
RING
SAM
DED
TM
C
Bcl-2 + BAR ΔSAM
Bcl-2+BAR
D
Bax + BAR
Bax+BAR ΔSAM
E
DEVD Hydrolysis (RFU)
2500
2000
1500
1000
500
0
0
250
500
750
1000
1250
Time (Seconds)
Bax
Bax+BAR ΔSAM
Bax+BAR
CNTL
F
BAR
BAR ΔSAM
% Apoptosis
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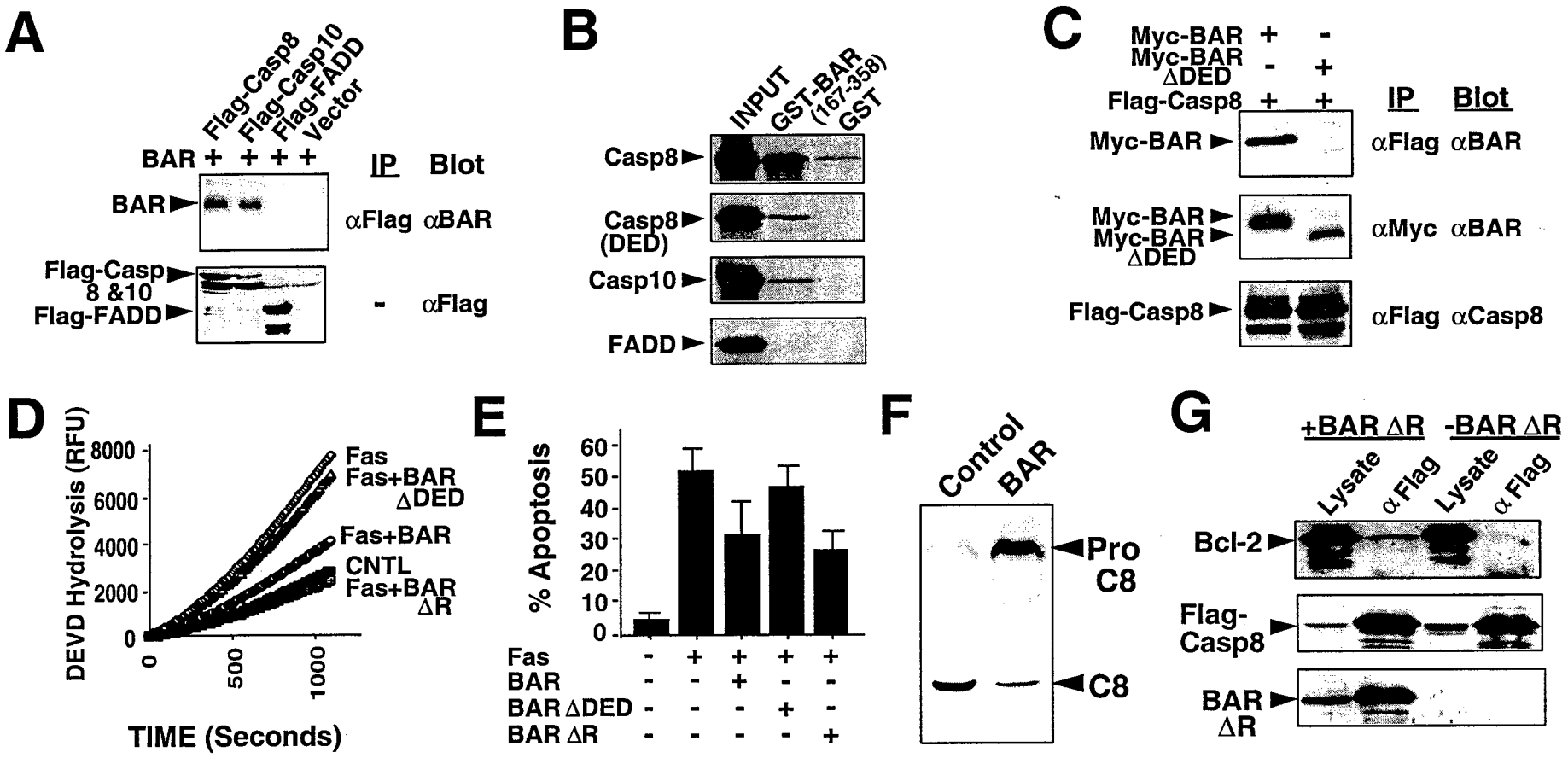
BAR
BARΔSAM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BAR
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Flag-Casp 8 &10
Flag-FADD
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B
INPUT
GST-BAR (167-358)
GST
Casp8
Casp8 (DED)
Casp10
FADD
C
Myc-BAR + -
Myc-BAR ΔDED - +
Flag-Casp8 + +
IP Blot
Myc-BAR
αFlag αBAR
Myc-BAR
Myc-BAR ΔDED
αMyc αBAR
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D
DEVD Hydrolysis (RFU)
8000
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0
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500
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TIME (Seconds)
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Fas+BAR
CNTL
Fas+BAR ΔR
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BAR - - + - -
BAR ΔDED - - - + -
BAR ΔR - - - - +
F
Control
BAR
Pro C8
C8
G
+BAR ΔR
-BAR ΔR
Lysate
αFlag
Lysate
αFlag
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BAR ΔR