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Apoptosis Searching for FLASH domains

During programmed cell death (apoptosis), a protein named FLASH is required to regulate the proteolytic cascade that ends in the death of the cell. Imai and co-workers have reported¹ that FLASH appears to be a functional analogue of two other apoptotic proteins, mammalian Apaf-1 and its nematode homologue CED-4, and that FLASH contains an amino-acid sequence motif that is homologous to the ATPase domain of Apaf-1, to the CED-4 sequence, and to a family of plant stress-resistant proteins that are apoptotic ATPases². Furthermore, FLASH contains two other domains (DRD) that are apparently related to the death-effector domain $(DED)^1$, an adaptor sequence that mediates interactions between proteins of the apoptosis machinery². These findings should help to explain the mechanism of action of this important protein. However, we have been unable to confirm the existence of these domains after re-examining the FLASH sequence.

We could identify no sequence similarity between FLASH and the Apaf-1/CED-4 or DED domains by searching the non-redundant protein sequence database at the NCBI using the gapped BLAST or PSI-BLAST programs^{3,4}, and over 1,000 sequences in the database were found to be more similar to the 'CED-4 homology'¹ and 'DED homology' regions of FLASH than were CED-4 or Apaf-1.

Searching databases, however, may only detect less than half of all similarities between sequences in proteins that are considered to be homologues on the basis of structural comparisons^{5,6}. Further analysis is needed, for example by direct comparison of functionally analogous proteins. We compared FLASH with Apaf-1/CED-4 and with DED-containing proteins by using the MACAW program⁷, but failed to detect any blocks of statistically significant sequence similarity (data not shown). We also used the PHI-BLAST program to assess the importance of the ATP-binding (P-loop) signature in FLASH (this program screens for similarity only those sequences that contain the specified signature), but found

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no similarity to the apoptotic ATPases even in this reduced search space. The other four motifs typical of the apoptotic ATPases² are not conserved in the published alignment of FLASH with these ATPases.

To confirm the presence of DED-related domains in the FLASH sequence, we searched it with the DED profile by using the SMART system⁸ and an independent method based on the PSI-BLAST program that detects all known DED domains⁹, but we were unable to find any similarity to DED.

These tests cannot rule out a subtle similarity, although we believe that structure predictions for FLASH and phylogenetic analysis may effectively do so, at least with regard to the purported ATPase domain. Compositional complexity analysis using the SEG program¹⁰ indicates that FLASH is largely a non-globular protein (Fig. 1). The entire 'CED-4 homology' region of FLASH is predicted to be non-globular, which is incompatible with the compact structure based on a parallel β -sheet with inserted α helices that is typical of ATPase domains¹¹. The P-loop in ATPases and GTPases is preceded by a hydrophobic β -strand, but this feature is lacking in FLASH.

The argument against structural similarity is supported by phylogenetic evidence. We have cloned and partly sequenced a human homologue of FLASH which has 67% amino-acid identity with FLASH in an alignment of 1,250 residues; the P-loop signature, however, is not conserved (data not shown; GenBank accession no. AF165161).

The structural and evolutionary evidence thus indicates that FLASH contains no globular domains with predictable functions and is not homologous to its functional analogues. FLASH does contain a predicted coiled-coil domain (Fig. 1) which may mediate functionally important protein–protein interactions¹² and so is probably the best available lead we have from the sequence for further experiments.

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Kimura, Imai and Yonehara reply — We do not consider that our inferences about FLASH function are misleading because they were deduced from functional analyses¹. We originally identified the deatheffector domain (DED)-binding activity of the DED-recruiting domain (DRD) of FLASH before analysing the structural homology between the DED and DRD domains. Moreover, we deduced the selfassociation activity of FLASH through its central region from functional analysis of several deletion mutants of mouse FLASH. We note that self-association activity of



Figure 1 Diagram of the predicted domain organization of FLASH (roughly to scale). The numbered bar indicates amino-acid residue positions. Boxes, predicted globular regions; lines, predicted non-globular domains; CC, coiled-coil. Regions of alleged similarity¹ to the apoptotic ATPases (CED-4) and DED domains (DRD, or DED-related domains) are indicated by broken lines. Predicted non-globular domains were detected by using the SEG program¹⁰, with the following parameters optimized for partitioning protein sequences into globular and non-globular domains: window length, 45; trigger complexity, 3.4; extension complexity, 3.7. Coiled-coil domains were predicted using the COILS2 program¹²; boundaries of the strongly predicted coiled-coil domain are indicated.

bplash 1 maadddngdgtsledvysasplknndegsldiyagldsavsds <mark>asks</mark> cypskncldiyeeilfeegtakeatyndloveygkoolomk <mark>el</mark> imkkreioro mflash 1 maadddngdgtsledvycbasplknndegsldiyagldsavsds <mark>tars</mark> cysfracldiyeeilfregtakeatindloievgcoomdiakerkeioro
bflash 101 NFSLINENQSLKKNISALIKTARVEINRKDEETSNLHQRISEFPHFRNNHKTARTEDTVKTKDLKSFSPHLDDCSKTDHRAKSDVSKDVHHSTSLPNLEK mFLASH 101 NLNLKNENQSLKKNISALIKTARVEINRKDEETNHLHQRLSEFPHFRNNHKAARTKDSQSTSPHLDDCSKTDHGVKSDVQKDVHPNTAQPNLEK
hPLASH 201 BGRPHS <mark>DKRS</mark> TSHLPTSVEKHCTNGVWSRSHYQVGEGSSNEDSRRGKDIRHSQFNRCTERVRKDLSTCCGDGEPRILEASORLQGHPEKYCKGEPK mFLASH 195 BGRSHSEAQNPLHLSTGVEKHCANNVWSRSPYQVGEGNSNEDNRGRSGTRHSQCSRGTDRTQKDLHSGCNDSEPRDKEANSRLQGHPEKHGNSEARTES
<i>hPlash 301</i> KSKrK <mark>SNS</mark> DSDYKGERINSSWEKETPGERSHSRVD SOSDKKL <mark>ERÖ</mark> SERSONINKKEVKSODKEERKVDOKPKSVVKDOD HWRRSERASLPHSKNEITFS m <i>Plash 295</i> KISESKSSTGMGYKSERSASSWEKETSHERPHTRVESOHDKNLEKONERLONMHRKELPSODKTERKVDVKFKPAGEBOGHRGRVDRALPPHFKNDVKH-
<i>hplash 401</i> <mark>HNS</mark> KYHLEERRGWEDCKRDKSVNSHSFQDGRCPSSLSINSRTHKNIDSKEVDAMHQWENTPLKAERHRTEDKRKRE <mark>QESKEENRHIRNEKRV</mark> PTEHLQKT mplash 394 vgfnkyhfeerrgredckrdromnshjeqdrrcSistLssnrnskyphskevsvahqwentprkaerhrtedrrkrerenkeesrhvksdkkseppehlqrt
hFLASH 501 NKETKKTTTDL <mark>KKONEPKTDKGEVLDNGVSEGADNKELAMKAESGPNETKNKDLKLSFNKKLNLTLSPAKKOPVSODNO<mark>HKITDIP</mark>KSGOVCDSESSMOV mFLASH 494 HKDTKKSTADGKROTEPKHGKGAVSNSELSKGTDSKEGATKVESGPNEAKKDKLKLSFNKKLNLTLSPAKKOPAGDNEHGITGVPESGTCDSRELETT</mark>
<i>hFLASH 601</i> KTVATVPSISEHILGEAAVSEHTMGETKSTÄLEPRVALLAVTEPRIGISETNKEDENSLIVRSVDNTMLCEEPICGTETSF <mark>PSPMEIQO</mark> TESEPPSTGMK mFLASH 594 GTVACLESGSEHNREETKSELPEPKEALLATSOLRISIPENKMKEEKRLEFKSVENTVPCELLACGTEISLPAPVEIEQARCILGSVEVE
<i>hflash 701</i> otinngraaryvmdviotdvisonfgleidtkrudnsdyggisegmemkvalstvysettesiigsisednifytnisednnerfepsvivtflvesks mflash 684 bigggartaasvvmhvipehasedasgeldtkrhdginagatsegvktkvilsekaraaseshiaelveepsisivngsgdnnerfepsieerdivetks
hFLASH 801 CHLEFCLPKETLDSSLÖGTELMDHRMATGETNSVYHDDDNSVLSIDLNHLRPIPEAISPINAVLRNESPPOVPVINNSHKDVFLFNSAHSISK mFLASH 784 CFLESCLPKETFVPSPOKTELIDHKIETGESNSVYQDDNSVLSIDFNNLRPIPDPISPINSPVCUVVSMESSCAIFLYDSSHKDEFPSNSTLSIFK
hFLASH 901 SOSDLNKENOKPTYKSDKCTEADTCKNSPLDELEEGEIRSDSETSKPOESFEKNSKRRVSADVRKSKTPRRGKSTVCLDKDSRKTHVRIHOTNNKWKR mFLASH 884 SOSDLNKENEKEVPKFDKCSEADSCKHLSLDELEEGEIRSDEESVAQKRLEKSARPKVSADVDCKSSPSSRSTVHVHKUNGRTAVKLPRDRLT#SKR
bFLASH 1001 PDKSSRSSKTEKKDKVWSTSSLEKIVPITAVPSSEQEIMHMLRMIRKHVRKNYMKFKAKFSLIOFHRIIESAILSFTSLIKHLNLHKISKSVTIQKNLC mFLASH 984 SSBS-RFONTERSSKTESISSLEKI1PLTIVPSSLWEVMHMLRLLCHVRKNYMKFKIKFSLTOFHRIIESAILSFTSLIKULDISKTCKSUSTLQKSLC
hFLASH 1101 DIIESKLKQVKKNGIVDRLFEQQLFDMKKKLWKFVDDQLDYLFAKLKKILVCDSKSFGRDSDEGKLEKTSKONAQYSNSOKRSVDNSNRELLKEKLSK mFLASH 1083 EVIESMLEQVKKNGIVDRLFEQQDTDMKKKWKFVDEQLDYLFEKLKKILLKFCDSVNFENENSEGKLGKKYKERTQHSNCQKKKMDNKEIFRENVIK
hFLASH 1199 SEDFVHYKSLYGCKKSEENYODONN <mark>SSIN</mark> TVKHDIKKNENIÖFDNIKNSOSEERSLEVHCPSTEKSEKNEGS <mark>SIEDAGTSOHATLKPERSFELLTEQGAS</mark> mFLASH 1181 LENTVNHESSIGGEKSEEKHODONKTNASIVKHDVKRTESTOSDNIKNAECKEGFLEKSCPSTERPCKDEGHTEEBAGAAQHASAKSERSFELLTEGGAS
hflash 1299 Slifnlvsdaqmgeifksllqgsdlld <mark>ssvncfeksewelktpekqllet</mark> ikcesifactteelvsgväspcpkmisddnwsllssekgpslssglslpv mplash 1281 Slifnlvsdaqmgeifksllqgsdlldtsgtekaewelktpekqllesikcesapacateelvsegaslcpkvisddnwsllssekgpslssglslpv
hflash 1399 Hpdvld <mark>escmfevstnlp</mark> üskdnvc&vekskpc <mark>v</mark> ssilledlavsltvpsplksdghlsflkp <mark>dm</mark> S <mark>S</mark> StpeevisahfsedalleeedaseQdihlale mflash 1379 HpdvldenCmfevSsntalGkdnvySsekskpcissilledlavsltvpsplksdghlsflkpeviststpeevisahfsedalleeedaseQdihlale
hFLASH 1499 SDNSSSKSSCSSSWTSRSVAPGFQYHPNLPMHAVIMEKSNDHFIVKIRRATPSTSSGLKQSMMPDELLTSLPRHGKEADGGPEKEYISCONTVFKSVEEL mFLASH 1479 SDNSSSKSSCSS-WTSRSVASGFQYHPNLPMHAVIMEKSNDHFIVKIRRATPSTSPGLKHGVVAEESLTSLPRHGKEAGVATEKEPNLFQSTVIKFVKDL
hFLASH 1599 ENSMKNVDGSKSTHEEOSSMIQTOVPDIYEFLKDASDKMCHSDEVADECFKLHQVWETKVFESIEELPSMEEISHSVGEHLPNTYVDLTKDPVTETKNLG mFLASH 1578 ENTOKNIDKKKLTHEEONSIVQTVVDIYEFLKDASNKVVHCDQVVDICEKLHQVWEPKVSENLQELPSMEKIPHSLDNHLPDTHIDLTKDSATETKSLG
hFLASH 1699 EFLEVTUHIDDDCCSGGNINGSAGTLDNSLGADTVGAFIDLTQDASSEAKSEGNHFALAVEDLGCGVTQVDEDNCKEERAQVANRPIKGIVEETYIDLT mFLASH 1678 EIMCVTVINVDHIECSQTNLDGIAFITCSSLQPUTIDAFIDLTHDASSESENEGSEPVLAVECMGCQVTCIDEDTNKEGMGRANSPIESIVEETOIDLT
hFLASH 1799 TESPSSCEVKKDELKSEPGSNCENSELFGTLENSHKKRRNISDLNH-PHKKORKETDLTNKKKKKPTODSCENTEAHOKKASKKKAEFVTKOPSSLKAT mFLASH 1778 TESPSSCEVKKDELKSEPFSKLDCLELFETLGNGHKKRKNSPGVSHSSQKKORKDIDLSS-EKKORLSPNSDRNGDAHRKOASKKRBPAVNETSLSSEA
hFLASH 1898 BE <mark>IK</mark> ÖS <mark>S</mark> ÄLATS-TSLSAKNVIKKKGEII <mark>IL</mark> ÄTRNDDREILLECOKRGPSFKTEAYLÄÄKLDKNPNOVSERFOOLMKLFEKSKCR hFLASH 1982 mFLASH 1877 BEVKASTAVLAASPASLSAKNVIKKKGEIIVSWIRNDDREILLECOKRAPSLKTETYLÄVKLNKNPNOVSERFOOLKKLFEKSKCR mFLASH 1962

Figure 1 Comparison of deduced amino-acid sequences of mouse and human FLASH. Green and yellow boxes indicate identical and similar residues, respectively. Deletion mutants ΔB and ΔD containing 'CED-4-homologous' and DRD domains, respectively, which showed self-association and DED-recruiting activity, respectively¹, are underlined. Walker's A- and B-box-containing regions are underlined (W-A and W-B), as are the DRD domains. The amino-acid residues of mouse FLASH (red) in W-A, W-B and DRD domains are identical or similar to residues in CED-4-homologous and DED-containing proteins, as indicated previously¹. Human FLASH was cloned by using a cDNA library of human KT cells⁶ (a gift from S. Nagata). The cDNA library was screened by using fragments of human FLASH cDNA (EST clones N68740 and H50582) as probes and by standard colony hybridization procedures. The nucleotide sequence corresponding to the amino-acid sequence of human FLASH has been deposited in the GenBank database (accession no. AF154415).

CED-4-homologous proteins is required for the activation of caspases².

To determine the significance of the conserved amino-acid residues in mouse FLASH and in other apoptotic proteins, we cloned human FLASH to see whether these residues are still conserved in human FLASH (Fig. 1). Human FLASH complementary DNA showed 80% identity in the nucleotide coding sequence with mouse FLASH cDNA; in the amino-acid sequence, human FLASH shares 66% identity and 78% similarity with mouse FLASH.

In human FLASH, however, the ATP/GTP-binding motif homologous to the Walker's A-box consensus sequence³ is not conserved, with a serine residue being substituted for an essential glycine in the ATP/GTP-binding motif (arrowhead in Fig. 1). In addition, amino-acid residues that are conserved between mouse FLASH and CED-4-homologous proteins in the Walker's A-box-containing region are not conserved in human FLASH (W-A in Fig. 1). These results indicate that the ATP/GTP-binding motif of mouse FLASH is not essential and that FLASH is not a CED-4-

homologous protein. As Koonin *et al.* suggest, we could have overestimated the structural homology of FLASH with CED-4, even though these proteins are functionally homologous¹. In the self-association region (ΔB in Fig. 1), there are some well conserved domains outside the W-A region, and it needs to be determined which of these conserved domains are required for the self-association activity of FLASH.

Contrary to the inference of Koonin et al. (a misunderstanding that may have arisen as the result of our calling DRD a DED-related domain to indicate a DEDrecruiting domain), we reported that the DRD domain of FLASH is functionally different from the DED domain¹ in that the DRD domain is unable to self-associate, whereas the DED domain can. However, there could be some conservation of amino-acid residues in the FLASH DRD domain with those in the DED domain, given that these residues are conserved between the DRD domain of mouse FLASH and the DED domain of human FLASH (Fig. 1). Moreover, Koonin et al. show that the DRD domain is a globular domain, so

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we presume that the DRD domain has a structure that is quite similar to the DED domain.

Although the EST database has been exclusively searched using the BLAST program for DED-containing proteins, this method has never been able to identify FLASH. Having established that FLASH can bind to the DED domain¹, we cloned FLASH by using this function to detect it. Such an approach complements cloning methods based on searches of the EST database using BLAST, which has been successful for many other important apoptotic proteins, such as DED-containing FLICE and FLIP^{4,5}.

To address a more general concern regarding the specificity of our antibody, we cloned human FLASH and expressed recombinant Flag-tagged human FLASH in 293T cells. Following immunoprecipitation of human FLASH with anti-Flag antibody, our affinity-purified anti-mouse FLASH antiserum¹ was able to detect human FLASH on a western blot. Furthermore, we have shown that our antibody, which was raised against the peptide LSPNSDRNG-DAHR (from mouse FLASH), can bind the peptide PTQDSCENTEAHQ (from human FLASH), albeit with a lower affinity than to mouse FLASH. We have also obtained monoclonal antibodies that recognize both mouse and human FLASH with associated characteristics to our affinity-purified polyclonal antibodies, and we shall report these results elsewhere.

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Materials

Transformation of diamond to graphite

Despite almost forty years of trying, no one has managed to transform diamond into graphite under pressure¹, or find out what the pressure limit for diamond might be². If diamond were to behave like other group IV elements, such as silicon, germanium or tin, it would transform under compressive indentation to the β -tin structure³, but it does not^{2,4}. Here we use micro-Raman spectroscopy to determine what happens to diamond when it is subjected to high contact compression as a result of pressing a

NATURE | VOL 401 | 14 OCTOBER 1999 | www.nature.com