Interaction with Sug1 enables Ipaf ubiquitination leading to caspase 8 activation and cell death

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INTRODUCTION

Molecules involved in regulation of apoptosis are of paramount importance in maintaining cell and tissue homeostasis. The major effectors of apoptosis belong to a class of cysteine proteases known as the caspases. They are expressed as inactive pro-enzymes to keep the apoptosis programme under control. Apoptotic stimuli (whether extrinsic or intrinsic) result in activation of initiator caspases, such as caspases 8, 1, 2 and 9, through oligomerization and formation of multimolecular complexes, leading to cleavage and activation of effector caspases, such as caspases 3, 6 and 7 [1–3]. Although Apaf (apoptotic protease-activating factor) aids in formation of the apoptosisosome and activation of caspase 9, adaptor proteins, such as FADD (Fas-associated death domain), aid in the formation of DISC (death-inducing signalling complex) and caspase 8 activation [4,5]. Molecules, such as IAPs (inhibitor of apoptosis proteins) and c-FLIP (cellular FLICE [FADD-like IL-1β-converting enzyme]-inhibitory protein), form a second level of regulation and determine the extent of caspase activation [6,7]. Initial activation of caspase 8 during death receptor signalling involves proximity-induced dimerization. However, full activation and autocatalytic processing of caspase 8 requires further aggregation [8,9].

Ipaf [ICE (IL-1β-converting enzyme)-protease-activating factor], also known as CLAN (CED-4-like gene)/CARD12 (caspase activation and recruitment domain protein 12)/NLRC4 [NLR (Nod-like receptor) family CARD-containing 4], belongs to the family of NBS-LRR (nucleotide-binding site and leucine-rich repeat) proteins [10,11]. It is a component of the ‘inflammasome’ and an activator of caspase 1 [12]. It is a transcriptional target of p53 and p73 and is required for p53-mediated apoptosis [13–15]. Ipaf plays an essential role in responses induced by intracellular pathogens, and macrophages from Ipaf-knockout mice are deficient in IL-1β secretion and bacterial clearance [16,17]. Ipaf contains an N-terminal CARD, a central NBD (nucleotide-binding domain) and LRR domain containing 13 LRRs at its C-terminus. The NBD is essential for activation of caspase 1, and the CARD is involved in homodimerization and interaction with other CARD-containing proteins [18]. The LRR domain is a negative regulatory domain, as its deletion results in a truncated protein which is constitutively active in caspase 1 activation [11]. Therefore activators of Ipaf may bind to LRRs to relieve auto-inhibition and activate Ipaf-mediated signalling pathways. The LRR domain is required for sensing the flagellin protein of intracellular pathogens, such as Salmonella typhimurium and Legionella pneumophila [16,19]. However, the

Abbreviations used: aa, amino acid(s); Ac-Ipaf, LRR-deleted Ipaf; ASC, apoptosis-associated speck-like protein containing a CARD; BAG, Bcl-2-associated athanogene; CARD, caspase activation and recruitment domain; Cdk, cyclin-dependent kinase; c-FLICE, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; CMV, cytomegalovirus; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; FADD, Fas-associated death domain; GFP, green fluorescent protein; GST, glutathione transferase; HA, haemagglutinin; HDAC, histone deacetylase; HEK-293 cell, human embryonic kidney cell; IL-1β, interleukin 1β; Ipaf, ICE (IL-1β-converting enzyme)-protease-activating factor; LRR, leucine-rich repeat; mCaspase, dominant-negative caspase mutant; mSug1, Sug1(K196M); NA, numerical aperture; NBD, nucleotide-binding domain; PARP, poly(ADP-ribose) polymerase; RFP, red fluorescent protein; RIP, receptor-interacting protein; shRNA, small-hairpin RNA; Sug, suppressor of gal; TNF, tumour necrosis factor; Ub, ubiquitin; Ub1, Ub2 or Ub4, mono-, di- or tetra-Ub; UPS, Ub–proteasome system.

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mechanism by which the LRR domain negatively regulates Ipaf is not known.

To gain insights into cellular activators and effectors of Ipaf function, a yeast two-hybrid screen was performed and Sug1 (suppressor of gal1)/Rpt6 (regulatory particle 6)/S8 (subunit 8)/TRIP1 (thyroid receptor-interacting protein 1) was identified as an interacting protein. Sug1 is one of the six AAA (ATPase associated with various cellular activities) ATPase complexes in the base of 19S proteasome and has been shown to interact with various transcription factors [20,21]. It is up-regulated during developmental cell death in the moth Manduca sexta, and Sug1 modulates p53 transactivation and target gene expression [20,22]. Ubiquitination represents a key modulator of a variety of cellular processes, such as cell cycle regulation, transcription, signal transduction and apoptosis. This modification aids in protein–protein interaction and proteasome-mediated degradation [23,24]. Although it is well established that adaptor proteins regulate caspase activation, the mechanism of regulation of adaptor molecules, such as Ipaf, is poorly understood. We therefore examined the intra- and inter-molecular mechanisms involved in the regulation of Ipaf to induce cell death in A549 lung carcinoma cells. Our results show that Ac-Ipaf (LRR-deleted Ipaf) induces caspase-8-dependent cell death in A549 cells. We also showed that ubiquitination mediated through the Sug1 interaction regulates Ipaf function to induce caspase 8 activation and cell death.

EXPERIMENTAL

Cell culture and transfections
A549, HEK-293 (human embryonic kidney) and MCF-7 cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin) at 37°C in a CO2 incubator. Transfections with purified plasmids (purified with the Qiagen Plasmid Midi kit) were carried out using LipofectamineTM PLUS reagent or LipofectamineTM 2000 (Invitrogen) according to the manufacturer’s instructions. The stable clones, P2-Yama MCF-7 (expressing caspase 3) or neomycin-resistant MCF-7 (control clone), were maintained in DMEM containing 100 μg/ml genetin (G418) as described previously [25]. The THP-1 cell line was grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. A549 cells were treated with 25 μM MG132 for 6 h in all experiments; a concentration which is not toxic to A549 cells, even when exposed for 24 h [26].

Antibodies and reagents
Mouse monoclonal anti-HA (haemagglutinin) and anti-Myc monoclonal antibodies were from Roche Molecular Biochemicals and Oncogene Research Products respectively. Anti-p53 antibody (Ab-6 clone DO-1, mouse monoclonal) was from Neomarkers. Antibodies against caspase 1 (rabbit polyclonal), GFP (green fluorescent protein) (mouse monoclonal), GFP (rabbit polyclonal), α-tubulin (mouse monoclonal), Cdk2 (cyclin-dependent kinase 2) (rabbit polyclonal), caspase 8 (goat polyclonal), caspase 8 p20 (rabbit polyclonal), IL-1β (rabbit polyclonal), HA (rabbit polyclonal) and RIP (receptor-interacting protein) (rabbit polyclonal), and GFP–agarose-conjugated beads and Protein A/G PLUS–agarose beads were purchased from Santa Cruz Biotechnology. Cleaved caspase 8 (Asp-391-18C8, rabbit monoclonal) and cleaved caspase 3 (Asp-175, rabbit polyclonal) antibodies were from Cell Signaling Technology. Anti-PARP [poly(ADP-ribose) polymerase] antibody (rabbit polyclonal) was from Roche. Anti-Sug1 antibody (mouse monoclonal) was from BD Biosciences. Anti-Ub (ubiquitin) antibody (6C1, mouse monoclonal) was from Calbiochem. Anti-Ipaf (rabbit polyclonal) and anti-HDAC7 (histone deacetylase 7) (rabbit polyclonal) antibodies were purchased from Abcam. Glutathione–agarose beads were purchased from Sigma. Yeast culture reagents and media were obtained from Himedia and Sigma.

Expression vectors
All cloning experiments were performed according to standard procedures [26a]. Full-length Ipaf cDNA was subcloned from pCDNA3 containing full-length Ipaf [11] by BamHI/XhoI digestion in pEGFP-C1 (Clontech) mammalian expression vector to derive GFP–Ipaf. Ac-Ipaf, without the C-terminal LRR domain, has been described previously [13]. Ipaf and its deletion constructs expressing various domains were amplified with gene-specific primers containing restriction sites at ends and cloned in the yeast two-hybrid expression vector pGBK7T (Clontech) and also in the GFP-expression plasmid pEGFP. The Myc–LRR construct was generated by subcloning the LRR fragment into the pCDNA3.1-myc vector at the EcoRI and XbaI sites. HA–Sug1 and GST (glutathione transferase)–Sug1 were provided by Dr Takeshi Inoue (Department of Life Sciences, University of Tokyo, Tokyo, Japan). Mutant Sug1 was generated by replacing Lys-196 (AAG) with a methionine residue (ATG) by PCRBASE-directed mutagenesis (Stratagene). RFP (red fluorescent protein)-Sug1 was generated by restriction digestion of the NheI and XhoI sites of pCI-HA-Sug1 to release the HA tag, which was replace with the RFP cassette from pDsRed-Monomer-HyG-C1 (Clontech) digested with HhaI and SalI in-frame upstream of pCI-Sug1. The C-terminal deletion constructs of Sug1, i.e. 1–173 aa (amino acids) or 1–315 aa, were generated by restriction-digestion-based cloning into pACT2 (Clontech). The plasmid expressing the catalytically inactive mutant of human caspase 1 has been described previously [27]. The plasmid expressing mCaspase 8, an active-site mutant, C560A, which acts to dominantly inhibit endogenous caspase 8 activity, was provided by Professor D.V. Kalvakolanu (Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, U.S.A.) [28]. The K48R and K63R mutants for ubiquitination were generated by replacing a lysine residue (AAG) with an arginine residue (AGA) at respective positions in pCI-HA-Ub by PCR-based site-directed mutagenesis (Stratagene). RFP (red fluorescent protein)-Sug1 was generated by restriction digestion of the NheI and XhoI sites of pCI-HA-Sug1 to release the HA tag, which was replaced with the RFP cassette from pDsRed-Monomer-HyG-C1 (Clontech) digested with HhaI and SalI in-frame upstream of pCI-Sug1. The C-terminal deletion constructs of Sug1, i.e. 1–173 aa (amino acids) or 1–315 aa, were generated by restriction-digestion-based cloning into pACT2 (Clontech). The plasmid expressing the catalytically inactive mutant of human caspase 1 has been described previously [27]. The plasmid expressing mCaspase 8, an active-site mutant, C560A, which acts to dominantly inhibit endogenous caspase 8 activity, was provided by Professor D.V. Kalvakolanu (Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, U.S.A.) [28]. The K48R and K63R mutants for ubiquitination were generated by replacing a lysine residue (AAG) with an arginine residue (AGA) at respective positions in pCI-HA-Ub by PCR-based site-directed mutagenesis (Stratagene). Mono-, di- and tetra-Ub-Ipaf (GFP–Ipaf–Ub1, GFP–Ipaf–Ub2 and GFP–Ipaf–Ub4) were constructed by restriction digestion of GFP-Ipaf at the SpeI and BamHI restriction sites, to release the stop codon, and ligated with linkers containing the Ub1, Ub2 or Ub4 sequences in tandem.

Construction of adenoviral vectors
All adenoviral vectors were generated using the AdEasy System provided by Dr Bert Vogelstein (Howard Hughes Medical Institute and The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins Medical Institutions, Baltimore, MD, U.S.A.). GFP–Ipaf or GFP–Ac-Ipaf cDNA was isolated from the pEGFP-C1-Ipaf or pEGFP-C1-Ac-Ipaf plasmid by NheI/BamHI digestion and cloned into the pShuttle-CMV (cytomegalovirus) plasmid digested with XbaI/EcoRV under the control of the CMV promoter terminated by the SV40 (simian virus 40) polyadenylation signal, resulting in pShuttle-CMV-GFP–Ipaf or Ac-Ipaf. The pAdtrack-CMV plasmid was utilized as a control vector. Recombinant plasmids were generated by homologous recombination in AdEasier cells. HEK-293 cells
were transfected with the recombinant adenoviral plasmids using Lipofectamine™ 2000 (Invitrogen), and adenoviruses were collected.

**Construction of vectors expressing shRNA (small-hairpin RNA)**

The shRNA expression vector targeting Sug1 (Sh A) was constructed using the U6 promoter-based vector as described previously [13,29,30]. The Sug1 sequence targeted by shRNA was from nucleotides 911 to 929 (GenBank® accession number NM_002805). The sequence of the constructs was confirmed by automated DNA sequencing. This vector expressed sense, hairpin and antisense sequences. A vector expressing shRNA of unrelated sequence (C-Sh) of the same length was used as a control. RFP–Sug1-Sh A/Sh D [Sh D is an alternate shRNA targeting nucleotides 235–254 bp of Sug1 (GenBank® accession number NM_002805)] and RFP–C-Sh were generated by cloning the respective shRNA cassettes in pDsRed-Monomer-Hyg-C1 vector (Clontech).

**Yeast two-hybrid screening**

A Gal4-based yeast two-hybrid screen of a human placental cDNA library was performed, using 1–561 aa (Ac-Ipaf) residues of Ipaf as bait, as described previously [31]. The yeast strain PJ69-4A was used for the two-hybrid analysis. The expression of Ac-Ipaf fused to the Gal4 DNA-binding domain (expressed by the construct in a pGBK-T-7 vector) was checked by Western blotting. The Ac-Ipaf (pGBK-T-7)-expressing PJ69-4A strain was then transformed with a human placental cDNA library (Clontech). Transformants were plated on to yeast dropout medium (HiMedia) lacking Trp, Leu and Ade. A small sample (0.1%) of the transformant mixture was plated on to the Leu⁻ and Trp⁻ dropout medium plate to determine the efficiency of the transformation. The positives obtained on Trp⁺, Leu⁻ and Ade⁻ plates were checked for the activation of the β-galactosidase reporter gene by plating them on to plates containing Trp⁺ and Leu⁺ dropout medium, adjusted to pH 7.0, and the β-galactosidase substrate. Plasmids were isolated from positive yeast colonies, propagated in *Escherichia coli* and then sequenced. The interactions were confirmed by retransformation of positive clones with Ac-Ipaf or control plasmids.

**Indirect immunofluorescence and confocal microscopy**

Indirect immunostaining of cells and microscopy was carried out essentially as described previously [25,32]. The secondary antibodies used were rabbit or mouse IgG coupled with Cy3 (indocarbocyanine) (Amersham), Alexa Fluor® 488 or Alexa Fluor® 633 (Molecular Probes) fluorescent dyes. Immunofluorescence staining was observed and digital images were captured with an LSM 510 Meta confocal microscope (Carl Zeiss) or with an Axioplan 2 microscope (Zeiss) fitted with an ApoTome. The confocal images were captured using ×63/1.4 NA (numerical aperture) oil immersion objectives. The images from the Axioplan 2 microscope were captured using a ×40/0.75 NA dry objective. Similar parameters of image capture were used for analysis of coverslips belonging to a particular experiment. Images were further processed using Adobe Photoshop software.

**Quantification of cell death**

Quantitative analysis of dead cells was carried out essentially as described previously [25,32,33]. Cells grown on coverslips were transfected with the required plasmids and fixed after 24 h (or later, as indicated) and stained with the required antibodies as described above. Cells were mounted in 90% glycerol containing 1 mg/ml paraphenylenediamine (anti-fade) and 0.5 mg/ml DAPI (4’,6-diamidino-2-phenylindole) to stain the DNA. Cells showing immunofluorescence staining were counted and those cells that showed cytoplasmic shrinkage, loss of refractility and condensed chromatin or the presence of apoptotic bodies were scored as dead cells. At least 200 expressing cells were counted in each coverslip. Cells not expressing the transfected protein were scored in each coverslip for determining the background level of cell death. The data are expressed as the means ± S.D. of the percentage of dead cells among the total number of expressing cells after background subtraction from at least three independent experiments carried out in duplicate. The Student’s *t* test was used as a test of significance.

**Caspase-8-like activity assay**

Caspase-8-like activity was measured by the Caspase-Glo™ 8 assay kit (Promega) that provides a luminesogenic caspase 8 substrate. It contains the tetra-peptide sequence LETD, which is cleaved to release aminoluciferin. Briefly, A549 cells transfected with the indicated plasmids for 24 h were washed with 1× PBS, and 80 μl of DMEM and 80 μl of Caspase-Glo™ 8 reagent was added to each well. The contents were gently mixed using a plate shaker at 500 rev/min for 1 min and incubated for 1 h at room temperature (25°C) to allow the reaction. Finally, the activity in 100 μl of the reaction solution was measured using a TD-20/20 luminometer (Turner BioSystems).

**Co-immunoprecipitation and Western blotting**

HEK-293 or A549 cells transfected with the required plasmids for 36 h, were lysed at 4°C for 20 min in buffer containing 25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 1 mM PMSF, 0.1% BSA, 5 mM EDTA and protease inhibitor cocktail (Roche Biochemicals). Lysates were centrifuged out at 10000 g for 10 min at 4°C and the supernatant was used for immunoprecipitation with 2 μg of anti-c-Myc monoclonal antibody or 2 μg of normal mouse IgG as the control antibody. Complexes were precipitated using Protein A/G PLUS–agarose beads, washed and lysed in 20 μl of 3× SDS sample buffer. Anti-GFP antibody complexed with agarose beads were used to immunoprecipitate the GFP-tagged proteins. The samples were resolved by SDS/PAGE (10% gel) and subjected to Western blot analysis, as described previously [32].

**GST pull-down assays**

Cultures of *E. coli* BL-21 DE-3 cells expressing GST or GST–Sug1 were induced by 1 mM IPTG (isopropyl β-D-thiogalactoside) for 4 h at 37°C. Cells were lysed by addition of ice-cold PBS containing 1 mM PMSF and protease inhibitors (Roche Biochemicals) and sonicated. To this, 1% Triton X-100 was added for 30 min at 4°C for solubilization and then centrifuged out at 10000 g, 10 min, 4°C) to remove insoluble materials. To the supernatant, glutathione–agarose beads (50% slurry) were added and incubated with end-to-end shaking on a Roto-Torque at 4°C for 1 h. The beads were pelleted (3000 g, 2 min, 4°C), washed with PBS containing 0.1% Triton X-100 and incubated for 6–8 h with lysates from HEK-293 cells that had been transiently transfected with the indicated plasmids. The bound proteins were eluted by boiling in 3× SDS sample buffer and subjected to immunoblotting.
RESULTS

Characterization of Ac-Ipaf-induced cell death in A549 cells

The pro-apoptotic activity of Ipaf is known to be negatively regulated by its C-terminal LRR domain, but the mechanism of this intramolecular regulation is not known. Our previous study showed that Ipaf is induced in the lung carcinoma cell line A549 upon p53 expression or upon treatment with doxorubicin [13]. We therefore investigated the consequence of enhancing Ipaf protein levels through exogenous expression on survival of these cells. GFP-fusion proteins for full-length Ipaf and the LRR-domain-deleted form (Ac-Ipaf) (Figure 1A) were transiently expressed for 24 h, and A549 cells were examined for the localization pattern and morphological changes. Full-length Ipaf showed diffuse cytoplasmic localization, whereas Ac-Ipaf was present predominantly in cytoplasmic aggregates (Figure 1B). These structures were of irregular size and shape. Several cells showed the presence of Ac-Ipaf in a single large spherical structure of 2–5 μm in diameter, localizing in the juxtanuclear region. The observation of morphological changes in cells after staining the DNA with DAPI showed that Ac-Ipaf induced cell death in 33.8 ± 6.21 % of expressing cells, whereas full-length Ipaf induced cell death in only 5.44 ± 0.13 % cells, as determined by nuclear fragmentation, chromatin condensation, cytoplasmic shrinkage and membrane blebbing (Figures 1B and 1C). The difference in localization pattern or in the extent of cell death induced was not due to a difference in the expression level of the two proteins, as determined by Western blotting (Figure 1C). Similar localization patterns and phenotypic changes were observed upon expression of T7-tagged constructs (results not shown).

To determine which initiator caspases are engaged in Ac-Ipaf-induced cell death, dominant-negative mutants of caspase 1 (mCaspase 1, a catalytically inactive mutant) and caspase 8 (mCaspase 8, a catalytically inactive mutant) were co-transfected with GFP–Ac-Ipaf into A549 cells. Interestingly, mCaspase 1 had no inhibitory effect on Ac-Ipaf-induced cell death, whereas mCaspase 8 showed a 70 % inhibition (Figure 1D, upper panel). The expression level of the various constructs transiently transfected in A549 cells was confirmed by Western blotting (Figure 1D, lower panel). These results suggested that, in A549 cells, Ac-Ipaf-induced cell death is dependent on caspase 8 and is independent of caspase 1. Activation of caspase 8 was tested in A549 cells infected with adenoviruses expressing GFP–Ipaf and GFP–Ac-Ipaf. Caspase 8 activation was seen in cells expressing GFP–Ac-Ipaf, whereas no significant activation was observed in the case of GFP–Ipaf, as determined by Western blotting with an antibody which recognizes the cleaved form of caspase 8 (Figure 1E). A decrease in the level of PARP, a substrate of caspase 3, was observed in a time-dependent manner in cells expressing Ac-Ipaf. Caspase 8 activation, assayed using a synthetic peptide-based substrate, showed a 6.16-fold increase in caspase-8-like activity in Ac-Ipaf-expressing cells compared with control (Figure 1F). Activation of caspase 8 and caspase 3 in Ac-Ipaf-expressing cells was also confirmed by indirect immunofluorescence (results not shown). It has been documented that use of peptide substrates does not enable determination of the activation of specific caspases in the cellular context [34]. Since the presence of cleaved caspase 8 does not always correspond with its activation [7], we monitored the levels of HDAC7, a cellular protein described as a substrate of caspase 8 [35], in Ac-Ipaf-expressing cells. We observed a significant reduction in HDAC7 levels upon Ac-Ipaf expression, which was abrogated by co-expressing mCaspase 8 (Figure 1G).

To rule out the possibility that activation of caspase 8 was not specific to A549 cells, we examined the consequence of Ipaf and Ac-Ipaf expression in differentiated THP-1 cells, which express both caspase 1 and caspase 8. THP-1 cells were differentiated with PMA for 48 h prior to the addition of GFP–, GFP–Ipaf- or GFP–Ac-Ipaf-expressing adenoviruses for 18 h. Lysates were tested for expression of caspases and IL-1β (Figure 2A). Compared with GFP– or GFP–Ipaf-expressing cells, GFP–Ac-Ipaf-expressing cells showed significant caspase 8 activation. Activation of caspase 1 was also seen, as detected by the presence of cleaved IL-1β. Expression levels of caspase 1 or pro-IL-1β remained unchanged. A reduced level of another caspase-8-specific substrate, RIP [36], was also observed upon Ac-Ipaf expression in these cells (Figure 2B). To rule out the possibility of caspase 8 and its targets HDAC7 and RIP being cleaved by caspase 3, MCF-7 cells (which lack functional caspase 3) were infected with the control, Ipaf- or Ac-Ipaf-expressing adenoviruses. Figure 2(C) shows the presence of cleaved caspase 8 and a corresponding reduction in levels of HDAC7 and RIP only in Ac-Ipaf-expressing cells. These results show that Ac-Ipaf expression can cause caspase 8 cleavage in the absence of caspase 3. We tested the requirement of caspase 3 in Ac-Ipaf-induced cell death using MCF-7 cells and a MCF-7 stable clone that expresses caspase 3 (P2-Yama) [25]. Ac-Ipaf expression did not result in cell death in a neomycin-resistant control clone of MCF-7 cells, but did so in P2-Yama cells (Figure 2D).

Negative regulation of Ipaf by the LRR domain

In order to determine how the the LRR domain negatively regulates Ipaf activity, the effect of co-expressing the LRR domain on Ac-Ipaf-induced cell death was analysed in A549 cells. Co-expression of LRR inhibited Ac-Ipaf-induced cell death by 60 % (Figure 3A). This reduction in cell death was not due to the effect of LRR expression on Ac-Ipaf protein expression levels (Figure 3B). The LRR domain also inhibited the activation of caspase 8 by Ac-Ipaf. This suggests that the independent LRR domain of Ipaf acts as a negative regulator to inhibit the cell-death-inducing function of Ac-Ipaf.

We further examined whether the LRR domain acts as a negative regulator by interacting with other domains in Ac-Ipaf. A plasmid expressing the N-terminally Myc-tagged LRR domain was co-transfected with GFP–Ac-Ipaf or a deletion construct with aa 91–253 in HEK-293 cells, and lysates were subjected to immunoprecipitation with anti-Myc and control antibodies. Ac-Ipaf (Figure 3C), as well as aa 91–253 of Ipaf (Figure 3D), were detected in the anti-Myc antibody immunoprecipitates, but not in control immunoprecipitates, suggesting that the LRR interacts with other domains in Ac-Ipaf. The region between aa 91–253 is sufficient for interaction with the LRR domain. Therefore intramolecular interaction between the LRR domain and the region at aa 91–253 might be responsible for regulation of Ipaf.

Identification of Sug1 as an Ipaf-interacting protein

To identify novel Ipaf-interacting proteins, a Gal4-based yeast two-hybrid screen of a human placental cDNA library was performed, using 1–561 aa (Ac-Ipaf) residues of Ipaf as bait. One of the positive clones contained a cDNA fragment encoding Sug1, a component of the 19S regulatory complex of 26S proteasome (Figure 4A). The cDNA clone obtained through screening encoded the full-length Sug1 protein (1–406 aa). To map the domain requirement of Ipaf for interaction with Sug1, we made various deletion constructs of Ipaf and tested their interaction with Sug1 in
yeast (Figure 4B). Sug1 interacted only with Ac-Ipaf and not with the independent CARD, NBD and LRR domains (Figure 4C). This deletion analysis revealed that the Sug1-binding site is present within the 90–561 aa region of Ipaf, and aa 90–160 play a crucial role in this interaction. We also tested the domain requirements of Sug1 for an interaction with Ac-Ipaf. mSug1 [Sug1(K196M)], which lacks ATPase activity, did not show any interaction with Ac-Ipaf, suggesting that a functional ATP-binding site is crucial for interaction (Figures 4D and 4E). Furthermore, C-terminal deletion constructs, 1–173 aa and 1–315 aa, did not show any interaction, suggesting the involvement of both the C-terminal domain and ATPase site for interaction with Ac-Ipaf (Figure 4E).

The interaction of Sug1 with Ipaf and Ac-Ipaf was examined in mammalian cells. cDNAs expressing GFP-Ipaf and GFP-Ac-Ipaf 
Figure 2  Ac-Ipaf-induced caspase 8 activation is not specific to A549 cells

(A) Ac-Ipaf expression results in activation of caspase 8 in differentiated THP-1 cells. THP-1 cells were differentiated into a macrophage-like phenotype by the addition of 20 ng/ml PMA (TPA) for 48 h and infected with adenoviruses (AdV) expressing GFP, GFP–Ipaf or GFP–Ac-Ipaf for 18 h. Lysates were subjected to Western blotting to detect pro-caspase 1, IL-1β, cleaved (Cl) caspase 8 (Casp-8), GFP and tubulin (loading control). The presence of cleaved caspase 8 (an indicator of caspase 8 activation) is significantly increased only in Ac-Ipaf-expressing cells. UT, untransfected. (B) Ac-Ipaf expression results in cleavage of RIP, a caspase 8 substrate, in differentiated THP-1 cells. THP-1 cells were differentiated as described in (A) and lysates were subjected to Western blotting to detect RIP, GFP and tubulin (loading control). (C) Ac-Ipaf activates caspase 8 independently of caspase 3. MCF-7 cells were infected with adenoviruses expressing GFP, GFP–Ipaf or GFP–Ac-Ipaf for the indicated times. Western blot analysis for GFP, cleaved caspase 8, RIP, HDAC7 and Cdk2 (loading control) is shown. (D) Ac-Ipaf-induced cell death is caspase 3 dependent. Quantitative representation of the extent of cell death induced by Ipaf/Ac-Ipaf in neomycin-resistant control MCF-7 cells (NrMCF7) and the caspase-3-expressing stable clone P2 Yama. Western blot for caspase 3 (Casp-3) expression in the MCF-7 parent cell line and stable clones is shown. Results are means ± S.D.; **P < 0.01. Cyto., cytoplasmic.
Interaction with Sug1 regulates Ipaf

Figure 3  LRR domain acts as an auto-inhibitory domain through intramolecular interaction

(A) The LRR domain of Ipaf negatively regulates the cell-death-inducing function of Ipaf. A549 cells were transfected with the indicated plasmids and the extent of cell death determined in antigen-expressing and non-expressing cells after 24 h. Results are means ± S.D.; * P < 0.05. (B) A549 cells were transfected with the indicated plasmids as in (A) and protein expression from various constructs and activation of caspase 8 was determined by Western blotting. Cdk2 was used as a loading control. The lysate of cells treated with TNF+CHX (cycloheximide) was used as a positive control for activated caspase 8 (Casp-8). Cl, cleaved; UT, untransfected. (C) A549 cells transiently expressing GFP–Ac-Ipaf and Myc–LRR were immunoprecipitated (IP) with control rabbit IgG or anti-Myc polyclonal antibody. The immunoprecipitates were subjected to Western blotting (WB) to detect Ac-Ipaf and LRR. WCL, whole-cell lysate. (D) HEK-293 cells transiently expressing GFP–Ipaf (91–253 aa) and Myc–LRR were immunoprecipitated with control mouse IgG or anti-Myc monoclonal antibody. The immunoprecipitates were subjected to Western blotting to detect Ipaf and LRR.

Figure 4  Identification of Sug1 as an interacting partner of Ac-Ipaf

(A) The PJ69-4A yeast strain was co-transformed with Ac-Ipaf Gal4-DNA-binding-domain-fusion construct and hSug1 (human Sug1) Gal4-activation-domain-fusion construct obtained in the yeast two-hybrid screen. Transformants were grown on plates with (Ade+) or without (Ade−) adenine. (B) Representation of Ipaf and Ac-Ipaf and various deletion constructs cloned into the yeast vector pGBK7-T7. (C) Interaction of Ipaf constructs with hSug1. The yeast strain PJ69-4A was co-transformed with pACT2 hSug1 or empty pACT2 and various deletion constructs of Ipaf. Transformants were grown on plates with (Ade+) or without (Ade−) adenine. (D) Representation of wild-type Sug1 and various mutants cloned into the yeast vector pACT2. (E) Interaction of Sug1 deletion constructs with Ac-Ipaf. hSug1 cDNA fragments cloned in the pACT2 vector were transformed into the yeast strain PJ69-4A with pGBK7-T7 vector containing Ac-Ipaf (1–561 aa) or empty pGBK7-T7. Transformants were grown on plates with (Ade+) or without (Ade−) adenine.
mutant caspase 8 resulted in a significant reduction in the number of cells undergoing cell death relative to cells that did not express mCaspase 8 (Figure 6C). These results suggest that the Ipaf–Sug1 interaction induces caspase-8-dependent cell death.

As Sug1 enhanced Ac-Ipaf-induced cell death, we wished to determine whether cellular Sug1 was a mediator of Ac-Ipaf-induced cell death. The K196M mutant of Sug1 (mSug1) has been demonstrated to eliminate its ATPase activity and this mutant functions as a dominant-negative in the proteasome-dependent degradation pathway [21]. Upon expression in A549 cells, mSug1 localized throughout the cell, with predominantly nuclear staining. Its expression did not affect cell survival. When co-expressed at a ratio of 1:2 (to ensure maximum co-expression), mSug1 inhibited GFP–Ac-Ipaf-induced cell death by 70%, indicating that endogenous Sug1 is an effector of cell death (Figure 6D). Expression of shRNA targeting Sug1 was also used to determine a role for Sug1 in Ac-Ipaf-induced cell death. Relative to shRNA of an unrelated sequence (C-Sh), Sug1 shRNA (Sh A) reduced the co-expressed Myc–Sug1 protein by approx. 80% (Figure 6E). Co-expression of Sh A at a 1:3 ratio inhibited Ac-Ipaf-induced cell death in A549 cells by 55% compared with C-Sh (Figure 6F). C-Sh had no effect on Ac-Ipaf-induced cell death and aggregate formation. Expression of these shRNAs had no inhibitory effect on the level of Ac-Ipaf or another protein, syntaxin-17 that was used as a transfection control (TEC) (Figure 6G). Down-regulation of Sug1 reduced Ac-Ipaf-induced aggregate formation (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/427/bj4270091add.htm), indicating that endogenous Sug1 enables formation of aggregates by Ac-Ipaf.

**Caspase 8 forms a complex with Ipaf and Sug1**

Since caspase 8 was activated upon co-expression of Ipaf and Sug1, we examined the presence of caspase 8 in cellular complexes containing Ipaf and Sug1. To prevent caspase-8-induced cell death, mCaspase 8 and Ipaf were expressed in cells with or without Sug1. In the caspase 8 immunoprecipitates, Ipaf, as well as Sug1, were observed, suggesting that caspase 8, Ipaf and Sug1 interact within cells to form multimolecular complexes (Figure 7A). The overexpression of Sug1 promoted this complex formation. It was also observed that in cells co-expressing Ipaf and Sug1, mCaspase 8 was recruited into the cytoplasmic aggregates containing Ipaf and Sug1. When expressed alone, these proteins showed diffuse cytoplasmic staining (Figure 7B). We observed the presence of active caspase 8 in cells positive for both Ipaf and Sug1 that showed the morphological features of cell death (Figure 7C).

**Ipaf-mediated cell death is dependent on ubiquitination**

Since ubiquitination is the primary modification for regulating proteasomal degradation, we examined whether the UPS (Ub–proteasome system) regulates Ipaf. Enhanced endogenous Ub signals were seen in cells expressing Ac-Ipaf compared with those expressing full-length Ipaf (see Supplementary Figure S3A at http://www.BiochemJ.org/bj/427/bj4270091add.htm). Modification of Ac-Ipaf by Ub was tested directly by subjecting Ac-Ipaf immunoprecipitates to immunoblotting using an anti-Ub antibody. Ub signals were seen in cells expressing Ac-Ipaf compared with those expressing full-length Ipaf (see Supplementary Figure S3A). Ub signals were observed on Ac-Ipaf, which increased in the presence of MG132 (Figure 8A). In the presence of MG132, Ipaf immunoprecipitates showed very weak Ub reactivity (see Supplementary Figure S3B). We studied the effect of blocking Ub chain formation for Ac-Ipaf-induced cell death, by co-expressing the ubiquitination mutants of Ac-Ipaf, K48R and K63R [37]. Expression of the K48R mutant resulted in significant reduction in cell death induced by Ac-Ipaf (Figure 8C), suggesting that its ability to induce cell death is dependent upon formation of multiple Ub chains at Lys-48. The K63R mutant did not show significant reduction. Expression of these mutants had no inhibitory effect on Ac-Ipaf levels (Figure 8C).

The main function of the proteasome is ubiquitination-dependent degradation of proteins. Since Sug1, a component of proteasome, interacts with Ipaf, we examined the requirement...
Interaction with Sug1 regulates Ipaf

Figure 6  Sug1 co-operates with Ipaf to induce caspase 8 activation and cell death

(A) Quantitative representation of the extent of cell death induced by co-expressing Sug1 with Ipaf or Ac-Ipaf. A549 cells were transfected with the indicated expression constructs in a ratio of 1:1 for 24 h and the extent of cell death quantified in Sug1 and Ipaf/Ac-Ipaf co-expressing and non-expressing cells. Cyto., cytoplasmic. (B) Co-expression of Sug1 with Ipaf activates caspase 8. A549 cells were transfected with indicated plasmids and Western blot analysis performed for cleaved caspase 8 (Cl-Casp8), Ipaf, Sug1 and Cdk2. CHX, cycloheximide. (C) Effect of mCaspase 8 on Ipaf- and Sug1-induced cell death. A549 cells were transfected with Ipaf and Sug1 with or without mCaspase 8 and analysed for cell death after 24 h. (D) Effect of mSug1 on Ac-Ipaf-induced cell death. A549 cells expressing HA–mSug1 and GFP–Ac-Ipaf for 24 h were analysed for cell death. Lower panel: protein level of various constructs determined by Western blotting. Cdk2 was used as a loading control. (E) Efficacy of shRNA directed against Sug1. A549 cells were transfected with Myc–Sug1 along with Sug1 shRNA expressing vector (Sh A) or control vector (C-Sh) at a 1:3 ratio for 30 h and subjected to Western blotting for Sug1 (anti-Myc antibody) and Cdk2. UT, untransfected. 1 and 0.21, extent of Sug1 knockdown upon Sh A expression relative to control RNAi (RNA interference) expression as determined by densitometric analysis. (F) shRNA directed against Sug1 compromises Ac-Ipaf-induced cell death in A549 cells. A549 cells were transfected with GFP–Ac-Ipaf, along with pcDNA3, Sug1 shRNA (Sh A) or a control shRNA (C-Sh) expressing vectors in a ratio of 1:3, and after 30 h the extent of cell death was determined in GFP–Ac-Ipaf-expressing and non-expressing cells. (G) A549 cells were transfected with the indicated plasmids as in (E), and protein levels were determined by Western blotting. Syntaxin-17 expression plasmid was included as a control for the tranfection efficiency and specificity; TEC, transfection efficiency control; * P < 0.05; ** P < 0.01.

of proteasome-mediated degradation for Ipaf–Sug1-induced cell death. MG132 is an inhibitor of protease activity of proteasome and it does not inhibit ubiquitination. MG132 treatment inhibited cell death induced by expression of Sug1, along with full-length Ipaf, suggesting that Sug1 potentiates cell death through proteasome-mediated degradation (Figure 8D). MG132 treatment of cells also inhibited Ac-Ipaf-induced cell death. Since caspase 8 is activated during cell death induced by Ac-Ipaf, we tested whether inhibition of the proteasome affected caspase 8 activation. Treatment of cells with MG132 decreased caspase 8 activation by 80%, as detected by Western blotting using cleaved caspase 8 antibody (Figure 8E). Treatment of Ac-Ipaf-expressing cells with MG132 resulted in polypeptides showing slower mobility, possibly corresponding with ubiquitinated forms. These results suggest that during Ac-Ipaf-induced cell death, the UPS functions upstream of caspase 8.

The role of ubiquitination has also been tested by tagging Ub molecules to proteins. [9]. In order to test whether ubiquitination of Ipaf is required for its activation to cause cell death, we generated C-terminally fused GFP–Ipaf–Ub1, –Ub2 and –Ub4 constructs and tested their localization pattern and effect on cell survival in A549 cells at 30 h post-transfection (Figure 9A). Interestingly, GFP–Ipaf–Ub2 and GFP–Ipaf–Ub4 showed a localization pattern similar to that of Ac-Ipaf, i.e. cytoplasmic aggregates, whereas GFP–Ipaf–Ub1 showed an expression pattern similar to GFP–Ipaf (Figure 9B). Both GFP–Ipaf–Ub2 and GFP–Ipaf–Ub4 showed significant induction of cell death and involved activation of caspase 8.
Figure 7 Sug1 enables complex formation of Ipaf with caspase 8

(A) Ipaf and mCaspase 8 (mC8) plasmids were transiently expressed with or without the Sug1 construct in A549 cells for 30 h and lysates immunoprecipitated (IP) with control rabbit IgG or anti-caspase 8 (Casp-8) polyclonal antibody. The immunoprecipitates were subjected to Western blotting (WB) to detect Sug1, Ipaf and caspase 8. WCL, whole-cell lysate. (B) mCaspase 8 (mCasp-8) is sequestered in structures formed by Ipaf and Sug1. A549 cells were transfected with GFP–Ipaf, RFP–Sug1 and mCaspase 8 in the ratio of 1:1:4, and immunostained using anti-caspase 8 p20 rabbit polyclonal antibody and analysed by confocal microscopy. Lower panels: pattern of expression of Ipaf, Sug1 and mCaspase 8 when expressed alone. (C) Activation of caspase 8 in Ipaf-Sug1-expressing cells. A549 cells transfected for 24 h as indicated, were fixed and immunostained with antibodies that specifically recognize the cleaved form (Cl) of caspase 8 (Casp-8) and analysed by confocal microscopy. White areas in merged image indicate co-localization. Scale bars, 10 μm.

(Figures 9C and 9D, and see Supplementary Figure S4 at http://www.BiochemJ.org/bj/427/bj4270091add.htm). GFP–Ipaf–Ub2 showed a better interaction with caspase 8 (Figure 9E), indicating that ubiquitination aids complex formation with caspase 8. These results therefore suggest that ubiquitination of Ipaf regulates its cell-death-inducing function.

Cell death mediated by p53 is dependent on Sug1

Previous studies from our laboratory have shown that Ipaf is one of the transcriptional targets of p53 and plays a role in p53-mediated cell death [13]. It has also been shown that p53 physically interacts with Sug1 in vitro and in vivo [20]. We therefore tested the effect of mSug1 on p53-mediated cell death in A549 cells. Co-transfection of mSug1 with p53 resulted in a 73% inhibition of p53-induced cell death, suggesting a role for Sug1 as one of the mediators of p53-induced cell death (Figure 10A). The expression of mSug1 did not significantly affect p53 expression (Figure 10B). The expression of mSug1 also protected against cell death induced by doxorubicin, a DNA damaging agent, which is known to induce p53 and Ipaf (Figure 10C). In response to doxorubicin treatment we observed an enhancement in Ipaf protein levels, but no change in the Sug1 protein level. Increase in Ipaf protein corresponded with an increase in caspase 8 activation, as seen by increased levels of cleaved caspase 8 (Figure 10D). Previously, it has been shown that apoptosis induced by TNF-α (tumour necrosis factor-α) requires caspase 8 activation and proteasome function [37a]. We found that MG132 treatment inhibited TNF-α-induced cell death almost completely (Figure 10E). TNF-α-induced cell death was also inhibited by mSug1, suggesting that TNF-α also engages Sug1 to activate cell death pathways (Figure 10F).

DISCUSSION

Ipaf is an adaptor protein involved in the activation of caspase 1 through the formation of the inflammasome. It is known to interact directly with caspase 1 and not with any other caspase [10,11]. However, in the present study, we have provided evidence that Ipaf interacts with Sug1 and this interaction leads to activation of caspase-8-dependent cell death. Our results suggest that the activation of caspase 8 by Sug1 involves ubiquitination of Ipaf and the ubiquitination-dependent recruitment of caspase 8 in a molecular complex containing Ipaf. This is supported by the observation that tagging two or more Ub molecules to Ipaf enhances its interaction with caspase 8, leading to its activation. Ubiquitination of Ipaf also causes its aggregation and these aggregates contain caspase 8. Aggregation of caspase 8 is believed to be a requirement for its full activation and autocatalytic processing [9]. How ubiquitination of Ipaf results in recruitment of caspase 8 is yet to be determined. Since Ipaf does not interact directly with caspase 8 [10], an adaptor or intermediary protein which binds to ubiquitinated Ipaf and caspase 8 would be required for the formation of a complex between ubiquitinated Ipaf and caspase 8. Such a protein is yet to be identified.

Deletion analysis showed that, in addition to the NBD, sequences between the CARD and NBD (i.e. aa 90–160) of Ipaf are required for the interaction with Sug1. The CARD, NBD or LRR domain by themselves could not interact with Sug1. It has been postulated that the LRR domain serves as a recognition motif for bacterial components, leading to Ipaf activation, and that LRR-mediated interactions are responsible for Ipaf-mediated caspase 1 activation [11,19]. Our results, showing that Sug1 interacts with sequences other than the LRR domain, provide...
Evidence for an alternative and so-far-undescribed means of Ipaf regulation, which leads to caspase 8 activation and cell death. Catalytic activity, as well as C-terminal sequences of Sug1, is required for interaction with Ipaf. Knockdown of Sug1 or expression of the K196M mutant had an inhibitory effect on Ipaf-mediated cell death. Sug1 is also required for cell death induced by p53 expression or treatment with doxorubicin or TNF-α, indicating its role in many signalling pathways leading to cell death.

The intramolecular mechanism by which Ipaf is retained in an inactive state dependent on its LRR domain was not known. The present study identified the ability of the LRR domain to interact with a previously undefined region in Ipaf, i.e. aa 91–253, which comprise the region between the CARD and NBD. Overexpression of the LRR domain inhibited Ac-Ipaf-induced cell death. Although the LRR domain interacts with the same region of Ipaf that binds to Sug1, it does not seem to abolish binding of Sug1 to Ipaf in mammalian cells. Therefore it is likely that the LRR domain through intramolecular interaction inhibits ubiquitination of Ipaf or alters Ipaf-mediated interactions with other cellular proteins necessary for activation of caspase 8. Levels of endogenous Sug1 appear insufficient to activate full-length Ipaf, whereas they can mediate Ac-Ipaf-induced cell death. As overexpressed Sug1 binds and activates full-length Ipaf and the overexpression of the LRR domain inhibits Ac-Ipaf-induced signalling, it may be suggested that inter- and intra-molecular interactions of Ipaf are dependent on levels of Sug1 and also Sug1-regulated modifications. It is clear that the Sug1 interaction leading to release of the negative regulation of the LRR domain may not be sufficient for full activation of Ipaf, as Ac-Ipaf, which lacks the LRR domain, is further activated by Sug1. Therefore the Sug1 interaction may function to dislodge the LRR domain and also enable ubiquitination of Ipaf, which is required for complex formation with caspase 8 that results in caspase 8 activation.

We observed that when expressed in A549 cells, full-length Ipaf distributed diffusely throughout the cytoplasm, whereas Ac-Ipaf predominantly localized to prominent juxtanuclear aggregates that showed features of aggresomes, which have functions in
Figure 9  Ub tagging of Ipaf activates it to induce cell death

(A) Representation of Ipaf and various C-terminally fused Ipaf–Ub constructs. (B) Ipaf–Ub2 and Ipaf–Ub4 show cytoplasmic aggregates. A549 cells were transfected with the indicated plasmids and, after 30 h, analysed by confocal microscopy at ×63 magnification to show details of subcellular localization. Scale bar, 10 μm. (C) A549 cells were transfected with the indicated plasmids as in (B) and quantitative analysis of cell death was carried out. Results are means ± S.D.; *P < 0.05. Cyto., cytoplasmic. (D) Poly-Ub–Ipaf expression activates caspase 8. A549 cells were transfected with the indicated plasmids as in (B) and, after 30 h, the lysates were subjected to Western blotting with the indicated antibodies. Cl-Casp-8, cleaved caspase 8. (E) Ipaf ubiquitination enhances interaction with caspase 8. HEK-293 cells transiently expressing mCaspase 8 (mCasp8), along with GFP–Ipaf or GFP–Ipaf–Ub2, were immunoprecipitated (IP) with control rabbit IgG or anti-caspase 8 (Casp8) polyclonal antibody. The immunoprecipitates were subjected to Western blotting (WB) to detect mCaspase 8 and Ipaf or Ipaf–Ub2. WCL, whole-cell lysate. (F) Scheme showing the Ipaf–Sug1-induced cell death pathway. Sug1 interaction with Ipaf removes auto-inhibition of the LRR domain and enables Ipaf ubiquitination. Ubiquitinated Ipaf recruits caspase 8, forming a multimolecular complex wherein caspase 8 is activated, resulting in cell death dependent on caspase 3.

various physiological contexts [38]. Ipaf was also localized to similar structures when co-expressed with Sug1 or when tagged with two or more Ub molecules. Blocking of the protease activity of the proteasome resulted in an increase in size of aggregates, which co-localize with Ub. Aggregation and formation of speckles might therefore be mediated by ubiquitination. It is probable that the Sug1 interaction with and activation of Ipaf enables formation of aggresomes. Cell death induced under these conditions was caspase-8-dependent, and we also observed that mCasp8 was recruited into these cytoplasmic aggregates. Activated caspase 8 was seen in these cells, indicating thereby that activation of caspase 8 (by Ipaf–Sug1-mediated interactions) occurs through multimolecular complex formation. Active caspases have previously been shown to be sequestered into spheroidal cytoplasmic inclusions during TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis [39]. It has been suggested that, although initial activation of caspase 8 occurs by dimerization, commitment to apoptosis depends on full activation and autocatalytic processing of caspase 8, which requires its further aggregation [9].

Our results showing that inhibition of proteasome-dependent degradation suppresses caspase 8 cleavage caused by expression of Ac-Ipaf is also indicative of the involvement of a downstream effector, whose degradation by the proteasome is important for caspase 8 activation. This component(s) is/are yet to be identified. We examined whether c-FLIP was a candidate, but did not find its degradation in response to Ac-Ipaf expression (results not shown). Another possibility is that the proteasome inhibitor may induce an anti-apoptotic protein, such as BAG3 (Bcl-2-associated athanogene 3), which may inhibit Ac-Ipaf-induced apoptosis and caspase 8 activation. BAG3 is known to be induced by MG132 in certain cell types [40].

Although Ipaf can directly interact with caspase 1, ASC (apoptosis-associated speck-like protein containing a CARD) is required for Ipaf-mediated caspase 1 activation in mouse models of infection by Salmonella, Shigella and Pseudomonas [17,41,42]. However, ASC-deficient mice are not defective in Ipaf-mediated cell death, indicating therefore that caspase 1 does not mediate this cell death. The majority of reports describe Ipaf as an activator of caspase 1, although Ipaf has been shown to interact with another
inflammasome component, ASC, to induce caspase-8- and not caspase-1-dependent cell death in HEK-293 cells [43]. In fact, it was hypothesized that Ipaf can engage other caspases in addition to caspase 1 [44]. Therefore it is pertinent to ask what determines the Ipaf activation of caspase 1 or caspase 8. On the basis of the present study, we conclude that Sug1 interaction and Ipaf ubiquitination enables activation of caspase 8, leading to cell death in A549 cells, which is a lung carcinoma cell line with an epithelial phenotype. These cells constitutively express caspase 8, but not caspase 1. In THP-1 cells, which express both caspase 8 and caspase 1, we observed caspase 8 activation upon Ac-Ipaf expression, indicating that caspase 8 can be engaged by Ipaf irrespective of the presence or absence of caspase 1. It is probable that in cells, which possess caspase 1 as well as caspase 8, Ipaf activates one or both of the initiator caspases, depending on the stimulus. The results of the present study provide a basis for investigating this hypothesis.

In conclusion, our results show that Ipaf interacts with Sug1 to activate caspase 8 leading to cell death. The LRR domain negatively regulates caspase 8 activation through an intramolecular interaction with Ipaf. Our results suggest a novel mechanism of caspase 8 activation leading to cell death, which involves Sug1-mediated ubiquitination of Ipaf for the assembly of the caspase 8 activation complex (Figure 9F).

**AUTHOR CONTRIBUTION**

The project was conceived by Ghanshyam Swarup and Vesegna Radha. Yatender Kumar carried out all the experiments and Vesegna Radha helped with the cell death assays. All of the authors contributed to the experimental design, data interpretation and writing of the manuscript.

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SUPPLEMENTARY ONLINE DATA
Interaction with Sug1 enables Ipaf ubiquitination leading to caspase 8 activation and cell death

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Figure S1  Ipaf localizes to cytoplasmic aggregates when co-expressed with Sug1
(A) Expression pattern of Ipaf, Ac-Ipaf and HA–Sug1 in A549 cells. (B) A549 cells co-transfected with HA–Sug1 and GFP–Ipaf or Ac-Ipaf in the ratio of 1:1 were immunostained for HA–Sug1 and analysed by confocal microscopy. Co-localization is indicated by the yellow. Scale bar, 10 μm.

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Figure S2  Requirement of endogenous Sug1 for aggregate formation by Ac-Ipaf

(A) Representative fields of A549 cells transfected GFP-Ac-Ipaf along with pcDNA3 or RFP–Sug1 shRNA-expressing vectors (Sh A)/(Sh D) or control vector (C-Sh) at a 1:3 ratio for 30 h. Cells were analysed with an Axioplan2 microscope fitted with an ApoTome (Zeiss). DAPI was used to stain the nucleus. Scale bar, 50 μm. (B) shRNA directed against Sug1 compromises Ac-Ipaf-induced aggregates in A549 cells. A549 cells were transfected with GFP–Ac-Ipaf along with pcDNA3, RFP–Sug1 shRNA (Sh A)/(Sh D) or a control RFP–shRNA (C-Sh) expressing vector in a ratio of 1:3 and after 30 h the extent of aggregate formation was quantitated in GFP–Ac-Ipaf and RFP–shRNA co-expressing cells. Con, control. (C) Efficacy of shRNA directed against Sug1. A549 cells were transfected with Myc–Sug1 along with RFP–Sug1 shRNA-expressing vectors (Sh A)/(Sh D) or control vector (C-Sh) at a 1:3 ratio for 30 h and subjected to Western blotting for Sug1 (anti-Myc antibody) and Cdk2. Values indicate the extent of Sug1 down-regulation. UT, untransfected.
Interaction with Sug1 regulates Ipaf

Figure S3  Aggregates formed by Ac-Ipaf show enhanced Ub staining

(A) A549 cells transfected with GFP–Ipaf or GFP–Ac-Ipaf for 24 h were fixed and immunostained with an anti-Ub antibody, and analysed by confocal microscopy. Scale bar, 10 μm. Bottom panels: Ac-Ipaf-expressing cells exposed only to secondary antibody as a blank. (B) MG132 treatment enhances Ub-containing aggregate formation in cells co-expressing Ipaf with Sug1. A549 cells were transfected with GFP–Ipaf and HA–Sug1 for 24 h and were treated either with DMSO or MG132 (25 μM) for 6 h. Cells were fixed and immunostained with an anti-HA antibody and an anti-Ub antibody, and analysed by confocal microscopy. Scale bar, 10 μm.
**Figure S4 Expression of poly-Ub-tagged Ipaf activates caspase 8**

Representative field of A549 cells transfected with the indicated plasmids and stained for cleaved caspase 8 after 30 h. DAPI was used to stain the nucleus. DIC (differential interference contrast) images of the corresponding fields are shown. Arrows indicate cells showing morphological features of cell death. Scale bar, 20 μm.