Supporting Online Material for

HIN-200 Proteins Regulate Caspase Activation in Response to Foreign Cytoplasmic DNA


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Corrected 20 February 2009: The corrected SOM file includes details of the anti-p202(S-19) antibody on pages 4 and 5.
Supporting online material.

METHODS

Cells and Cell Culture.

Cell lines used were RAW264.7, NIH3T3, and J774, purchased from American Type Culture Collection, and RAW264.7 stably expressing p202 with a C-terminal V5 tag. Plasmid for the latter cell line was prepared by PCR amplification of full length coding sequences of p202a and insertion into pEF6-V5/HisTOPO (Invitrogen). RAW264.7 cells were stably transfected with this plasmid. p202 is antiproliferative, and consequently can only be expressed at modest levels. Bone marrow-derived macrophages (BMM) were prepared and cultured as described from BALB/c, C57BL/6, NZB mice or TLR9−/− mice backcrossed 12 times onto C57BL/6 background. NZB mice were supplied by Kew animal house (Walter and Eliza Hall Institute, Australia). TLR9−/− mice were obtained from Shizuo Akira, Research Institute for Microbial Diseases, Osaka University. Animal work was done under approval and in accordance to guidelines from the University of Queensland Animal Ethics Committee. RAW264.7 and J774 cells were cultured in RPMI1640 medium (Invitrogen) containing 5% foetal calf serum (FCS), 2mM L-glutamine, 20U/ml penicillin, and 20µg/ml streptomycin. NIH3T3 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FCS, 2mM L-glutamine, 20U/ml penicillin, and 20µg/ml streptomycin.

Nucleic Acids. Calf thymus DNA (CT DNA) was purchased from Sigma and poly(dA) and poly(dA):(dT) were purchased from GE Healthcare and purified to remove LPS as described. pBluescript plasmid DNA was prepared using Qiagen Endo-free kits according to manufacturer’s instructions. Calf thymus or plasmid DNA was labelled with Cy3 or biotin using a labelIT kit (Mirus). 100bp and biotinylated 250bp DNA fragments used for EMSA and protein purification respectively were amplified by PCR from the β-actin gene and purified to remove LPS as described. Poly(I:C) was purchased from Invivogen. Oligonucleotides were purchased from Geneworks (Adelaide, Australia). The sequences of oligonucleotides used for activation and binding studies were:

44bp 5′-GCCTTAGGGACTAGCTCATAAGCTCTCCGTAGCTGTAGTAGG-3′;
22bp 5′-GCTCATGAGCTTCCTGATGCT-3′;

These were made ds by annealing with complementary strands, but also used in ss form in some experiments. The degree of secondary structure of the two single stranded oligonucleotides comprising the 44bp oligonucleotide was checked using the mfold structural prediction program (4). The only structure of any significance was a 4bp stem-loop (AGCT stem) which may form under physiological conditions. Some oligonucleotides were purchased with 5′ Cy3 or biotin labels.

Electroporation. Cells were electroporated with DNA in 0.25ml full medium at room temperature as described previously except at lower voltage (Bio-Rad Gene Pulser, 240V, 1000µF).

Assay of MTT Cleavage. Cleavage of the tetrazolium dye MTT was used as an indication of cell viability. MTT is cleaved by the mitochondrial enzyme succinate dehydrogenase, and the degree of MTT cleavage is a measure of mitochondrial activity. Although this is therefore a measure of both metabolic activity and cell viability, microscopic inspection of cells confirmed that DNA transfection had a profound effect on cell viability (fig. S1). MTT was assayed on cells
plated at 120,000 per well as described previously (5), except that the blue cleavage product was dissolved in 10% SDS, 50% isopropanol before measurement of absorbance at 570nm.

**Measurement of caspase activation.** Cells were lysed at 5-30 minutes post electroporation in either boiling 2% SDS, 50mM Tris pH 7.4, or 5mM PIPES, pH 6.5, 0.2mM EDTA, 0.01% CHAPS, 0.5mM DTT with complete mini-protease inhibitor cocktail (Roche Applied Science). Caspase activation was more pronounced when cells were incubated at room temperature rather than 37°C post-electroporation. Caspase activation was detected by western blotting. Samples were separated on Invitrogen 4-12% pre-cast Bis-Tris gels run with MES buffer and transferred onto PVDF membrane. Membranes were blocked in 5% skim milk powder in Tris buffered saline + 0.5% Tween 20 for more than 4 hours at 4°C. Membranes were incubated with primary antibody for 2 hours at room temperature or overnight at 4°C. Primary antibodies used were 1/2000 rabbit polyclonal anti-caspase 3 (Cell Signaling Technology), 1/2000 rabbit polyclonal anti-cleaved caspase-3 (Asp 175) (Cell Signaling Technology), 1/3000 mouse anti-caspase 1 (Calbiochem), and 1/2000 mouse anti-S6 ribosomal protein (54D2) (Cell Signaling Technology). Membranes were washed and then incubated with secondary antibody (1/2000 anti-rabbit HRP (Cell Signaling Technology) or 1/2000 anti-mouse HRP (Cell Signaling Technology)). Membranes were washed again and signal detected using ECL reagent (Amersham Biosciences) or for caspase 1 blots Immobilon Western HRP substrate (Millipore). The total caspase 3 antibody detects pro-caspase 3 (35kD) and two active cleaved forms (19 and 17kD). The cleaved caspase 3 antibody detects only cleaved forms, but relative amounts of 19 and 17kD bands detected varied with batches of antibody.

**Cytoplasmic Extracts.** BALB/c BMM were harvested and resuspended at 160 million cells/ml in cold hypotonic wash buffer (HWB- 10mM HEPES, pH 7.4, 1.5mM MgCl₂, 10mM KCl with complete protease inhibitors (Roche)), and left to swell for 5 minutes before addition of two volumes of lysis buffer (HWB + 0.1% NP-40). After 10 minutes on ice, lysate was centrifuged twice at 1000g for 5 minutes to remove nuclei; supernatant from this step is cytoplasmic extract. To obtain the cytoplasmic pellet fraction, extract was ultracentrifuged at 100,000g for 1 hour and the pellet was resuspended in HWB. The DNA binding complex observed on EMSA was not always present in extract prepared as described, perhaps being lost when there was any nuclear disruption.

**Recombinant protein production and purification.** Full-length p202 was cloned into pET-Sumo (Invitrogen) for bacterial expression. Rosetta 2 cells (Novagen) were transfected with pET-Sumo-p202, and a 1L culture of cells (in LB broth containing chloramphenicol and kanamycin) was induced with IPTG and grown at 25°C for 24hr. Cells were lysed in Buffer A (20mM Sodium Phosphate, pH 7.5, 0.5M NaCl, 10mM imidazole and EDTA-free protease inhibitors (Roche)), sonicated and insoluble protein removed by centrifugation. Tagged protein was isolated from the supernatant by binding to a Talon Cobalt column (Clontech). The column was washed in Buffer A and bound proteins eluted in Buffer A with 150mM imidazole. Recombinant protein was detected by western blot using 1:10,000 anti-His antibody (Novagen) and 1:5000 anti-mouse HRP (Cell Signaling Technology).

**Electromobility Shift Assays (EMSA).** Total cytoplasmic extract, the pellet fraction following ultracentrifugation (100,000g 1h), the supernatant following ultracentrifugation or purified recombinant protein were used in EMSA with a 32P-labelled DNA probe. Probes used were either ss or ds 44mer described above under nucleic acids. To make ds probe 5 pmoles of 44 base ss oligonucleotide was end labelled using polynucleotide kinase and 32P-ATP, then
separated from nucleotide by gel filtration on a sephadex G25 NAP column (GE Healthcare). Recovery of probe was estimated at 4.5 pmoles. This was then mixed with a 1.1-fold molar excess of the unlabelled complementary strand, to ensure that probe was all double stranded. The mixed oligonucleotides were heated to 60°C in the presence of 50 mM KCl, then annealed by cooling slowly to room temperature. Labelled probe was incubated with protein extract in Binding Buffer (20mM Hepes pH 7, 140mM KCl, 13mM NaCl, 5mM MgCl₂, 12% glycerol (v/v)) with or without unlabelled competitor DNA at room temperature (RT) for 15 minutes. Samples then underwent electrophoresis through a non-denaturing Tris-Glycine 6.5% polyacrylamide gel. Gels were dried and exposed to X-ray film. Supershifts were performed by addition of polyclonal goat anti-p202(S-19), anti-p204 or anti-vimentin antibodies (Santa Cruz) to the binding reaction.

**Affinity Purification and Mass Spectrometry.** Either 250bp biotinylated DNA derived from mouse β-actin cDNA sequence (amplified by PCR using a biotinylated primer) or biotinylated ss 44 base oligonucleotide was bound to streptavidin-sepharose beads (Amersham Biosciences). 250bp dsDNA was used, as the DNA binding protein clearly had higher affinity for longer DNA (Fig. 2C). The ss44 oligonucleotide was used to control for proteins binding to both ss and dsDNA, and also for non-specific electrostatic interactions. The amount of ss44 bound to beads was half the mass of the 250bp DNA, so that the total length of ss and dsDNA available for protein binding was the same. Beads with or without DNA were incubated with the ultracentrifuged pellet fraction of BALB/c BMM cytoplasmic extract for 1 hour at RT in glycerol-free Binding Buffer containing an additional 300mM KCl. Beads were washed in this same buffer, and bead-bound proteins were electrophoresed on 12% SDS-PAGE (Bis-tris Nu-PAGE gel, Invitrogen) and silver stained using the Silver Quest staining kit (Invitrogen). Proteins binding specifically to dsDNA were isolated from the gel and standard in-gel trypsin digestion and peptide isolation was performed. Peptides were analysed by LCMS using an HP1100 HPLC equipped with a C₁₈ RP HPLC coupled to an Applied Biosystems QTRAP linear ion trap mass spectrometer. Protein identification was carried out using Mascot.

**Biotinylated DNA pulldowns from transfected cells.** RAW264.7 stable cell lines expressing p202-V5 were examined for ability of proteins to endogenously bind to introduced dsDNA. 30 million cells were electroporated with 4µg of biotinylated-pBluescript, unlabelled pBluescript or 20µg biotinylated-ss44. Electroporations were performed in triplicate and then combined. Cells were incubated at 37°C for 30 minutes or 1 hour and then lysed in 200µl RIPA buffer (0.1% SDS, 0.5% Na DOC, 150mM NaCl, 1% NP-40 substitute (Fluka), 50mM Tris-HCl pH8). Cell debris was removed by centrifugation (14 000g, 1 minute) and cleared cell lysate incubated with streptavidin-sepharose beads for 1 hour at RT. Beads were pelleted and supernatant fraction removed and kept for analysis; Beads were then washed with RIPA buffer, and bead-bound proteins and supernatants run on SDS-PAGE and analysed by Western blot. Antibodies used were 1:1000 anti-V5 (Serotec), 1:2000 anti-mouse-HRP (Cell Signaling Technology). Cell number to volume of lysis buffer was important, as increased buffer volumes led to decreased p202 binding to DNA, potentially due to increased effective concentration of free detergent breaking up the protein:DNA complex.

**Immunofluorescence.** For microinjection, cells were plated overnight onto coverslips and then microinjected with Cy3-labelled DNA in 10mM KH₂PO₄, pH 7.2, 75mM KCl using an automated microinjection system (CompiC INJECT, AIS2; Cellbiology Trading, Hamburg, Germany). Cells were incubated at 37°C for indicated times and then fixed with either ice-cold
methanol or 4% paraformaldehyde (w/v). BMM electroporated with 2.5µg Cy3-labelled DNA were washed after electroporation, and incubated for 1 hour before harvest and treatment with DNasel in PBS for 5 minutes to reduce DNA bound to the cell surface. Cells were fixed for 30 minutes in 1% paraformaldehyde, then cytospun onto superfrost slides. Slides were fixed for a further 15 minutes with 1% paraformaldehyde, then washed. Prior to immunostaining cells were permeabilised with 0.1% TritonX-100. Mitotracker, anti-tubulin and secondary antibodies (chicken anti-goat Alexa 488 or goat anti-mouse Alexa 488) were purchased from Molecular Probes, anti-vimentin and anti-V5 from Sigma and anti-p202(S-19) and anti-p204 from Santa Cruz. Antibodies against LAMP1 and EEA1 were a gift from Rachael Murray, Institute for Molecular Bioscience. Slides were viewed using Zeiss LSM-510 META confocal microscopes at x100 magnification.

**siRNA Knockdown.** 10 million BALB/c BMM were electroporated in a total of 385µl with 620 nM Stealth siRNA duplexes (Invitrogen). Electroporation conditions were 270V, 1000µF. Sense siRNA sequences were:

- p202#1 5′-UGUCAACUGAGAUUGGUAAC-3′
- p202#2 5′-GCAAAGGUGCUGUUCUCAUGAGA-3′
- p202#3 5′-GGGAACCAUAUUACACUCUGGA-3′
- AIM2#1 5′-CCACAUCACGAGGAAGACUC-3′
- AIM2#2 5′-CCACCCGCAGUGACAAUGCUU-3′
- p204 5′-AGGUGUGAACCAGCGAGAU-3′
- TLR9 5′-GCCAGCCCCUGAUGGAAGGU-3′

Twenty-four hours post siRNA introduction, cells were challenged by electroporation with 10µg CT DNA or 1µg poly(dA):(dT). Cells were lysed after 20 minutes for analysis of caspase activation by western blot. TLR9 siRNA was considered an appropriate control, since it does not have an expressed target in BMM but is not involved in the response to cytoplasmic DNA. Cells were also harvested 24 hours post siRNA electroporation for analysis of the degree of mRNA knockdown, by real-time PCR. siRNAs against p202 and AIM2 were designed to regions of maximum dissimilarity between HIN-200 factors. The p204 siRNA is a pre-designed siRNA from Invitrogen. AIM2 is a relatively divergent HIN-200 factor and multiple different siRNAs are possible. siRNAs likely to be specific for p202 are more limited in number. The effects of p202 siRNAs on expression of other HIN-200 factors was assessed by real time PCR to ensure that observed effects of knockdown correlated only with p202 knockdown and not with effect on any other factor.

**Real time PCR analysis of mRNA levels.** RNA was isolated from cells using RNA miniprep kits (QIAGEN). cDNA was synthesised and levels of gene expression relative to HPRT were quantified by real-time PCR using the ΔCT method (6) as described previously (7). The absolute levels of expression of the different genes cannot be directly compared due to small differences in the efficiency of each round of amplification for each primer set. Assays were performed in duplicate for individual genes on a particular cDNA sample and mean and SD for cycle thresholds (C_T) for each gene determined. The difference in mean cycle threshold (ΔC_T) between the test gene and HPRT was determined. The conversion to expression values = 2^{-ΔCT} assuming a doubling of PCR product in each round of amplification. Standard deviations (SD) in the expression values were calculated by the following equations. SD_{gene} C_T^2 + SD_{HPRT} C_T^2 = Σ. Plotted SD = 2^{\sqrt{Σ + ΔCT}_{ΔC_T}}. Amplification of the single exon gene IFNβ from reactions containing no reverse transcriptase was always done as a control to ensure lack of genomic DNA contamination.
Realtime PCR primer sequences:
p202 forward 5′-GAGAAAGGAATGGGAAACC-3′
p202 reverse 5′-TCAATGCCACCACCTTTGTTG-3′
p204 forward 5′-TGGAGAACACACTTTTCAAGATATC-3′
p204 reverse 5′-ACTTGTTTGGGACCATGATGGT-3′
p210 forward 5′-GAATGGGGCTGTTTTAAAGTCCAGAAG-3′
p210 reverse 5′-CCTTCCTCGCCTTTGTTTGTC-3′
HPRT forward 5′-CAGTCCCAGCGTCGTGATTAG-3′
HPRT reverse 5′-AAACACTTTTCCAAATCTCGG-3′
Supplementary Figures

Figure S1. Transfected DNA causes death of BMM. Light microscopic examination of cells which were either untreated, electroporated in the absence of DNA, or electroporated with 10µg pBluescript DNA demonstrates the toxic effect of transfected DNA, 24 hours post-electroporation.

Figure S2. Transfected dsDNA of only 44bp is toxic to BMM at high doses. BALB/c BMM were electroporated either without DNA or with 20µg of salmon genomic DNA, the indicated doses of 44bp oligonucleotide or 200µg of the component 44 base single stranded oligonucleotides (ss44 and ss44R). MTT cleavage was assayed after 48 hours. Previous work showed that a transfected 29bp oligonucleotide had minimal cytotoxic effect (5). Results here establish that 44bp can mediate a cytotoxic effect. In addition, the requirement for dsDNA for toxicity is clearly established by the survival of most cells following transfection with either of the single stranded oligonucleotides.
Figure S3. Caspase 3 is activated by transfected dsDNA in BMM and J774 mouse macrophage cell line, but not in NIH3T3 fibroblasts or RAW264.7 mouse macrophage cell line. Consequently the activation of caspase 3 is seen in cells in which transfected DNA is highly toxic (Fig. 1)(5, 8), and not in RAW264.7 and NIH3T3 which survive the introduction of DNA and are generally considered readily transfectable (5). In many cell lines such as RAW264.7, the inhibition of cell death pathways in response to cytoplasmic DNA is likely to be the result of the viral transformation used to generate the cell line. A. RAW264.7 cells and BMM were either untreated (“no zap”) or electroporated with or without 20 µg of CT DNA. They were lysed at 10 min or 30 min post electroporation and caspase 3 activation assessed by western blot for cleaved caspase 3. S6 ribosomal protein is shown as a loading control. B. BMM, NIH3T3 and J774 were either untreated (“no zap”) or electroporated with or without 20 µg of CT DNA. They were lysed at 15 min post-electroporation and caspase 3 activation assessed by western blot for cleaved caspase 3. Total ERK MAPkinase protein is shown as an indication of protein loading.

Figure S4. Transfection of dsDNA does not induce IL-1β mRNA. BMM were electroporated with 5 µg CT DNA or treated with 10 ng/ml lipopolysaccharide (LPS) from Salmonella minnesota Re595 and harvested for RNA extraction at the indicated times. The relative level of IL-1β mRNA to HPRT mRNA was assessed by real time PCR. Bars show mean and SD as described in materials and methods.
Figure S5. p202 is a dsDNA-specific binding factor found in the ultracentrifuge pellet of BMM cytoplasmic extract. A. Ultracentrifugation of BMM cytoplasmic extract reveals a discrete DNA-binding factor. Cytoplasmic extract was prepared from BALB/c BMM. When this was assessed for binding to a 44bp DNA probe in EMSA as per Figure 2A, a high MW complex was formed, which was retained in the well of the gel, with some evidence for formation of a smaller complex (lane “total”). The extract was ultracentrifuged at 100,000g for 1 hour, and supernatant and pellet fractions were assessed for DNA binding. This showed a discrete migrating band in the resuspended pellet fraction. This suggests that the DNA binding factor is bound to an ultrastructure such as membrane or cytoskeleton that pellets, but upon binding DNA, the factor is released from this site and can run as a discrete complex on the gel. In the total cytoplasmic extract, other factors appear to be present which increase the size of the complex. We chose to study the pellet fraction, as it is evidently a simpler complex, amenable to antibody supershift for identification. B. The dsDNA binding protein does not bind dsRNA. Labelled 44bp probe was mixed with 10 or 100-fold μg excess of unlabelled dsDNA (CT DNA) or dsRNA (poly(I:C)) prior to addition of cytoplasmic extract pellet fraction. The effect of this competition is compared to probe alone (“no comp.” lane). C. The dsDNA binding protein binds more strongly to long DNA. The labelled 44bp probe was mixed with a 660-fold excess (on a weight basis) of unlabelled dsODN 44bp or 22bp prior to addition of cytoplasmic extract pellet fraction. The effect of this competition is compared to probe alone (“no comp.” lane). Together with Figure 2C this shows that although the p202 complex has a higher affinity for longer DNA, a vast excess of short DNA can compete for binding. D. EMSA on purified recombinant SUMO-p202 shows that full-length p202 binds specifically to dsDNA. SUMO-p202 was prepared for Figure 2G, and
contains full length protein as well as some proteolytic cleavage products. SUMO-p202 was incubated with ss or ds 44mer ³²P-labelled probes in the presence or absence of various amounts of unlabelled 44mer ds or ss competitor DNAs. The slowest migrating complex, which is likely to correspond to full length SUMO-p202, bound to ds probe but not ss probe. Binding to ds probe was competed for by unlabelled ds competitor, but only a very high concentrations of ss competitor reduced binding. Competition for SUMO-p202 by ds 44bp was less efficient than for the native protein (Fig. 2A), indicating some loss of DNA affinity by addition of the SUMO tag. Lower bands on the gel bound to both ds and ss DNA. Bands marked with asterixes were observed in some preparations of other proteins expressed in E.coli, and hence are likely to be contaminating E.coli DNA binding proteins. The other lower bands were seen uniquely in preparations of p202 protein and suggest that the truncated forms of p202 in the preparation (Fig. 2G) can bind ssDNA. This is consistent with recent work showing that a single HIN domain of the human HIN-200 protein IFI16 binds preferentially to ssDNA (9). Previous work has shown that p202 bound to both dsDNA and ssDNA- sepharose (10). However this work used commercial ssDNA sepharose which is prepared from calf thymus DNA. There is no indication of quality control on this product, and it seems unlikely that it would be possible to get completely ss calf thymus DNA due to rapid re-annealing of repeat sequences. E. Binding of control SUMO protein to ss or ds DNA probe is not detectable by EMSA.

Figure S6: p202 co-localises with microinjected dsDNA after 5 minutes. NZB BMM were microinjected with Cy3-labelled CT DNA and rapidly fixed with methanol. Cells were then stained for p202 localisation. The green in the left hand panel shows p202 localisation, the red in the centre panel shows the microinjected Cy3-labelled CT DNA and yellow or orange in the right hand panel indicates co-localisation. In contrast, the diffuse localisation of p202 in non-DNA treated cells is shown in figures S7, S8 and S9.
Figure S7: p202 co-localised with electroporated DNA. NZB BMM were electroporated with Cy3-labelled plasmid DNA, and 20 minutes later were fixed with paraformaldehyde and stained for p202 localisation and with a DAPI nuclear stain (blue). Top row: Colocalisation between p202 (green, left hand panel) and electroporated DNA (red, centre panel) can be seen from the enrichment of p202 in regions where DNA has entered the cell. Co-localisation is indicated in yellow (right hand panel). Bottom row: controls for antibody specificity and channel bleed through. In this row, red, green and blue channels are superimposed. Cy3 DNA injected cells stained with secondary antibody alone showed no signal in the green channel, i.e. no bleed through of the Cy3-DNA signal into the green channel and no background staining from the green secondary antibody. Cells stained for p202 in the absence of DNA injection ("no DNA control") showed only faint diffuse green cytoplasmic staining using the same microscope settings used in the top panel. This shows the extent to which p202 was concentrated upon the introduction of DNA in the top panels. Staining with secondary antibody alone (green) showed only weak background staining in the green channel and no bleed through into the red channel. Such controls to ensure lack of bleed through and non-specific staining with secondary antibodies were performed in all similar experiments.
Figure S8: p202 is diffuse in the cytoplasm in resting cells. NZB BMM were methanol fixed and then stained for p202 (green left hand panels) and either the early endosomal marker EEA1, tubulin, vimentin or with mitotracker for mitochondria (red central panels). Merged images (right hand panels) show no strong co-localisation with any of these markers.
Figure S9: p202 is enriched in the LAMP1–containing compartment (late endosomal/lysosomal) prior to but not after introduction of dsDNA. A: NZB BMM (top row) and RAW264.7 cells (second row) were methanol fixed and stained for p202 and LAMP1 localisation. Co-localisation

enlarged area indicated in white box
between p202 (green) and LAMP1 (red) is indicated in orange (right hand panel). p202 staining in cells not treated with DNA is diffuse and weak, but regions of the cell where LAMP1 stained strongly also showed enrichment of p202 staining (see enlarged box). However p202 is also clearly found in some sites separate from LAMP1. B: 1 hour after injection of Cy3-dsDNA (red, third panel) into NZB BMM, p202 (green, second panel) had moved away from LAMP1 (blue, first panel) and co-localised with dsDNA (co-localisation between DNA and p202 shown in yellow, right hand panel). The DNA dependent re-location of p202 is also clearly seen in figure S7.

**Figure S10.** Knockdown of p202 enhances transfected DNA-dependent caspase 3 activation. Together with Figure 4A, these results show that 3 different siRNAs targeting p202 all boost DNA-dependent caspase activation. As noted in the methods section, the selection of siRNAs specifically targeting p202 is limited by the homology amongst HIN-200 family members. p202 siRNAs were screened for effects on other HIN-200 members. p204 is structurally the most closely related HIN-200 factor to p202, being the only other factor which has both HIN-A and HIN-B domains. Consequently in Figure 4A it was used as a control to ensure that p202 siRNA effect was actually mediated through p202 knockdown. The results in Figure 4B show that the p204 siRNA had a very minor effect on p202 mRNA levels, which was insufficient to have an effect on caspase activation. p202#1 siRNA, but not p202#2, had some effect on p204 mRNA levels. The much more effective knockdown of p204 with the p204-specific siRNA had no effect on the caspase activation. Consequently, effects of the p202 siRNAs can be attributed to p202 knockdown. A. BALB/c BMM were electroporated with the indicated siRNAs and left 24 hours before electroporation with either no DNA (“cont.”), 10µg of CT DNA (“CT”), or 1µg of poly(dA):(dT) (“AT”). After 20 minutes, cells were lysed and protein extracts analysed by western blotting for cleaved caspase 3 and S6 ribosomal protein as loading control. B. Knockdown of p202 mediated by siRNAs used in Figure S10A. Cells were harvested for RNA 24 hours post electroporation with siRNA. The level of p202 was determined relative to HPRT by real time PCR.
Figure S11. The degree of caspase activation by dsDNA is dependent on the balance between p202 and AIM2. dsDNA-dependent caspase 1 activation is known to require the adapter protein ASC (11) which recruits pro-caspase 1 via CARD (caspase activation and recruitment domain) interactions. Clustering of pro-caspase 1 is required for its activation via intermolecular cleavage. AIM2 is essential for both caspase 1 and 3 activation in response to transfected dsDNA (Fig. 4C), and would recruit ASC via pyrin domain interactions. The greater degree of clustering of AIM2, and consequently caspase activation, afforded by long DNA molecules explains the observed effect of DNA length on cell death (Fig. 1D). In contrast to results with AIM2, knockdown of p202 increased caspase activation (Fig. 4A and fig. S9), showing that it antagonises AIM2. p202 has two HIN domains but importantly, no pyrin domain. High levels of p202 binding to DNA would interfere with the pyrin domain-dependent clustering and activation of caspase 1. HIN-200 proteins are known to homo- and heterodimerise (12) and AIM2 can dimerise with p202 in vitro (13). A role for such heterodimerisation in the inhibitory role of p202 in vivo is yet to be established. Caspase 1 and 3 were activated in parallel in BMM by DNA transfection. Caspase 3 has no CARD domain, and is generally activated by an initiator caspase. Caspase 1 is known to cleave caspase 7, which is another executioner caspase similar to caspase 3 (14), but there is as yet no data indicating that caspase 3 is also a substrate for caspase 1. In fact, published work views caspase 1 and 3 activation as distinct pathways initiating pyroptosis and apoptosis respectively (15). Other work on responses to transfected dsRNA has shown co-activation of caspase 1 and 3 (16).
References for Supplementary Material