Baculovirus P35 inhibits NO-induced apoptosis in activated macrophages by inhibiting cytochrome c release

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Accepted 23 January 2004
Journal of Cell Science 117, 3031-3039 Published by The Company of Biologists 2004
doi:10.1242/jcs.01121

Summary

The baculovirus protein P35 inhibits apoptosis in a diverse range of animals such as insects, nematodes and mammals. Evidence suggests that P35 can inhibit members of caspase family proteases that are key mediators of mammalian apoptosis. We demonstrate that *p35* inhibits activation-induced nitric oxide (NO)-mediated apoptosis in the RAW 264.7 mouse macrophages. Parent or vector-transfected RAW 264.7 cells underwent apoptosis when treated with a combination of cisplatin and interferon-γ (IFN-γ) or LPS and IFN-γ in a NO-dependent manner. By contrast, RAW 264.7 cells stably expressing P35 did not undergo apoptosis when treated with a combination of cisplatin and IFN-γ or LPS and IFN-γ. Activation of parent, vector- or *p35*-transfected cells with cisplatin and IFN-γ or LPS and IFN-γ caused equivalent levels of inducible nitric oxide synthase (iNOS) expression and produced equal amounts of nitrite, which ruled out attenuated iNOS activity during P35-mediated protection. Rather, expression of P35 inhibited translocation of mitochondrial cytochrome c into cytosol, mitochondrial depolarization, activation of caspase-9 and caspase-3, and cleavage of poly(ADP-ribose)polymerase (PARP). These findings indicate that P35 inhibits NO-induced apoptotic cell death of activated macrophages by inhibiting mitochondrial cytochrome c release, which suggests that P35 has targets upstream of the caspase cascade in apoptosis.

Key words: Apoptosis, Cisplatin, Cytochrome c, Nitric oxide, P35, Macrophage

Introduction

Nitric oxide (NO) produced by activated macrophages has been implicated in a diverse range of (patho) physiological processes such as cytotoxicity against tumor cells and killing of intracellular pathogens (Nathan, 1992; Moncada, 1992; Stuehr and Nathan, 1989). Macrophages can be activated to produce a large amount of NO by a variety of agents, including cisplatin, a broad-spectrum anticancer drug used for its anti-tumor activity (Son, 1995; Sodhi and Suresh, 1992). The tumoricidal effect of macrophages has been shown to be further enhanced when these cells are treated with cisplatin in combination with interferon-γ (IFN-γ) (Geetha and Sodhi, 1990). The combination of cisplatin-based therapy with an immunostimulant, referred to as chemoimmunotherapy, has shown a synergistic effect in the in vivo antitumor activity of tumor-bearing mice (Johnson et al., 1993) and in the in vitro cytotoxic action of immune cells (Pai and Sodhi, 1992). Although NO production by activated macrophages is considered to be essential for various tumoricidal and microbicidal functions, macrophages themselves are not immune to the cytotoxic effects of NO. Previously we have reported that mouse peritoneal macrophages treated with a combination of cisplatin and IFN-γ undergo NO-induced apoptotic cell death (Ranjan et al., 1998). Others have also provided similar reports on the activation-induced apoptosis of mouse macrophages (Sarih et al., 1993; Albina et al., 1993). Although various biological effects of NO are known, the molecular mechanism(s) by which NO triggers apoptotic cell death is poorly understood.

Apoptosis is a tightly regulated process of cell death that plays an important role in development, host defense and immune regulation (Borges et al., 2002). It is an important biological process for the elimination of unwanted cells such as those with potentially harmful genetic mutation, autoreactive lymphocytes or virally infected cells (Liston et al., 1997). Apoptotic events are morphologically distinguishable by cellular shrinkage, nuclear condensation, cytoplasmic vacuolization and membrane blebbing (Wyllie et al., 1980). At the biochemical level, apoptosis involves sequential activation of cysteine proteases that cleave after an Asp residue in their substrate, hence also known as caspases (Degterrev et al., 2003). In response to apoptotic stimuli, cytochrome c from the mitochondrial inner membrane space gets released into the cytosol, where it interacts with Apaf-1 to trigger autocatalytic processing of procaspase-9. Caspase-9 then activates caspase-3 and other effecter caspases, resulting in the proteolytic cleavage of substrate nuclear poly(ADP-ribose)polymerase (PARP) (Fesik, 2000). Importantly, PARP cleavage has been observed in a variety of apoptotic responses, including oxidative stress-mediated cell death (Tewari et al., 1995).

Direct evidence for the involvement of caspase family proteases in apoptosis comes from studies using the cowpox

virus protein CrmA and the baculovirus protein P35, which are direct inhibitors of at least certain members of this enzyme family (Clem and Miller, 1994). To circumvent host defense mechanisms, viruses have evolved mechanisms to antagonize host death signals so that viral propagation can continue unabated in infected cells (Oltvai and Korsmeyer, 1994). Baculovirus protein P35 has been shown to inhibit virally induced apoptosis, developmental cell death in *Caenorhabditis* and *Drosophila*, and neuronal cell death in mammals (Clem and Miller, 1994; Rabizadeh et al., 1993). Further, it has been shown to confer protection against various inducers of apoptosis including oxidative stress, TNF-α and Fas (Tewari et al., 1995). These reports suggest that P35 must interrupt a highly conserved and ubiquitous component of the death machinery. Recent findings suggest that P35 prevents cell death by inhibiting enzyme activity of several members of the caspase family proteases, including mouse caspase-1, human caspase-1 and human CPP32, by forming stable complex, thereby competitively inhibiting these proteases (dela Cruz et al., 2001). Despite the present understanding of P35-mediated inhibition of apoptosis, the precise mechanism involved therein is unclear.

In the present study, we investigated the mechanism of inhibition of NO-induced apoptosis in cisplatin and IFN-γ-, or LPS and IFN-γ-activated RAW 264.7 macrophages by baculovirus P35 and its potential targets.

Materials and Methods

Materials

RPMI 1640 medium, N^G-monomethyl L-arginine (L-NMMA), inhibitor of nitric oxide synthase, lipopolysaccharide (LPS; Escherichia coli 055:B5), and proteinase K were purchased from Sigma (St Louis, MO). Fetal Calf Serum (FCS) was purchased from Biological Industries (Haemek, Israel). [^{3}H]thymidine was purchased from Bhabha Atomic Research Center (Bombay, India). Murine recombinant IFN-γ and mouse monoclonal anti-poly(ADP-ribose)polymerase (PARP) antibody were purchased from Boehringer Mannheim (Mannheim, Germany), anti-(iNOS) and anti-(cytochrome c) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 antibody was purchased from Stratagene, CA. Sodium nitroprusside (SNP), a NO donor, was purchased from Hi Media (Bombay, India). 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) was purchased from Molecular Probes (Leiden, The Netherlands). All the reagents used were free of endotoxin contamination, as determined by Limulus amoebocyte lysate assay (sensitivity limit 0.1 ng/ml).

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 (National Tissue Culture Facility, Pune, India) as well as vector- or *p35*-expressing clones were maintained in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (20 μg/ml). Transfected clones were kept under the selection of G418 (Geneticin; Gibco) (200 μg/ml). RAW 264.7 cells/vector- or *p35*-transfected clone (1×10^6 cells/well) were cultured with or without activating agents as indicated: i.e. cisplatin (2 μg/ml) and recombinant murine IFN-γ (25 U/ml) in the wells of 6-well tissue culture plates in the presence or absence of L-NMMA (1 mM). A combination of LPS (1 μg/ml) and IFN-γ (25 U/ml) was used as standard activating agents. Macrophages were also treated directly with sodium nitroprusside, a NO donor.

Plasmid constructs and stable transfection

The *p35* cDNA was amplified by PCR on linearized baculovirus DNA from *Autographa californica* nucleopolyhedrovirus (BaculoGold, PharMingen, San Diego, CA) using the following primers (*Bam*HI restriction sites are underlined): *p35*-5′ primer, 5′-ATA <u>GGA TCC</u> CCA TAG CAA AAT GTG TGT AAT TTT TCC GGT-3′; *p35*-3′ primer, 5′-ATA <u>GGA TCC</u> TTA TTT AAT TGT GTT TAA TAT TAC ATT TTT GTT GAG-3′. The 927-bp cDNA fragment was cloned in-frame into the *Bam*HI site of the FLAG-tag mammalian expression vector, pCMV-Tag2 (Stratagene, CA).

RAW 264.7 cells were stably tranfected by electroporation with either 10 μg of a *p35*-encoding plasmid or the empty vector and selected by G418-resistance. G418-resistant cells were sub-cloned by limiting dilution. Cells were screened for the expression of the *p35* gene by RT-PCR (Fig. 1A) and P35 protein by immunoblotting using anti-FLAG antibodies. Representative clones (pCMV-FLAG/p35#3, pCMV-FLAG/p35#7 and pCMVFLAG/p35#9) or the control vector (pCMV-FLAG) are shown in Fig. 1B. The clone pCMV-FLAG/p35#3 demonstrated the strongest expression of P35 and was used in this study.

Quantitative estimation of DNA fragmentation.

Quantitative measurement of DNA fragmentation was determined as described previously (Ranjan et al., 1997). Briefly, parent RAW 264.7, vector- or *p35*-transfected clones in the logarithmic phase of growth were radio-labeled in culture medium with 0.5 μCi/ml [^{3}H]thymidine for 18 hours, washed thoroughly in RPMI 1640 and treated for various time periods using culture conditions as described. Cell-free culture supernatants (media; M) were collected at indicated time-points and preserved at 4°C. The cells were lysed with 50 μl of ice-cold lysis buffer (25 mM sodium acetate, pH 6.6) for 1 hour and DNA from cells was separated by centrifugation at 13,000 ***g*** at 4°C for 20 minutes into fragmented low molecular weight samples (supernatant; S) and intact high molecular weight (pellet; P) fractions. Radioactivity was determined by liquid scintillation counter (LKB, Wallac, Finland). Percent DNA fragmentation was calculated as:

$$\text{Percent DNA fragmentation} = \frac{\text{c.p.m. (M + S)}}{\text{c.p.m. (M + S + P)}} \times 100 \,.$$

Agarose gel electrophoresis of DNA

Extraction of cell DNA was performed as described previously (Ranjan et al., 1998). Treated or untreated cells were washed three times in PBS and lysed in 0.5 ml of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 75 mM NaCl, 10 mM EDTA, 0.5% SDS and 0.15 mg/ml proteinase K, and incubated for 3 hours at 50°C. Lysates were centrifuged at 10,000 ***g*** for 20 minutes at 4°C. The supernatant was collected carefully and a solution of 0.5 M NaCl and 50% absolute ethanol was added to precipitate the DNA. The precipitated DNA was resolublized in 30 μl of TE buffer (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0) for 1 hour at 65°C and then incubated with 200 μg/ml RNase A for 2 hours at 37°C. Thereafter, 10 μl of loading dye (0.025% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) was added and 40 μl samples were resolved in 1.5% agarose gels for 3 hours at 75 V in TBE buffer in the presence of 0.5 μg/ml ethidium bromide. DNA was visualized and photographed on a UV transilluminator.

RT-PCR

Total cellular RNA was isolated with Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. Reverse transcription was performed on the total RNA using Advantage RT-for-PCR kit (Clontech). PCR detection of *p35* mRNA was carried out by using the primers 5′-TTT TCC GGT AGA AAT CGA CG-3′ and 5′-GAA TCC ATC CAT TTG ATC GC-3′.

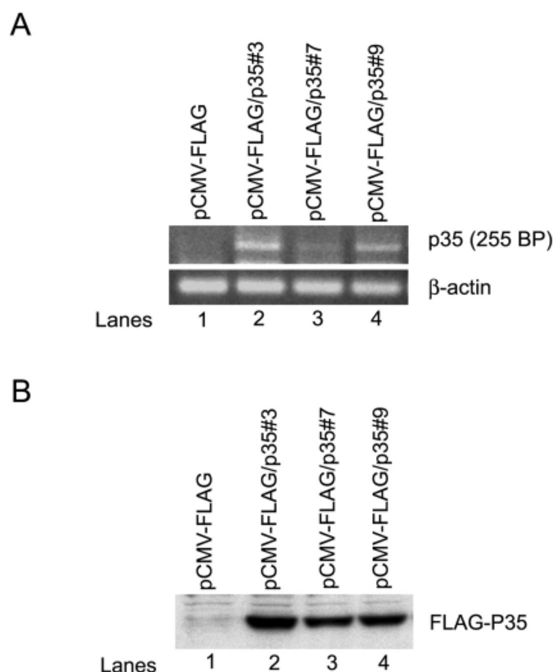

Fig. 1. Stable expression of P35 by transfected RAW 264.7 mouse macrophage cells as detected by RT-PCR of total RNA (A). Lane 1, clone pCMV-FLAG (vector control); lane 2, clone pCMV-FLAG/p35#3; lane 3, pCMV-FLAG/p35#7; lane 4, clone pCMV-FLAG/p35#9. (B) Immunoblot analysis of FLAG-P35 expression. Extracts (50 μg) from stably transfected vector or P35 RAW 264.7 cells were immunoblotted with anti-FLAG M2 antibodies to detect the expression of P35. Lane 1, clone pCMV-FLAG (vector); lane 2, clone pCMV-FLAG/p35#3; lane 3, pCMV-FLAG/p35#7; lane 4, clone pCMV-FLAG/p35#9.

Immunoblot analysis

Cells were washed with chilled PBS and then lysed in 50 μl of ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10% v/v glycerol, 1% v/v Triton X-100, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin containing aprotinin 0.15 μg/ml) for 20 minutes at 4°C. Protein content of different samples was determined by standard Bradford method. Equal quantities of solubilized protein were resolved by 12% or 15% SDS-PAGE, blotted to nitrocellulose membrane and probed with anti-PARP or other primary antibodies, and signals were detected by chemiluminescence using secondary antibodies conjugated to horseradish peroxidase and ECL detection kit.

Determination of percent apoptotic cells and cell viability

Quantitation of apoptosis was performed by counting apoptotic cells on the basis of their characteristic cellular and nuclear morphology. The percentage of apoptotic cells were determined by counting more than 200 cells for at least three separate determinations.

Relative cell viability was determined by MTT [3(4,5);dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay (Ranjan et al., 1998). Briefly, treated cells (5×10^4 cells/100 μl) were placed in each well of a 96-well tissue culture plate (Falcon, Becton Dickinson, Mountain View, CA). After 18 hours, 10 μl of 5 mg/ml MTT was added and the cells were incubated for 4 hours. The purple formazan assay product was solubilized by the addition of 100 μl of acidic isopropanol (0.04 N HCl in isopropanol) and the absorbance in each well was measured with a microplate ELISA reader using a wavelength of 570 nm. The relative cell viability was calculated according to the formula:

$$\text{Relative cell viability} = \frac{\text{Absorbance experimental}}{\text{Absorbance control}} \times 100\,.$$

Cytochrome c release

Mitochondrial and cytosolic fractions were prepared by a previously described method (Khaled et al., 1999). Cells were harvested, washed, resuspended in ice-cold buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose, homogenized with a Dounce homogenizer by using a type B (loose) pestle, and centrifuged at 1000 ***g*** for 10 minutes to separate nuclei and unbroken cells. The supernatant was then centrifuged at 10,000 ***g*** for 15 minutes to pellet heavy membranes (mitochondrial fraction). The pellet was washed three times with buffer A to eliminate contamination by other subcellular fractions. The supernatant from the 10,000 ***g*** spin fraction was further centrifuged at 100,000 ***g*** for 1 hour at 4°C to produce a supernatant corresponding to the cytosolic fraction (S100). Presence of cytochrome c was detected using α-cytochrome c antibodies.

Determination of mitochondrial transmembrane potential ($\Delta\Psi_m$)

Cells were washed and resuspended in medium containing $DiOC_6$ (20 nM) (Metivier et al., 1998). Cells were incubated for a further 30 minutes at 37°C and then analyzed by flow cytometry. As a control, the protonophore carbonyl cyanide m-chlorophenylhydrazine (20 μM) was added to the cells during equilibrium with $DiOC_6$ in order to ascertain that a decrease in $\Delta\Psi_m$ was reflected by a diminished $DiOC_6$ fluorescence. Results are expressed as the percentage of cells with depolarized mitochondria.

Caspase enzyme assay

Enzymatic assays were carried out in 96-well microtiter plates (Costar, Cambridge, MA). Treated or control cells were harvested by scraping into medium, washed in ice-cold PBS, and pellets were frozen at –80°C until analysis. Pellets were suspended in ice-cold lysis buffer obtained from Alexis (San Diego), placed on ice for 10 minutes, and then centrifuged at 13,000 ***g*** for 15 minutes at 4°C. 100 μg of protein was diluted in 50 μl of lysis buffer and added to 40 μl of reaction buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 20 mM β-mercaptoethanol). 10 μl of the appropriate caspase substrate was then added to give a final concentration of 100 μM. The plates were incubated for 3 hours in a 5% CO_2 incubator at 37°C. The substrates for each caspase were as follows: Ac-DEVD-*p*NA for caspase 3-like activity (Alexis) and Ac-LEHD-AFC (Calbiochem) for caspase 9 activity. These substrates are preferentially but not exclusively cleaved by the indicated caspases. The substrates were prepared as 1 mM stocks according to the manufacturer's instructions and stored at 20°C. Levels of released *p*-nitroanilide (*p*NA) were measured by measuring absorbance at 405 nm. Alternatively, levels of released 7-amido-4-methylcoumarin (AFC) were measured using excitation at 420 nm and emission at 517 nm.

Nitrite determination

Cell-free culture supernatants collected at different time-points as indicated, were assayed for nitrite by a microplate assay method according to Ding et al. (Ding et al., 1988). Briefly, 100 μl of culture supernatant was incubated with an equal volume of Griess reagent (one part of 1% sulphanilamide in 2.5% H_3PO_4 plus one part of 0.1% naphthyl-ethylene-diamine dihydrochloride in distilled water) at room

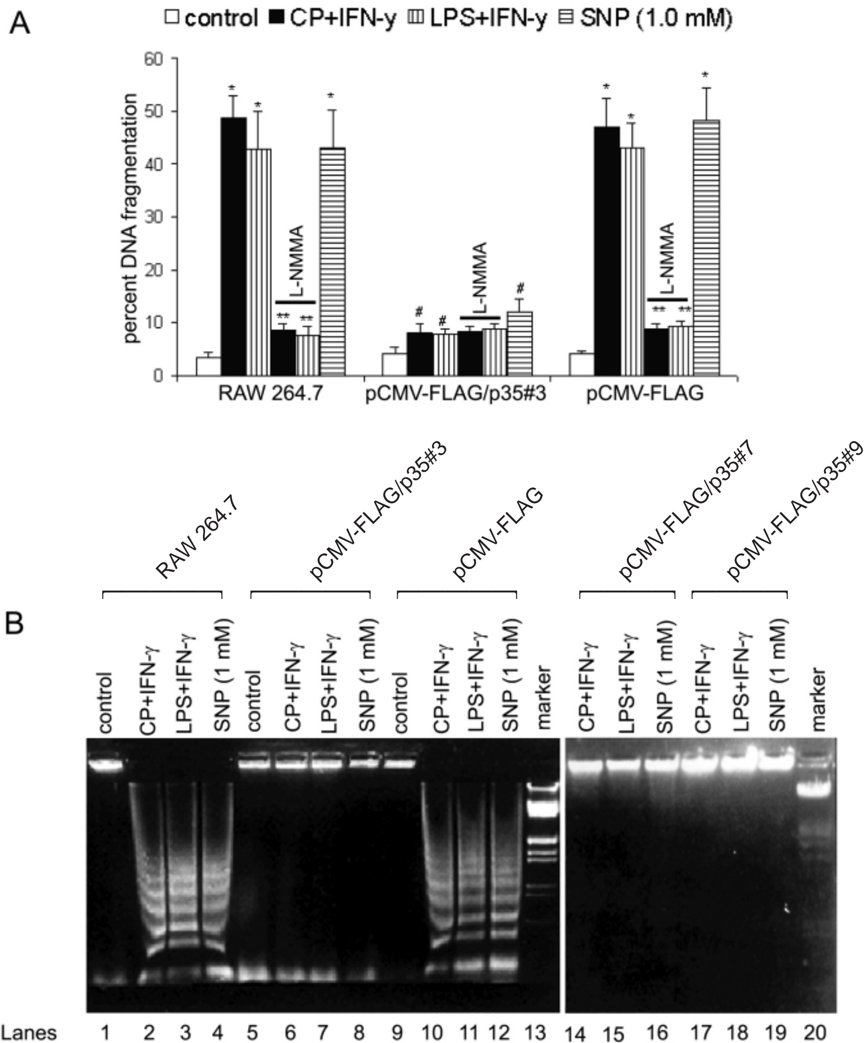

Fig. 2. P35 expression protects activated RAW 264.7 cells from undergoing NO-mediated oligonucleosomal DNA fragmentation. RAW 264.7 cells or vector- or *p35*-transfected clones (1×10^6 cells/well) in 6-well tissue culture plates were treated with cisplatin (2 μg/ml) and IFN-γ (25 U/ml) or LPS (1 μg/ml) and IFN-γ (25 U/ml) in the presence or absence of L-NMMA (1 mM) as indicated. Cells were also treated directly with NO donor, SNP (1 mM). After 36 hours of treatment, cells were assayed for DNA fragmentation using a radioisotope release assay (A) and agarose gel electrophoresis (B) as described in Materials and Methods. Data shown are mean±s.e.m. and are the representative of three independent experiments done in triplicates. *$P<0.05$ versus values of control cultures. **$P<0.05$ versus values of cisplatin and IFN-γ/LPS and IFN-γ-treated cultures. #$P<0.05$ versus values for cisplatin + IFN-γ/LPS + IFN-γ or SNP treated RAW 264.7/pCMV-FLAG cells.

temperature for 10 minutes. The absorbance was measured at 540 nm in a microtiter plate reader. Nitrite concentration as an index of NO release was quantified by using sodium nitrite as standard.

Statistical analysis

Results are expressed as means±s.e.m. of at least three independent experiments. Statistical significance of difference between test groups was analyzed by one-way ANOVA followed by Scheffe's test (post hoc). Statistical significance was defined at *$P<0.05$.

Results

Stable expression of P35 in RAW 264.7 macrophages

To study the role of P35 in attenuating NO-induced apoptosis of cisplatin and the IFN-γ-treated RAW 264.7 macrophage cell line, we transfected cells with plasmid encoding FLAG-tagged baculovirus *p35* or the empty vector. Following treatment with G418 (500 μg/ml), clones were isolated by limiting dilution and propagated. Fig. 1A shows expression of *p35* transcript in P35-transfected RAW 264.7 macrophages (lanes 2-4). Parent RAW 264.7 cells (data not shown) or cells transfected with empty vector did not express P35 (Fig. 1A, lane 1).

We also studied expression of P35 protein by immunoblotting. Fig. 1B shows the expression of FLAG-tagged P35 protein in selected clones (lanes 2-4). The clone pCMV-FLAG/p35#3 demonstrated the strongest expression of P35, and was selected for use in subsequent experiments. To document the uniformity of responses, other clones (pCMV-FLAG/p35#7 and pCMV-FLAG/p35#9) with lower levels of P35 expression were also examined.

P35 expression inhibits NO-induced apoptosis in activated macrophages

To study the role of P35 in NO-induced apoptosis of parent RAW 264.7 cells, empty vector- or *p35*-transfected clones were treated with cisplatin, IFN-γ or LPS alone, or with a combination of cisplatin and IFN-γ, or LPS and IFN-γ. Apoptosis was studied by analysis of DNA fragmentation and examination of corresponding cellular and nuclear alterations.

As shown in Fig. 2A, treatment with cisplatin and IFN-γ resulted in DNA fragmentation in RAW 264.7 cells and in cells transfected with empty vector. DNA fragmentation was detectable after 24 hours of activation (data not shown), and reached approximately 48% at 36 hours. Untreated cells or cells treated with cisplatin or IFN-γ alone did not show DNA fragmentation (data not shown). Further, to determine whether fragmentation of macrophage DNA reflected the specific pattern of oligonucleosomal DNA fragmentation found in apoptosis, DNA samples were analyzed by agarose gel electrophoresis. DNA extracted from parent cells or empty vector-transfected cells treated with cisplatin and IFN-γ showed oligonucleosomal cleavage that yielded a characteristic ladder pattern (Fig. 2B, lanes 2-3). Unlike parent or vector-transfected cells, the pCMV-FLAG/p35#3 clone expressing P35 showed no sign of DNA fragmentation even after 36 hours of treatment as assessed by both quantitative DNA fragmentation assay (Fig. 2A) and agarose gel electrophoresis (Fig. 2B, lanes 6-7).

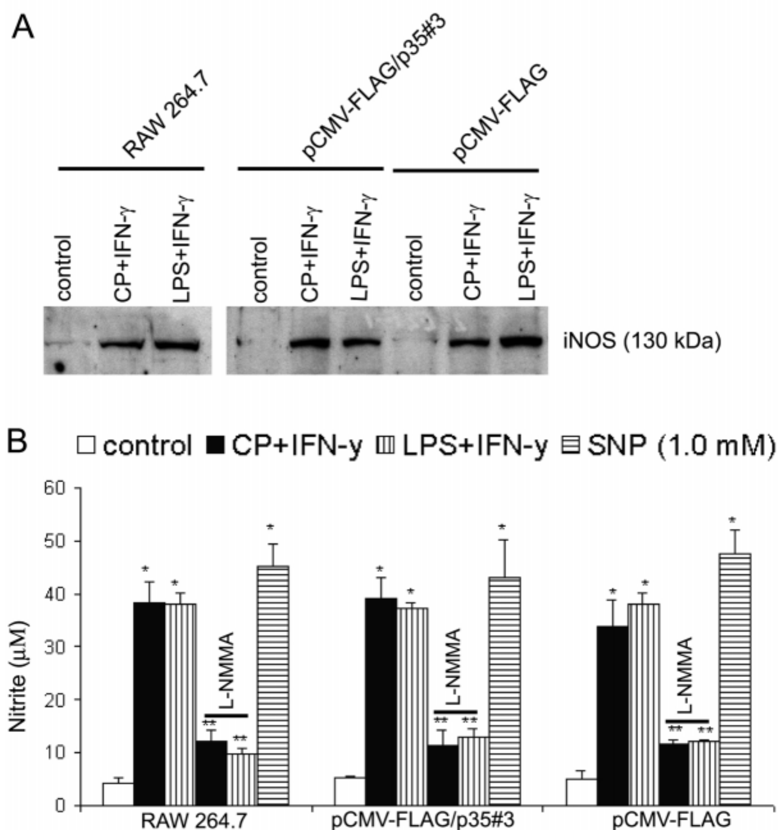

Fig. 3. P35 expression in RAW 264.7 cells does not inhibit iNOS expression and nitrite production. RAW 264.7 cells or vector- or *p35*-transfected clone (1×10^6 cells/well) in 6-well tissue culture plates were treated with cisplatin (2 μg/ml) and IFN-γ (25 U/ml), or LPS (1 μg/ml) and IFN-γ (25 U/ml) in the presence or absence of L-NMMA (1 mM) as before. Cells were also treated directly with NO donor, SNP (1 mM). After 18 hours of treatment, cell extracts were examined for α-iNOS expression by immunoblotting (A). Culture supernatants were assayed for nitrite after 18 hours of culture as described in Materials and Methods (B). Data shown are means±s.e.m. and are representative of three independent experiments done in triplicate. *$P<0.05$ versus values of control cultures. **$P<0.05$ versus values of cisplatin and IFN-γ/LPS and IFN-γ-treated cultures.

Addition of L-NMMA, a competitive inhibitor of NO synthase, to the macrophage culture treated with cisplatin and IFN-γ, or LPS and IFN-γ completely inhibited oligonucleosomal DNA fragmentation as determined by the DNA fragmentation assay or agarose gel electrophoresis (Fig. 2A and data not shown), indicating DNA fragmentation was completely NO-dependent. Confirmation that NO induces apoptosis in macrophages was obtained by treating cells directly with the NO donor, SNP. As shown by oligonucleosomal DNA fragmentation, parent cells and vector-transfected cells rapidly underwent apoptosis following SNP treatment (Fig. 2B, lane 4), whereas treatment of pCMV-FLAG/p35#3 clone with SNP cells did not induce apoptosis (Fig. 2B, lane 8). Interestingly, treatment of other clones that expressed P35 at lower levels (pCMV-FLAG/p35#7 and pCMV-FLAG/p35#9) with cisplatin and IFN-γ, LPS and IFN-γ, or SNP did not induce oligonucleosomal DNA fragmentation (Fig. 2B, lanes 14-20).

P35 expression in Raw 264.7 cells does not alter iNOS expression and nitrite/nitrate level

Activation-induced NO production depends on iNOS expression. To determine whether P35 mediates its protective effect by interfering with iNOS expression and therefore NO production, we studied the expression level of iNOS and NO production in parent, vector- or *p35*-transfected RAW 264.7 cells. iNOS expression was not detectable in untreated cells (Fig. 3A), and these cells produced a correspondingly low level of nitrite (Fig. 3B). Following treatment, iNOS expression was detectable by 12 hours, and a maximum level of nitrite concentration was detected in culture supernatant by 18 hours. Parent, vector-transfected, or *p35*-transfected (pCMV-FLAG/p35#3) RAW 264.7 cells did not show significant differences in the level of iNOS expression (Fig. 3A, lanes 1-9) or in nitrite accumulation upon treatment with cisplatin and IFN-γ, or LPS and IFN-γ (Fig. 3B). L-NMMA significantly reduced nitrite levels in these cells (Fig. 3B). Treatment with cisplatin, LPS or IFN-γ alone produced a comparatively low concentration of nitrite (data not shown), consistent with the failure of these agents to induce apoptosis in an NO-dependent manner. Nitric oxide production in other P35 clones (pCMV-FLAG/p35#7 and pCMV-FLAG/p35#9) also did not show any significant difference in nitrite accumulation pattern upon treatment (data not shown).

Percent apoptotic cells and viability

Parent, empty vector-transfected or *p35*-transfected cells were also assayed for apoptotic cells and loss of cell viability, the final outcome of apoptosis. As shown in Fig. 4A,B, untreated cells showed more than 95% viable cells, with less than 2% detected as an apoptotic cell population, even up to 36 hours of culture. Treatment of RAW 264.7 or vector-transfected cells with cisplatin and IFN-γ resulted in a time-dependent decrease in viability and the apoptotic cell population increased up to 41% of the culture at 36 hours. In contrast, *p35*-transfected clones treated with cisplatin and IFN-γ did not suffer a significant loss in cell viability, with an apoptotic cell population of less than 3%. As before, treatment of other *p35*-transfected clones (pCMV-FLAG/p35#7 and pCMV-FLAG/p35#9) with cisplatin and IFN-γ, or LPS and IFN-γ , or SNP did not result in apoptosis or loss in cell viability (data not shown).

P35 inhibited cytochrome c release and mitochondrial depolarization

Cytochrome c release from the mitochondrial intermembrane space is a central step during apoptosis and can occur through the action of NO (or its metabolites) on mitochondria or via upregulation of pro-apoptotic proteins. Treatment of parent cells or vector-transfected cells with cisplatin and IFN-γ, or LPS and IFN-γ resulted in a marked release of cytochrome c

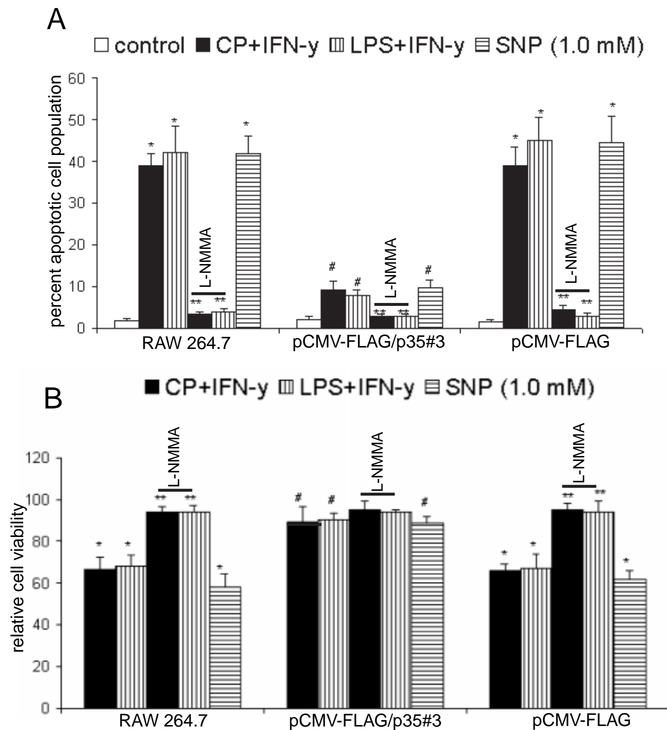

Fig. 4. P35 expression in RAW 264.7 cells inhibits apoptosis and loss of cell viability. RAW 264.7 cells or vector- or *p35*-transfected clones (1×10^6 cells/well) in 6-well tissue culture plates were treated with cisplatin (2 μg/ml) and IFN-γ (25 U/ml), or LPS (1 μg/ml) and IFN-γ (25 U/ml) in the presence or absence of L-NMMA (1 mM) as before. Cells were also treated directly with NO donor, SNP (1 mM). After 36 hours of treatment, cells were assayed for apoptosis (A) and relative cell viability (B). Data shown are mean±s.e.m. and are representative of three independent experiments done in triplicate. *$P<0.05$ versus values of control cultures. **$P<0.05$ versus values of cisplatin and IFN-γ /LPS and IFN-γ-treated cultures. $^{\#}P<0.05$ versus values for cisplatin and IFN-γ/LPS and IFN-γ or SNP-treated RAW 264.7/pCMV-FLAG cells.

from mitochondria after 18 hours of treatment (Fig. 5A, lanes 2-3). Addition of L-NMMA significantly inhibited this translocation even up to 36 hours of treatment (Fig. 5A, lanes 4-5). Direct treatment of these cells with SNP, however, resulted in increased translocation (Fig. 5A, lane 6). In contrast, no cytochrome c was detected in the cytosol of *p35*-transfected RAW cells upon treatment with cisplatin and IFN-γ or direct treatment with SNP (Fig. 5A, lanes 8-10).

Reduction of mitochondrial membrane potential ($\Delta\Psi_m$) is a consequence of cytochrome c release and commonly observed during apoptosis (Hortelano et al., 1999). To determine $\Delta\Psi_m$, we treated cells with cisplatin and IFN-γ, or LPS and IFN-γ for 24 hours and used $DiOC_6$ to access $\Delta\Psi_m$. FACS analysis revealed that the percentage of cells with depolarized mitochondria was significantly higher in parent or vector-transfected RAW cells upon cisplatin and IFN-γ or LPS and IFN-γ treatment compared with that of *p35*-transfected clones. L-NMMA significantly reduced the number of cells with depolarized mitochondria. Direct treatment of RAW or vector-transfected cells with SNP resulted in increased number of depolarized cells, but did not affect *p35*-transfected cells (Fig. 5B)

Activation of caspase-9 and caspase-3

Cytosolic cytochrome c induces oligomerization of Apaf-1 and caspase-9 in an ATP/ADP-dependent manner. In turn, caspase-9 becomes processed and subsequently activates the downstream caspase-3 (Klein and Brune, 2002). Therefore, we also investigated the caspase-9 and caspase-3 activity in parent, vector- or *p35*-transfected RAW cells. Parent or vector-transfected cells showed significant increases in caspase-9 (Fig. 5C) and caspase-3 (Fig. 5D) activity following 18 hour treatment with cisplatin and IFN-γ, or LPS and IFN-γ. L-NMMA was able to abrogate this response. *p35*-transfected pCMV-FLAG/p35#3 clone showed complete inhibition of caspase-9 and caspase-3 activity even up to 36 hours of cisplatin and IFN-γ, or LPS and IFN-γ treatment or after direct treatment with 1 mM SNP (Fig. 5C,D).

PARP cleavage

PARP is a target protein for caspase-3 that is cleaved during the apoptotic process. Treatment of parent, vector-transfected RAW cells resulted in cleavage of the 116 kDa PARP to the signature 85 kDa proteolytic fragment within 24 hour of activation with cisplatin and IFN-γ, or LPS and IFN-γ, as determined by western blot analysis (Fig. 6, lanes 2-3). Addition of L-NMMA prevented this proteolytic cleavage (data not shown). Further, direct treatment with SNP also resulted in PARP cleavage within 12 hours (data not shown). However, in the *p35*-tranfected cells, the 85-kDa cleavage product was not observed even after 36 hours of activation (Fig. 6, lanes 4-5) or direct treatment with SNP (data not shown).

Discussion

Despite recent progress in understanding the molecular mechanism(s) of program cell death, major aspects of the apoptotic pathway remain undefined. Furthermore, little is known about the death-effecter components involved in NO-induced apoptosis of macrophages. Recent evidence suggest that members of cysteine family protease family, also known as caspases, are key mediators of mammalian apoptosis (Degterev et al., 2003). These cysteine proteases are synthesized as proenzymes and require proteolytic processing to produce active, heterodimeric enzymes (Degterev et al., 2003). The baculovirus protein P35 has been shown to inhibit several caspase family proteases (Clem and Miller, 1994). The ability of P35 to act as a general apoptotic suppressor is correlated with the stoichiometric inhibition of the cellular caspases through cleavage of P35 and formation of a stable P35-caspase complex, which precludes subsequent caspase protease activity (dela Cruz et al., 2001). Despite the present understanding of P35-mediated interception of apoptotic pathway, it is not clear whether P35 acts on additional intermediate targets in apoptotic pathways induced by various stimuli. Given that the baculovirus protein P35 can inhibit cell-death in a response to a variety of stimuli, we explored whether expression of P35 can inhibit NO-induced apoptosis of activated macrophages.

Murine macrophages can be activated to produce a high level of NO by a variety of agents, including cisplatin, a broad spectrum anticancer drug that has proven effective in the

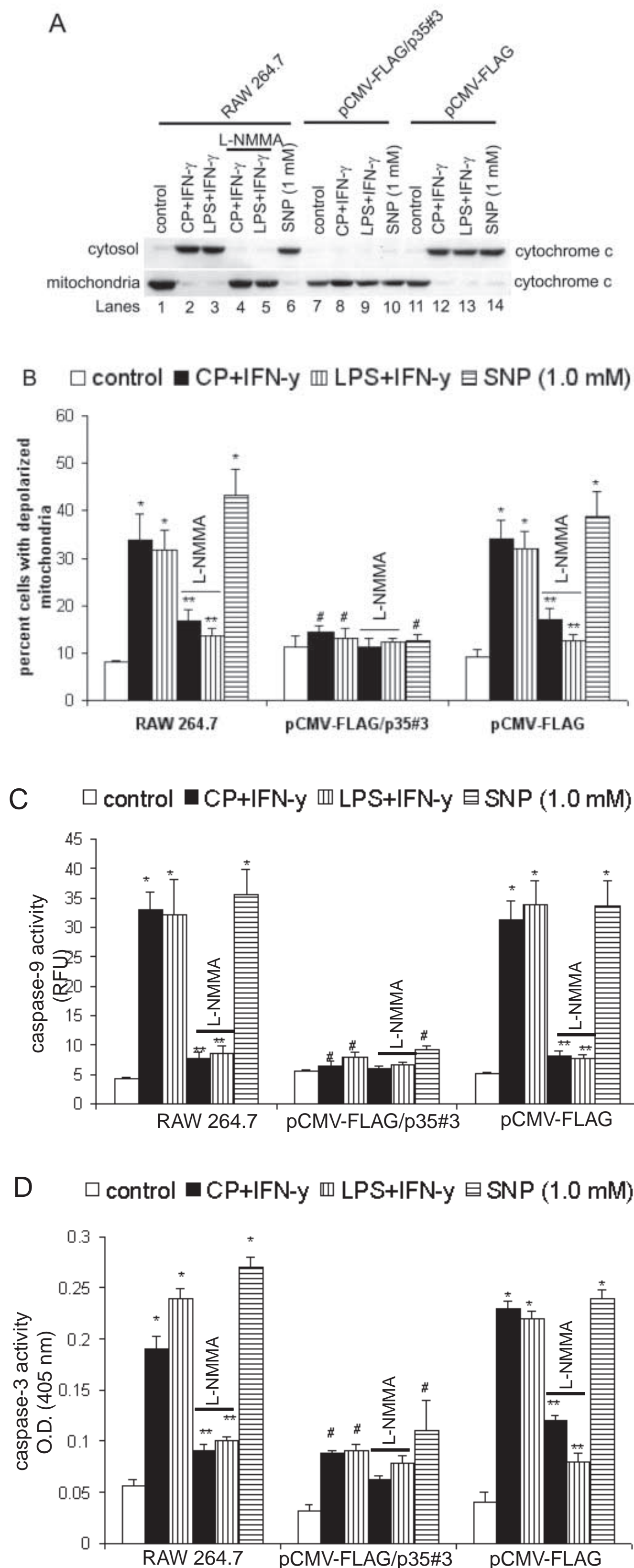

Fig. 5. P35 inhibits mitochondrial cytochrome c release, mitochondrial depolarization and caspase activation. RAW 264.7 cells or vector- or P35-transfected clones (1×10^6 cells/well) in 6-well tissue culture plates were treated with cisplatin (2 μg/ml) and IFN-γ (25 U/ml), or LPS (1 μg/ml) and IFN-γ (25 U/ml) in the presence or absence of L-NMMA (1 mM). Cells were also treated directly with NO donor, SNP (1 mM). Cytochrome c in the cytosol and mitochondria was detected by immunoblot analysis (A). Mitochondrial depolarization was determined as described in Materials and Methods, and results were expressed as the percentage of cells with depolarized mitochondria (B). Extracts were prepared as described in Materials and Methods. Caspase-9 (C) and caspase 3 (D) activities were measured in protein extracts as described in Materials and Methods. Data shown are mean±s.e.m. and are representative of three independent experiments done in triplicate. *$P<0.05$ versus values of control cultures. **$P<0.05$ versus values of cisplatin and IFN-γ /LPS and IFN-γ-treated cultures. #$P<0.05$ versus values for cisplatin and IFN-γ/LPS and IFN-γ or SNP-treated RAW 264.7/pCMV-FLAG cells.

treatment of bladder, lung, ovarian, head and neck, and breast cancer, and certain types of leukemia (Sodhi and Suresh, 1992; Geetha and Sodhi, 1990). NO production by murine macrophages is critical for their antitumor activity. For example, the tumoricidal effect of macrophages has been shown to be further enhanced when these cells are treated with cisplatin in combination with IFN-γ, and this effect is directly related with increased level of NO production (Ranjan et al., 1998). Although high levels of NO production by activated macrophages is required for its anti-tumor and anti-microbial activity (Keller et al., 1990), macrophages are themselves targets for NO and die prematurely when activated to express iNOS (Ranjan et al., 1998; Sarih et al., 1993; Albina et al., 1993).

To determine whether P35 expression was capable of blocking NO-induced apoptotic cell death in activated macrophages, the mouse macrophage cell line RAW 264.7 was stably transfected with a *p35*-expression construct, and clonal cell lines were derived. Clone pCMV-FLAG/p35#3 demonstrated the strongest expression of P35 other clones (pCMV-FLAG/p35#7 and pCMV-FLAG/p35#9) that expressed relatively low level of P35 (Fig. 1A,B). Treatment of cells with cisplatin and IFN-γ, or LPS and IFN-γ induced oligonucleosomal DNA fragmentation in parent or empty vector-transfected RAW 264.7 cells but not in *p35*-transfected RAW 264.7 clones. *p35* transfection also inhibited morphological and nuclear changes characteristic of apoptosis and prevented loss of cell viability. Addition of L-NMMA, an iNOS inhibitor, prevented parent RAW 264.7 cells from undergoing apoptosis, showing apoptosis in this setting is dependent on NO. Further, *p35*-transfected cells showed increased resistance to SNP, a NO donor.

Recent studies have suggested that P35 can act as an anti-oxidant by detoxifying free radicals, which consequently prevents cell-death (Sah et al., 1999). To examine the possibility that P35 may potentially affect NO production by activated macrophages, we studied iNOS expression and NO production by P35-expressing Raw 264.7 clones. Activation of parent, vector- or *p35*-transfected RAW cells with cisplatin and IFN-γ, or LPS and IFN-γ resulted in increased expression of iNOS and a corresponding increase in amounts of nitrite (Fig. 3A), a result which ruled out attenuated inducible NO synthase activity during P35-mediated protection. These results show that P35 does not interfere with cell signaling pathways leading to increased expression of iNOS or production of NO.

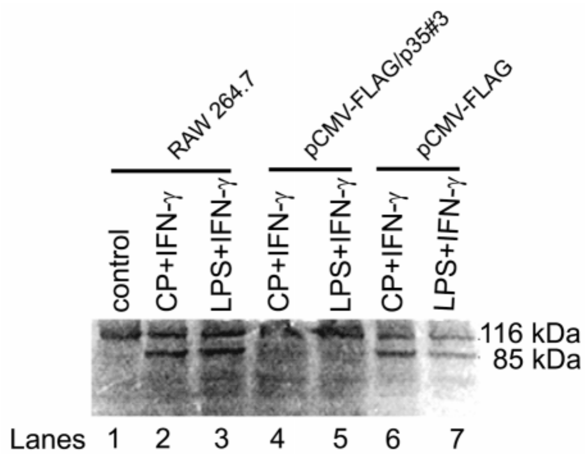

Fig. 6. P35 expression inhibits NO-mediated PARP cleavage in RAW 264.7 cells. RAW 264.7 cells or vector- or *p35*-transfected clones were treated with cisplatin (2 μg/ml) and IFN-γ (25 U/ml) or LPS (1 μg/ml) and IFN-γ (25 U/ml) in the presence or absence of L-NMMA (1 mM) as before. Cells were also treated directly with NO donor, SNP (1 mM). After 36 hours of treatment, cell extracts were analyzed by immunoblotting with anti-PARP antibody to assess cleavage of PARP.

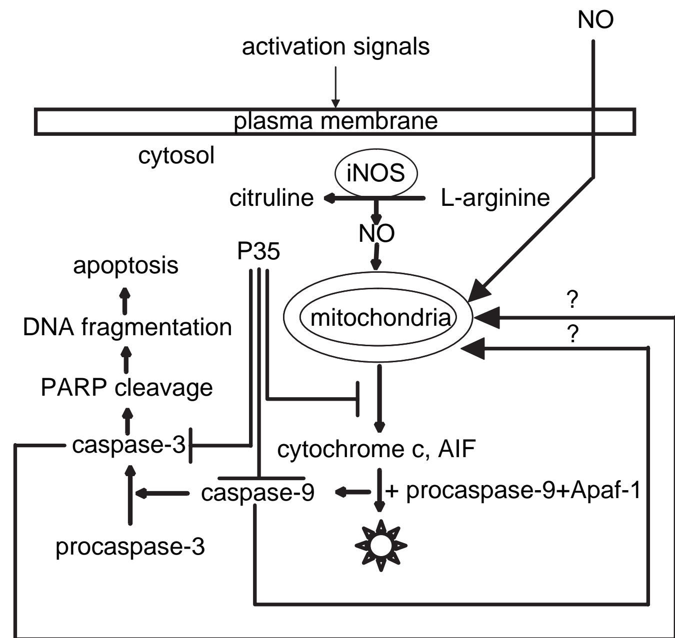

Fig. 7. Model for P35-mediated inhibition of NO-induced apoptosis in activated macrophages. Mitochondrial cytochrome c release is a critical event in molecular pathway of apoptosis that triggers subsequent caspases activation and apoptotic cell death. Baculovirus protein P35 inhibits mitochondrial cytochrome c release as well as caspase activation. This also raises the possibility that activation of caspases may be the cause rather than consequence of cytochrome c release. AIF, apoptosis inducing factor; Apaf-1, apoptotic protease activating factor-1.

Further study showed P35-mediated inhibition of apoptosis in activated macrophages is dependent on caspase activation. Caspase-9 and caspase-3 activities were compared in treated or untreated parent RAW 264.7 or vector- or *p35*-transfected cells. We observed increased caspase-9 and caspase-3 activity in parent or vector-transfected RAW cells upon treatment with cisplatin and IFN-γ, or LPS and IFN-γ or direct treatment with a NO donor, whereas *p35*-tranfected cells did not show significant increase in caspase-9 and caspase-3 activity (Fig. 5C,D), suggesting baculovirus protein P35 blocks apoptotic cell death in activated macrophages by inhibiting caspases and/or blocking steps leading to caspase activation.

The involvement of caspases in apoptosis arises from studies in which nuclear proteins such as PARP, lamins and the 70-kDa protein of U1 small nuclear riboprotein were shown to be processed proteolytically by unknown ICE-like cysteine proteases (Degterev et al., 2003; Fesik, 2000; Tewari et al., 1995). The results of the present study revealed that the death substrate PARP gets cleaved from native 116-kDa PARP to signature 85-kDa proteolytic fragment in the parent or vector-transfected RAW cells, but not in 35-transfected RAW clone even after 36 hr of CP and IFN-γ or LPS and IFN-γ treatment or after direct treatment with SNP.

The mechanism(s) for caspase activation in apoptotic pathways is incompletely understood. Several studies suggest that cytochrome c release from mitochondria into cytosol in response to apoptotic stimuli may cause caspase activation (Green and Reed, 1998). Cytosolic cytochome c forms an essential part of ‘apoptosome’, which is composed of cytochrome c, Apaf-1 and procaspase-9. This complex results in activation of caspase-9, which then cleaves and activates downstream executioner caspase-3 (Li et al., 1997). This prompted us to investigate whether cytochrome c release is inhibited by P35 in NO-induced apoptosis of activated macrophages. Parent RAW 264.7 or vector-tranfected cells showed release of cytochrome c from mitochondria to cytosol upon treatment with cisplatin and IFN-γ, or LPS and IFN-γ in a NO-dependent manner. However, P35 expression completely inhibited translocation of cytochrome c in to the cytosol (Fig. 5A), indicating P35 can act at the level of cytochrome c release in NO-mediated apoptosis.

The release of cytochrome c from mitochondria into cytosol results in mitochondrial depolarization (Green and Reed, 1998). Our data indicate that activation of macrophages with cisplatin and IFN-γ or LPS and IFN-γ altered $\Delta\Psi_m$ in a NO-dependent manner. *p35*-transfected cells did not show any change in $\Delta\Psi_m$. This finding is in agreement with others who report that cytochrome c release during apoptosis is accompanied by a reduction in $\Delta\Psi_m$ (Ricci et al., 2003).

The unique observation of this study is that baculovirus protein P35 can inhibit NO-induced apoptosis of activated macrophages by inhibiting cytochrome c release, an event upstream of caspase cascade. The present understanding of the precise mechanism by which cytochrome c is released from mitochondria in response to NO or apoptotic stimuli is not clearly understood. Though many reports suggest that caspases are downstream of cytochrome c release in the apoptotic pathway (Green and Reed, 1998), some studies suggest caspase inhibitors block cytochrome c release. Hence, the consequence of cytochrome c release may depend on cell type and stimuli (Green and Reed, 1998), or activated caspases may act in feedback loop on cyctochrome c release (Fig. 7).

Further, other apoptosis mediators are also released from

mitochondria (Mancini et al., 1998). Mitochondria in some cells contain procaspase-3 (Mancini et al., 1998) that is released into cytosol during apoptosis, although it remains unclear whether it becomes activated before release. Another caspase-activating protein that is released from mitochondria is apoptosis-inducing factor (AIF), which has been shown to process purified procaspase-3 in vitro (Susin et al., 1996). Its activity is blocked by zVAD-fmk, a general caspase inhibitor, raising the possibility that AIF is another caspase. It is possible that P35 can directly inhibit the anti-apoptotic activities of AIF. Further studies are required to judge whether P35 acts on these targets. It will be interesting to study whether or not the same mechanism operates in human macrophages that do not produce significant quantities of NO, but undergo apoptosis in response to other apoptotic stimuli (Flad et al., 1999).

In summary, this study demonstrates that baculovirus protein P35 can inhibit NO-mediated apoptosis in activated mouse macrophages via inhibition of mitochondrial cytochrome c release, suggesting P35 has upstream targets in addition to caspases. Identification of those targets will not only help to understand the molecular pathway of apoptosis, but may also provide important insights for therapeutic intervention. Macrophages may need protective mechanisms analagous to P35 to prevent autotoxicity during production of NO required for anti-tumor activity.

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