Inhibition of Poly(ADP-ribose) Polymerase Activity by Bcl-2 in Association with the Ribosomal Protein S3a

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ABSTRACT: We screened a human lymphocyte cDNA library using the yeast two-hybrid system and an automodification domain of PARP as a probe. The DNA sequence of an isolated clone (clone 3-9) was identical to the partial cDNA sequence of the human ribosomal protein S3a. We confirmed that PARP interacts with clone 3-9 by performing binding studies using a GST−3-9 fusion protein as bait. We also demonstrated that native S3a in nuclear extracts of HL-60 cells interacts with the automodification domain of PARP and that PARP from nuclear extracts is coprecipitated with the GST−3-9 fusion protein. Furthermore, we demonstrated that Bcl-2 interacts with PARP in association with S3a and that the interaction of S3a and Bcl-2 with PARP causes a significant decrease in PARP activity. Since Bcl-2 failed to inhibit PARP activity in the absence of S3a, we suggest that Bcl-2 together with S3a prevents apoptosis probably by inhibiting PARP activity.

Apoptosis is a physiological process that plays important roles in development, homeostasis, and immunological competence. It is characterized by striking morphological changes such as membrane blebbing, aggregation of chromatin, and nuclear breakdown and terminates with cell fragmentation and internucleosomal DNA fragmentation as well as cleavage of poly(ADP-ribose) polymerase (PARP).[1] PARP, which is activated by binding to DNA strand breaks, catalyzes the poly(ADP-ribosyl)ation of various nuclear proteins, including itself, using NAD as a substrate (*1*, *2*). It has been suggested that PARP contributes to the onset of apoptosis induced by a wide variety of agents, including alkylating agents, active oxygen species, topoisomerase II inhibitors, and adriamycin. Apoptosis induced by these agents was blocked by inhibiting PARP activity using PARP inhibitors (e.g., 3-aminobenzamide and benzamide) (*3*−*7*). Recently, a transient burst of poly(ADP-ribosyl)ation of nuclear proteins has been detected in the initial stages of apoptosis in cells (*8*). These findings imply that PARP activity is vital in apoptosis, especially in the early stages.

We screened a human lymphocyte cDNA library using the yeast two-hybrid system and an automodification domain of PARP (PARP/AMD) as a probe to find a binding partner of PARP (*9*). We isolated several positive clones, and the DNA sequence of clone 3-9 was identical to the partial DNA sequence of the human ribosomal protein S3a. S3a is regarded as a multifunctional protein; apart from its function in protein synthesis as part of the ribosome, it is reported to have several functions involved in regulating cell growth (*10*), transformation (*10*, *11*), and death (*11*, *12*). Recently, S3a has been implicated in the process of apoptosis. Apoptosis was induced in certain cell lines by lowering constitutively high levels of S3a expression using actinomycin D (*13*) or antisense sequences of the S3a gene (*14*). However, the precise role of S3a in apoptosis is still unclear. Recently, it has been reported that S3a interacts with Bcl-2 (*15*). In this study, we demonstrate that Bcl-2 and S3a act cooperatively to inhibit PARP activity. Our results provide novel insight in understanding the mechanism of apoptosis.

EXPERIMENTAL PROCEDURES

Preparation of Fusion Proteins. A GST−3-9 construct was prepared by inserting an *Xho*I fragment of the 3-9 cDNA into the *Xho*I site of pGEX-4T-2. The pGEX-3-9 plasmid was transformed into *Escherichia coli* BL21 competent cells. The induction and purification of the fusion protein were as described in the GST Gene Fusion System manual (Pharmacia Biotech). His-tagged PARP/AMD was constructed and purified as described previously (*9*). The full-length cDNA of Bcl-2 was synthesized from total RNA extracted from HL-60 cells by RT-PCR. The RT-PCR product was ligated into the pGEM-T vector (Promega). The full-length Bcl-2 primer sequences were as follows: sense, 5′-GTTAAC-CCCGTTACTTTTCCTCT; and antisense, 5′-GAATTC-CAGGCATGTTGACTTCAC. The human Bcl-2 cDNA (amino acids 1−218), lacking its transmembrane domain, was synthesized by PCR using pGEM-Bcl-2 (full length) as a template. The primers for the Bcl-2 cDNA (amino acids 1−218) were as follows: sense, 5′-CGGAAATTCATATG-GCGCACGCTGGGAGA; and antisense, 5′-CCCTCGAG-GTCTTCAGAGACAGCCAGGA. The PCR products were ligated into the *Eco*RI−*Xho*I sites of the pGEX-4T-2 vector after digestion of the PCR product with *Eco*RI and *Xho*I. The resultant plasmid, pGEX-Bcl-2 (amino acids 1−218),

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[1] Abbreviations: PARP, poly(ADP-ribose) polymerase; S3a, ribosomal protein S3a; PARP/AMD, automodification domain of PARP; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PVDF membrane, polyvinylidene difluoride membrane; GST, glutathione *S*-transferase.

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was transformed into *E. coli* BL21 competent cells. pGEX-Bcl-2 (amino acids 1−218) fusion proteins were prepared as described above.

Cleavage of the GST−3-9 Fusion Protein by Thrombin. One milliliter of GST−3-9 fusion proteins (1.0 mg/mL) purified on a glutathione−Sepharose 4B affinity column was dialyzed against 1000 volumes of buffer HN [20 mM Hepes (pH 8.0) and 150 mM NaCl] at 4 °C overnight. After $CaCl_2$ had been added to a final concentration of 2 mM, the solution was mixed with 10 μg of thrombin (Sigma) and incubated on ice for 5 h. Then, 50 μL of a 50% slurry of glutathione−Sepharose 4B beads and 5 μL of benzimidine−Sepharose equilibrated with buffer HN were added and incubated with gentle agitation at 4 °C for 30 min. The suspension was centrifuged to sediment the matrix. The supernatant containing the purified 3-9 cDNA product was stored at −80 °C for further experiments.

Preparation of Antibodies against the His−PARP Fusion Protein and the 3-9 cDNA Product. Antibodies against the His−PARP fusion protein or the 3-9 cDNA product were prepared using the His−PARP or GST−3-9 fusion protein as an antigen. The antisera were purified by ammonium sulfate precipitation and chromatography over a protein G column (Pharmacia). The purified IgG against the GST−3-9 fusion protein was mixed with free GST protein and incubated at 4 °C for 30 min with end-over-end mixing. Then, a 50% slurry of glutathione−Sepharose 4B beads (100 μL) was added to the mixture and incubated for 30 min at 4 °C to remove GST-specific antibodies from solution. The suspension was centrifuged to sediment the matrix. The supernatant containing the antibody against the 3-9 cDNA product was stored at −80 °C for further experiments.

Purification of Nuclear Extracts from HL-60 Cells. Nuclear extracts were prepared according to the procedure of Dignam et al. (*16*). HL-60 cells (1×10^6 cells/mL) were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Cells were harvested and washed twice with PBS and then pelleted. These cells were then suspended in 5 volumes of buffer A [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 0.5 mM PMSF, and 0.5 mM DTT] and incubated on ice for 10 min. Afterward, the cells were spun down, resuspended in 4 volumes of buffer A, and homogenized with a Dounce homogenizer equipped with a B-type pestle. Homogenates were centrifuged at 1600 rpm for 10 min to precipitate the nuclei. The crude nuclear pellets were rinsed twice with buffer A, then resuspended in 4 volumes of buffer C [0.42 M NaCl, 1.5 mM $MgCl_2$, 20 mM Hepes (pH 7.4), 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF], and rotated on a tube rotator at 4 °C for 1 h. Chromatins were sedimented by centrifugation at 100 000 rpm and 4 °C for 20 min. The supernatant was employed as nuclear extracts and stored at −80 °C for further analysis. Protein concentrations were determined by the Bradford method (*17*).

Far-Western Blot Analysis. Far-Western blot analysis was performed as previously described (*9*) with some modifications. The GST−3-9 fusion protein and GST protein were subjected to SDS−PAGE and then electrotransferred onto a PVDF membrane. After being soaked in 5% skim milk and TBBN [20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 0.1% NP-40] for 1 h at 25 °C, the membrane was incubated for 2 h with peroxidase-labeled His-tagged PARS/AMD (*18*) in 0.1% skim milk and TBBN at 4 °C. Finally, the bands were visualized using the ECL system according to the manufacturer's instructions (Amersham).

Pull-Down Assays. (1) Pull-Down Assays of S3a with the His−PARP Fusion Protein. Nuclear extracts (300 μg) of HL-60 cells were desalted with a Sephadex G-50 spin column equilibrated with TBBN. Pretreated nuclear extracts (300 μg) were mixed with (lane 2) or without (lane 1) His−PARP/AMD (30 μg). The samples were rotated for 2 h at 4 °C. A 50% slurry of Ni^{2+}−NTA resin (30 μL) was added to the tubes and rotated for 30 min at 4 °C. After centrifugation, the beads were rinsed five times with ice-cold TBBN. The proteins bound to the His−PARP fusion protein or the resin were eluted by suspending the beads in 500 μL of modified buffer C [1 M KCl, 1.5 mM $MgCl_2$, 20 mM Hepes-KOH (pH 7.4), 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF] and rotated for 30 min at 4 °C. The mixtures were centrifuged, and the proteins in the supernatant were precipitated with 10% TCA. These samples were subjected to SDS−PAGE and electrotransferred onto a PVDF membrane. Immunological detection was performed according to the method of Domingo and Marco (*19*). After being blocked in 5% skim milk and TBS [0.15 M NaCl and 20 mM Tris-HCl (pH 7.6)] at 4 °C overnight, the membrane was incubated with the antibody against the 3-9 cDNA product for 2 h in GENT solution [0.15 M NaCl, 5 mM EDTA (pH 8.0), 50 mM Tris-HCl (pH 7.6), 0.25% gelatin, and 0.05% Triton X-100] and then with a horseradish peroxidase-labeled secondary antibody against rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA). The 3-9 cDNA product and its derivatives blotted onto the membrane were visualized using an enhanced chemiluminescence detection (ECL) system (Amersham).

(2) Pull-Down Assays of PARP with the GST−3-9 Fusion Protein. Nuclear extracts (200 μg) of HL-60 cells were desalted with a Sephadex G-50 spin column equilibrated with TBBN. Either the GST−3-9 fusion protein (15 μg) or the GST protein (15 μg) was added to pretreated nuclear extracts of HL-60 cells (200 μg). The tubes were rotated for 2 h at 4 °C. A 50% slurry of glutathione−Sepharose 4B beads (20 μL) was added to the tubes and incubated for 30 min at 4 °C in the same way. After centrifugation, the beads were rinsed five times with ice-cold TBBN. The proteins bound to the GST−3-9 fusion protein or the GST protein alone were eluted as described above and analyzed using the antibody against the His−PARP fusion protein.

(3) Pull-Down Assays of Bcl-2 and the 3-9 cDNA Product with the His−PARP Fusion Protein. Pull-down assays of products translated in vitro were performed as previously described (*20*) with some modifications. In vitro transcription and translation were performed with pGEM-Bcl-2 (full length) as a template using the TNT-coupled reticulocyte lysate system (Promega) and ^{35}S-labeled Met (ICN) according to the manufacturer's instructions. Either the 3-9 cDNA product (15 μg) alone or the 3-9 cDNA product (15 μg) and the His−PARP protein (15 μg) were added to ^{35}S-labeled Bcl-2. After incubation at 4 °C for 1 h with gentle agitation, a 50% slurry of Ni^{2+}−NTA resin (15 μL) was added to the protein mixtures and further incubated at room temperature for 1 h. After removal of the supernatant, the beads were rinsed five times with the binding buffer. Proteins associated with the His−PARP fusion protein and the 3-9 cDNA product were solubilized with SDS sample buffer and

subjected to SDS−PAGE. Fluorography of the gel was performed using the method described by Chamberlain (*21*). The recovery of the ^{35}S-labeled Bcl-2 precipitated by the pull-down assay was analyzed densitometrically by Kodak Digital Science 1D.

Enzyme Activity Assay. Nuclear extracts of HL-60 cells were fractionated on a Sephadex 200 PC3.2/3.0 column using the SMART system [50 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, and 0.5 M KCl] (Pharmacia), thereby eliminating S3a and Bcl-2 from the PARP fractions. PARP-enriched fractions were dialyzed against PBS thoroughly and then mixed with either the 3-9 cDNA product or the GST−Bcl-2 fusion protein alone or in combination and rotated at 4 °C for 30 min. The standard reaction mixture in a total volume of 0.1 mL contained 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM [^{3}H]NAD (26.6 dpm/pmol), 10 μg of calf thymus DNA and calf thymus histone, and PARP or the above mixtures. The reaction was carried out at 25 °C for 10 min and terminated by adding 2 mL of 10% trichloroacetic acid and 1% $Na_4P_2O_7$. Trichloroacetic acid insoluble materials with incorporated radioactivity were collected on a glass filter and washed three times with 5 mL of 5% trichloroacetic acid and quantitated with a spectrophotometer (Packard, model 2500TR) in 5 mL of a scintillation fluid containing 0.5% 2,5-diphenylozazole and 0.03% 1,4-bis[2-(2-methyl-5-phenyloxazolyl)]benzene in a toluene solvent.

RESULTS

Identification of Clone 3-9 cDNA as the cDNA of Ribosomal Protein S3a. To search for a binding partner of PARP, we screened a human lymphocyte cDNA library using the yeast two-hybrid system and an automodification domain of PARP (PARP/AMD) as the bait (*9*). We isolated several cDNA clones and determined their DNA sequences. One of the cDNAs, 3-9, was identical to that of the human ribosomal protein S3a (S3a, GenBank accession no. D28374) (*22*). The 3-9 cDNA is missing nucleotides 1−28 from the 5′ end of the S3a gene (data not shown).

Interaction of the 3-9 cDNA Product and PARP/AMD in Vitro. To demonstrate the interaction between PARP and the 3-9 cDNA product in vitro, we constructed a plasmid encoding the GST−3-9 fusion protein by ligating the *Xho*I fragment of clone 3-9 into the *Xho*I site of the pGEX-4T-2 vector. The affinity-purified GST−3-9 fusion protein was analyzed by far-Western blot analysis with a peroxidase-labeled His-tagged PARP/AMD protein as a probe. The fusion protein migrated with an apparent molecular mass of 56 kDa (Figure 1A, lane 1), which coincides with the size deduced from the cDNA sequence. Proteins from a gel identical to that shown in Figure 1A were transferred onto a PVDF membrane and subsequently incubated with peroxidase-labeled His-tagged PARP/AMD. The bands corresponding to the GST−3-9 fusion protein were visualized using the ECL system (Figure 1B, lanes 1−3). In contrast, no band was detected at the expected position of GST. A dose-dependent interaction between the GST−3-9 fusion protein and PARP/AMD was demonstrated. These results indicate that PARP/AMD interacts directly with the 3-9 cDNA product in vitro and that the GST domain does not mediate binding (Figure 1B, lane 4).

Interaction of Native S3a and PARP in Nuclear Extracts of HL-60 Cells. To investigate whether S3a exists in the nuclei of HL-60 cells and interacts with PARP, we prepared nuclear extracts of HL-60 cells. Nuclear extracts (300 μg) of HL-60 cells were treated with a Sephadex G-50 spin column for desalting. Pretreated nuclear extracts (300 μg) were mixed with or without His−PARP/AMD (30 μg) and precipitated with Ni^{2+}−NTA resin. Proteins interacting with His−PARP/AMD or the resin were eluted with 1 M KCl. Eluted proteins were analyzed immunologically using an antibody against the 3-9 cDNA product (Figure 2A), as described in Experimental Procedures. A 32 kDa band was visualized in the eluted fraction associated with His−PARP/AMD (Figure 2A, lane 2). This result indicates that S3a exists in the nuclei of HL-60 cells and interacts with His−PARP/AMD.

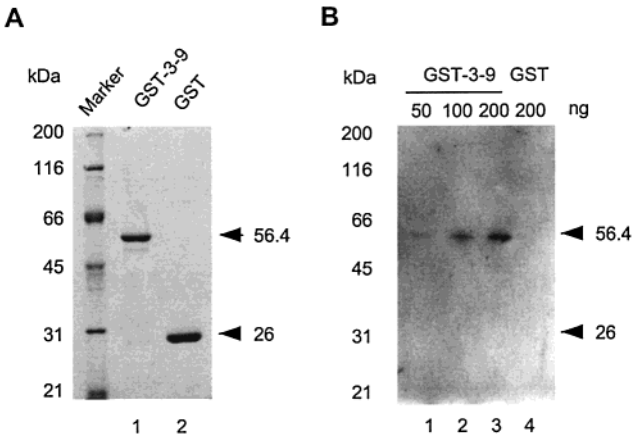

FIGURE 1: Interaction of the 3-9 cDNA product and the automodification domain of PARP in vitro. (A) The affinity-purified GST−3-9 protein and GST alone were subjected to SDS−PAGE and stained with CBB. (B) Proteins on the SDS gel were transferred onto a PVDF membrane, reacted with peroxidase-labeled His-tagged PARP/AMD, and visualized using the ECL system: 50 μg of GST−3-9 (lane 1), 100 μg of GST−3-9 (lane 2), 200 μg of GST−3-9 (lane 3), and 200 μg of GST (lane 4). The positions of the molecular size standards (in kilodaltons) are indicated.

To further confirm the binding between native PARP and the 3-9 cDNA product, we performed pull-down assays with the GST−3-9 fusion protein. Either the GST−3-9 fusion protein (15 μg) or GST (15 μg) was mixed with nuclear extracts (200 μg) of HL-60 cells and precipitated with the glutathione affinity resin. Proteins interacting with the GST−3-9 fusion protein or GST were eluted with 1 M KCl. Eluted proteins were analyzed immunologically using an antibody against the His−PARP protein, as described in Experimental Procedures. A 120 kDa band was visualized in the eluted fraction associated with the GST−3-9 fusion protein but not in that with GST alone (Figure 2B). This result suggests that the 3-9 cDNA product interacted with native PARP in nuclear extracts.

Interaction of PARP with Bcl-2 Is Mediated by the 3-9 cDNA Product, Forming a Heterotrimer Complex of PARP, the 3-9 cDNA Product, and Bcl-2. It has been reported that Bcl-2 interacts with S3a (*15*). Thus, we examined whether PARP was able to form a heterotrimer complex together with S3a and Bcl-2. The ^{35}S-labeled Bcl-2 protein, which was produced in an in vitro translation system, was incubated with the 3-9 cDNA product and His-tagged PARP/AMD or His-tagged PARP/AMD alone and precipitated with Ni^{2+}−NTA resin. Proteins interacting with His-tagged PARP/AMD were subjected to SDS−PAGE and visualized by autoradi-

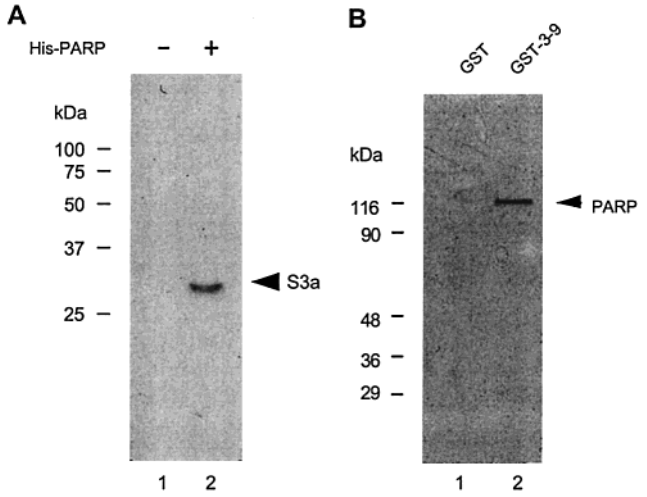

FIGURE 2: Interaction of S3a and PARP in nuclear extracts of HL-60 cells. (A) Pull-down assays of S3a with the His−PARP protein. Nuclear extracts (300 μg) of HL-60 cells in binding buffer were incubated with (lane 2) or without (lane 1) His−PARP/AMD (30 μg) and precipitated with Ni^{2+}−NTA resin. The proteins which interacted with the His−PARP protein or the resin were eluted with 1 M KCl and analyzed immunologically with the antibody against the 3-9 cDNA product as described in Experimental Procedures. (B) Pull-down assays of PARP with the GST−3-9 fusion protein. GST (15 μg) or the GST−3-9 fusion protein (15 μg) was incubated with nuclear extracts (200 μg) of HL-60 cells in binding buffer and precipitated by the glutathione affinity resin. The proteins which interacted with the GST−3-9 fusion protein (lane 2) or GST alone (lane 1) were eluted with 1 M KCl and analyzed immunologically with an antibody against the His−PARP protein as described in Experimental Procedures. The positions of the molecular size standards (in kilodaltons) are indicated.

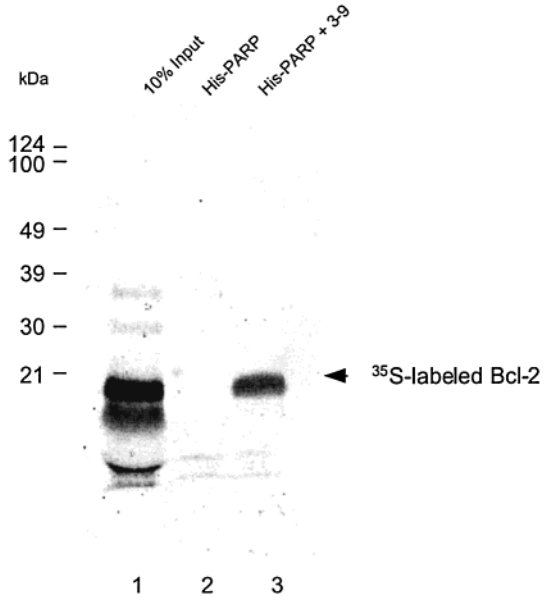

FIGURE 3: Interaction of PARP and Bcl-2 is mediated by the 3-9 cDNA product. The ^{35}S-labeled Bcl-2 produced by the in vitro translation system was incubated with either the 3-9 cDNA product alone (lane 2) or the 3-9 cDNA product and His-tagged PARP/AMD (lane 3) and then precipitated with Ni^{2+}−NTA affinity resin. The precipitates were eluted using an SDS sample buffer and subjected to SDS−PAGE. Then, 10% input of the ^{35}S-labeled Bcl-2 (lane 1) was loaded as a positive control. Densitometric analyses of the corresponding band in lanes 1 and 3 indicate that the quantity of ^{35}S-labeled Bcl-2 precipitated by both His-tagged PARP and the 3-9 cDNA product was 4.1% of the total Bcl-2. The positions of the molecular size standards (in kilodaltons) are indicated.

ography (Figure 3). The ^{35}S-labeled Bcl-2 was not precipitated with the His−PARP fusion protein alone (Figure 3, lane 2), but it was precipitated in a reaction mixture containing both the His−PARP fusion protein and the 3-9 cDNA product (Figure 3, lane 3). Densitometric analyses of the corresponding band in lanes 1 and 3 indicate that 4.1% of in vitro-translated ^{35}S-labeled Bcl-2 was precipitated with both the His−PARP fusion protein and the 3-9 cDNA product. These results suggest that S3a might act as a bridge protein for the interaction between PARP and Bcl-2.

Inhibition of PARP Activity by Bcl-2 in Association with the 3-9 cDNA Product. To determine the effect of Bcl-2 on PARP activity, we examined PARP activity in the presence of either the 3-9 cDNA product alone, the Bcl-2 protein alone, or a combination of these two proteins (Figure 4). Nuclear extracts of HL-60 cells were fractionated on a Sephadex 200 PC3.2/3.0 column (Pharmacia). Under the high-salt conditions, S3a and Bcl-2 were separated from the PARP fractions. S3a was not detected in PARP-enriched factions analyzed immunologically using the antibody against the 3-9 cDNA product (data not shown). The GST moiety of the GST−3-9 fusion protein was removed by thrombin digestion. PARP was inhibited by 30% in the presence of the 3-9 cDNA product. In addition, PARP activity was further decreased in the presence of both the 3-9 cDNA product and the GST−Bcl-2 fusion protein (amino acids 1−218) by 69%. PARP activity was unaffected by the GST−Bcl-2 protein (amino acids 1−218) or GST alone (Figure 4A). Even though the amounts of the 3-9 cDNA product were increased, PARP activity did not decrease more than 30% (Figure 4B). When PARP-enriched fractions were mixed with the 3-9 cDNA product (20 ng) and increasing amounts (10, 20, and 40 ng) of the GST−Bcl-2 protein (amino acids 1−218), PARP activity was inhibited depending on the amount of the GST−Bcl-2 protein (Figure 4C). These results indicate that Bcl-2 in association with the 3-9 cDNA product plays an important role in inhibiting PARP activity.

DISCUSSION

PARP has been implicated in apoptosis because inhibition of PARP activity with 3-aminobenzamide and benzamide blocks apoptosis (*3*−*6*). Moreover, we have demonstrated that 3-aminobenzamide blocks etoposide-induced apoptosis in THP-1 cells or actinomycin D-induced apoptosis in HL-60 cells (data not shown). In addition, Simbulan-Rosenthal et al. have reported that a transient burst of poly(ADP-ribosyl)ation of nuclear proteins occurs in the early stages of apoptosis and that inhibition of PARP activity at this time by expression of antisense RNA expression blocks various biochemical and morphological changes associated with apoptosis (*8*, *23*). Wang et al. produced PARP-knocked out mice, which display no phenotypic abnormalities (*24*), and PARP (−/−) cells apoptosized normally in response to treatment with anti-Fas (*25*). Smulson and his group, however, demonstrated that exposure to anti-Fas and cycloheximide induced marked internucleosomal DNA fragmentation in PARP (+/+) fibroblasts and PARP (−/−) cells stably transfected with a plasmid expressing wild-type PARP, whereas no apoptotic DNA ladders were evident in PARP (−/−) cells (*8*), which were originally produced by Wang et al. (*25*). Taken together with our findings that PARP

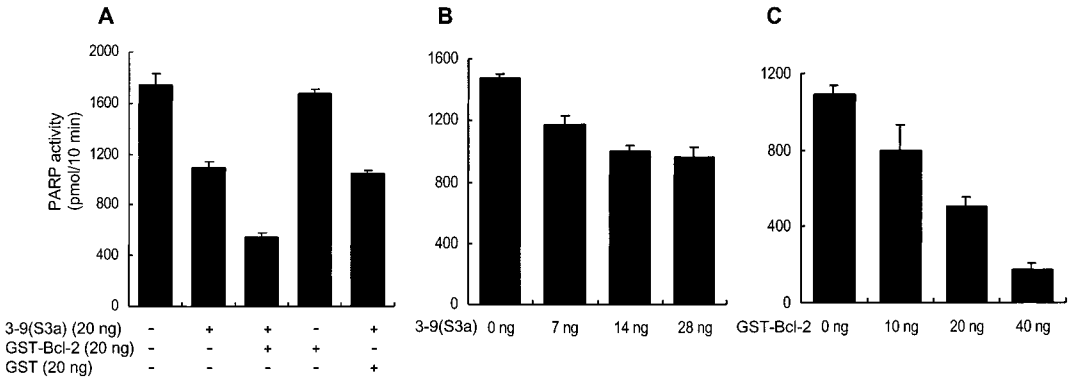

FIGURE 4: Effects of Bcl-2 and the 3-9 cDNA product on PARP activity. (A) Inhibition of PARP activity by Bcl-2 in association with the 3-9 cDNA product. PARP-enriched fractions of nuclear extracts from HL-60 cells were mixed with the 3-9 cDNA product (20 ng), GST−Bcl-2 fusion protein (20 ng), or both. PARP activity was examined as described in Experimental Procedures. (B) Inhibition of PARP activity with varying amounts of the 3-9 cDNA product. PARP-enriched fractions were incubated with varying amounts of the 3-9 cDNA product (7, 14, or 28 ng), and the PARP activity was examined. (C) Inhibition of PARP activity with the varying amounts of Bcl-2 in association with the 3-9 cDNA product. PARP-enriched fractions were mixed with the 3-9 cDNA product (20 ng) and varying amounts of the GST−Bcl-2 fusion protein (10, 20, or 40 ng), and the PARP activity was examined. The bars represent standard errors of the mean calculated from results derived from three different experiments.

activity was inhibited ~90% by Bcl-2 in association with S3a (Figure 4C), the apoptotic pathway is not always coincident in different cell types and apoptotic inducers, and PARP activity participates in the apoptotic cascade at least in certain cell types. PARP was also suggested to be involved in the later stages of apoptosis. Caspase-3, an aspartate-specific cysteine protease, is activated in the later stages of apoptosis and is responsible for the cleavage of PARP into 89 and 24 kDa fragments, thereby playing a role in the execution of the apoptotic program (*26*). The functions of the two degraded fragments of PARP in apoptosis have been extensively studied (*27*, *28*); however, the precise role of PARP in the process of apoptosis is still unclear.

S3a is a multifunctional protein involved in apoptosis (*29*, *30*). Naora et al. have reported that a decrease in the level of S3a expression occurs in the early stages of apoptosis (*13*, *14*, *31*). We have also detected decreased levels of the S3a protein in the early stages of etoposide-induced apoptosis in HL-60 cells (data not shown). These observations, taken together with data provided by Simbular-Rosenthal et al. (*8*), suggest that the reduction in S3a protein levels seems to coincide with the burst of poly(ADP-ribosyl)ation of nuclear proteins during apoptosis. In this study, we have demonstrated that S3a associates with PARP in vitro and partially inhibits PARP activity. Thus, our results, together with the findings described above, suggest that a decrease in S3a protein levels in the early stages of apoptosis contributes to a burst of PARP activity, resulting in a commitment to cell death.

Recently, Hu et al. have shown that S3a interacts with Bcl-2 (*15*). Here, we have demonstrated that S3a acts as a bridge protein to mediate the interaction between Bcl-2 and PARP and that Bcl-2 facilitates the inhibition of PARP activity by S3a (Figure 4). Bcl-2 is an antiapoptotic regulator and has been shown to block cell death induced by various cytotoxic agents (*6*, *32*−*34*). It has been proposed that Bcl-2 may inhibit cell death by interfering with the function of proapoptotic Bcl-2 homologues, by repressing the release of cytochrome *c* from mitochondria, by the sequestration of caspase activators, such as Apaf-1, by interfering with the production of free radicals by cytotoxic agents, or by regulating intracellular calcium homeostasis (*35*−*37*). However, Bcl-2 does not protect cells from DNA damage elicited by DNA-damaging drugs (*6*, *38*, *39*). DNA damage is known to activate PARP (*40*), but in the cells overexpressing Bcl-2, PARP activity does not increase during apoptosis (*6*). Therefore, the results shown in Figure 4 suggest that Bcl-2 functions to inhibit apoptosis by blocking PARP activity in an S3a-dependent manner. This function of Bcl-2 is different from the previous belief that Bcl-2 governed the mitochondria-dependent apoptosis pathway by restraining the release of cytochrome *c* (the intrinsic pathway) (*41*, *42*). Thus, our findings may provide a novel insight into understanding how Bcl-2 inhibits apoptosis.

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