

# Proteomics Characterization of the Cytotoxicity Mechanism of Ganoderic Acid D and Computer-automated Estimation of the Possible Drug Target Network\*<sup>§</sup>

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Triterpenes isolated from *Ganoderma lucidum* could inhibit the growth of numerous cancer cell lines and were thought to be the basis of the anticancer effects of *G. lucidum*. Ganoderic acid D (GAD) is one of the major components in *Ganoderma* triterpenes. GAD treatment for 48 h inhibited the proliferation of HeLa human cervical carcinoma cells with an  $IC_{50}$  value of  $17.3 \pm 0.3 \mu M$ . Flow cytometric analysis and DNA fragmentation analysis indicated that GAD induced  $G_2/M$  cell cycle arrest and apoptosis. To identify the cellular targets of GAD, two-dimensional gel electrophoresis was performed, and proteins altered in expression level after GAD exposure of cells were identified by MALDI-TOF MS/MS. The regulation of proteins was also confirmed by Western blotting. The cytotoxic effect of GAD was associated with regulated expression of 21 proteins. Furthermore these possible GAD target-related proteins were evaluated by an *in silico* drug target searching program, INVDOCK. The INVDOCK analysis results suggested that GAD could bind six isoforms of 14-3-3 protein family, annexin A5, and aminopeptidase B. The direct binding affinity of GAD toward 14-3-3  $\zeta$  was confirmed *in vitro* using surface plasmon resonance biosensor analysis. In addition, the intensive study of functional association among these 21 proteins revealed that 14 of them were closely related in the protein-protein interaction network. They had been found to either interact with each other directly or associate with each other via only one intermediate protein from previous protein-protein interaction experimental results. When the network was expanded to a further interaction outward, all 21 proteins could be included into one network. In this way, the possible network associated with GAD target-related proteins was constructed, and the possible contribution of these proteins to the cytotoxicity of GAD is discussed in this report. *Molecular & Cellular Proteomics* 7:949–961, 2008.

*Ganoderma lucidum* is a medicinal mushroom known to the Chinese as “Lingzhi.” It has been used as a home remedy in traditional Chinese medicine (TCM)<sup>1</sup> for over 2000 years (1). In TCM, it was believed to preserve the human vitality and to promote longevity. More recently, it has been used for the prevention or treatment of a variety of diseases including cancer. And in Western countries, the dried powder of *G. lucidum* is also popularly used as a dietary supplement (2).

Among the reported biological/pharmacological properties of *G. lucidum*, their antitumor activities are of particular interest. Investigations into the anticancer activity of *G. lucidum* have been performed in both *in vitro* and *in vivo* studies, supporting its application for cancer treatment and prevention (for reviews, see Refs. 3 and 4). Polysaccharides and triterpenes are two major categories of the bioactive ingredients from *G. lucidum*, and it has been found previously that polysaccharides exert their anticancer effect mainly via an immune-modulatory mechanism, whereas triterpenes directly suppress growth and invasive behavior of cancer cells (5). Triterpenes were reported to be able to inhibit growth, induce apoptosis, and cause cell cycle arrest of cancer cells (6–9). However, the cytotoxicity mechanism of *Ganoderma* triterpenes is still far from clear. In the present study, ganoderic acid D (GAD), a main component of *Ganoderma* triterpenes, with purity greater than 99% was used. We checked the GAD-mediated response on the proliferation of HeLa human cervical carcinoma cells. Then for a comprehensive analysis of the molecular targets of GAD, a proteomics approach was used for identifying proteins altered in steady-state levels after exposure of HeLa cells to GAD for 48 h. 2-DE was conducted, and then differentially expressed proteins were identified by

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<sup>1</sup> The abbreviations used are: TCM, traditional Chinese medicine; GAD, ganoderic acid D; SPR, surface plasmon resonance; RU, response unit(s); eIF5A, eukaryotic translation initiation factor 5A; PRDX3, thioredoxin-dependent peroxide reductase mitochondrial precursor; 14-3-3E, 14-3-3  $\epsilon$ ; EB1, microtubule-associated protein RP/EB family member 1; AHA1, activator of heat shock 90-kDa protein ATPase homolog 1; PDI, protein-disulfide isomerase; 2-DE, two-dimensional gel electrophoresis; 3-D, three-dimensional; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMF, peptide mass fingerprint; PPI, protein-protein interaction.

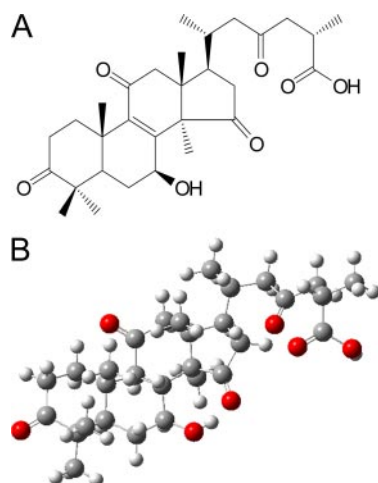


FIG. 1. **Structure of GAD.** A, The chemical structure (A) and 3-D structure (B) of GAD are shown.

MALDI-TOF MS/MS and further confirmed by Western blot analysis. Moreover a computational program, INVDOCK, was applied to verify the possible direct targets of GAD. The predicted binding between GAD and 14-3-3  $\zeta$  was then confirmed by using surface plasmon resonance (SPR) biosensor analysis. And finally a comprehensive network analysis was conducted to mine the functional association between the experimentally defined proteins.

#### EXPERIMENTAL PROCEDURES

##### Chemicals

GAD was isolated and purified from *G. lucidum* by the laboratory of TCM chemistry, Shanghai Research Center for Modernization of Traditional Chinese Medicine, Shanghai Institute of Materia Medica, Chinese Academy of Sciences as reported before (10). The structure of GAD (including the chemical structure and 3-D structure) is shown in Fig. 1. GAD was identified by spectral analyses, primarily NMR and MS, and comparison with previous literature (11). After identification, it was further purified by HPLC to yield authorized compound with a purity of at least 99%. The result of spectral analyses and HPLC analysis of GAD is shown in supplemental Figs. 1–4. All reagents used in 2-DE were purchased from Bio-Rad. Other chemicals, except where specially noted, were purchased from Sigma-Aldrich.

##### Cell Culture

The HeLa human cervical carcinoma cell line (CCL-2) was obtained from the American Type Culture Collection (Manassas, VA), and cells were cultured in minimum essential medium (Invitrogen) with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin (Invitrogen).

##### Cytotoxicity Assay

The cytotoxicity of GAD was determined by a calorimetric tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) assay as reported before (12). Briefly cells were plated in 96-well flat bottomed plates at a density of  $1 \times 10^3$  cells/well in complete medium and incubated overnight. Then the media were changed into fresh media containing various amounts of GAD for 24,

48, or 72 h. At the end of the incubation, 20  $\mu$ l of the dye MTT (5 mg/ml) was added to each well, and the plates were incubated for 3 h at 37  $^{\circ}$ C. Then 100  $\mu$ l of lysis buffer (20% SDS in 50% *N,N*-dimethylformamide containing 0.5% (v/v) 80% acetic acid and 0.4% (v/v) 1 N HCl) was added to each well and incubated overnight (16 h). Cell viability was evaluated by measuring the mitochondria-dependent conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The optical density (proportional to the number of live cells) was assessed with a Bio-Rad 550 microplate reader at 570 nm. Each experiment was performed in triplicate. Results of three independent experiments were used for statistical analysis.  $IC_{50}$  value (half-maximal inhibitory concentration) was calculated by the Logit method.

##### Flow Cytometric Analysis of Cell Cycle

Flow cytometric analysis of cell cycle was conducted as reported before (13). Briefly adherent and detached cells were harvested with trypsin, washed with PBS three times, and then fixed in ice-cold 70% ethanol at 4  $^{\circ}$ C for 2 h. After centrifugation at  $100 \times g$  for 2 min, cells were resuspended in propidium iodide stain buffer (0.1% Triton X-100, 10  $\mu$ g/ml DNase-free RNase A, and 50  $\mu$ g/ml propidium iodide in PBS) for 30 min in the dark. Flow cytometric analysis was conducted using a BD Biosciences FACStar Plus flow cytometer.

##### Imaging of Morphological Changes of GAD-treated Cells

To detect morphological changes in the apoptotic process, nuclear staining was performed as reported before (13). Briefly after treatment with GAD (10 or 50  $\mu$ M) for 48 h, cells were washed with PBS, and then fixed with 4% paraformaldehyde (pH 7.4) for 30 min at room temperature. After PBS washes, cells were stained with a 0.5 mg/ml solution of 4,6-diamido-2-phenylindole hydrochloride in PBS for 10 min at room temperature. The cells were washed twice with PBS and photographed using an Olympus UV light fluorescence microscope.

##### DNA Fragmentation Assay (DNA Ladder)

The integrity of the genomic DNA of the cells was assessed by agarose gel electrophoresis. Briefly after treatment with GAD (10 or 50  $\mu$ M) for 48 h, cells were washed with PBS and then collected by scraping. The cell genomic DNA was extracted using DNAzol (Invitrogen) and then loaded on 2% agarose gels for electrophoresis. The gels were stained with ethidium bromide (0.5 mg/l) and photographed under UV illumination.

##### 2-DE Analysis

**Sample Preparation**—For sample preparation, cells were cultured in 75-cm<sup>2</sup> flasks at a density of  $2 \times 10^5$  cells/flask. Cells at 70% confluency were incubated for 48 h with medium containing 0.1% DMSO (solvent control) or in addition with 10  $\mu$ M GAD. Subsequently cells were washed three times with ice-cold PBS and then scraped off with a cell scraper. Cells of two flasks were combined and subsequently centrifuged for 10 min at  $2500 \times g$ . The supernatant was discarded, and cell pellets were dissolved in 200  $\mu$ l of lysis buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 0.8% Pharylate, and protease inhibitor (all from Bio-Rad). Homogenization of the cells was achieved by ultrasonication (10 strokes, low amplitude) on ice. The lysed cells were centrifuged at  $15,000 \times g$  for 30 min at 4  $^{\circ}$ C, and the supernatant containing the solubilized proteins was used directly or stored at  $-80^{\circ}$ C. Protein samples from at least three independent experiments were collected for 2-DE assay.

**2-DE**—2-DE was carried out similarly to that described by Roberts *et al.* (14) using a Bio-Rad 2-DE system following the Bio-Rad handbook (15). Briefly a 150- $\mu$ g protein sample was applied for IEF using

the ReadyStrip IPG strips (17 cm, pH 4–7; Bio-Rad). The strips were placed into a Protean IEF cell (Bio-Rad) and were rehydrated at 50 V for 12 h, and then the proteins were separated based on their pI according to the following protocol: 250 V with linear climb for 30 min, 1000 V with rapid climb for 60 min, 10,000 V with linear climb for 5 h, and 10,000 V with rapid climb until 60,000 V-h was reached. After IEF, the IPG strips were equilibrated for 15 min in a buffer containing 50 mM Tris-HCl, pH 8.8, 30% glycerol, 7 M urea, 2% SDS, and 1% DTT followed by further treatment in a similar buffer (but containing 4% iodoacetamide instead of DTT) for 15 min and then directly applied onto 12% homogeneous SDS-PAGE gels for electrophoresis using a Protean II xi cell system (Bio-Rad). Furthermore two kinds of electrophoresis conditions, which were suitable for the separation of proteins with higher molecular weight (10 mA/gel for 30 min followed by 30 mA/gel for 5.5 h) and for the separation of proteins with lower molecular weight (10 mA/gel for 30 min followed by 20 mA/gel for 8 h), respectively, were both used. The gels were then silver-stained using Bio-Rad Silver Stain Plus kit reagents (Bio-Rad) according to the manufacturer's instructions.

#### Image Analysis and MALDI-TOF MS/MS

The silver-stained gels were scanned using a GS-800 densitometer (Bio-Rad) and then analyzed using PDQuest software (Bio-Rad). Paired (control and GAD-treated) protein samples from three independent experiments were analyzed by 2-DE. And for each pair of protein samples, triplicate electrophoreses were performed to ensure reproducibility. Comparisons were made between gel images of protein profiles obtained from the GAD-treated group and control group. The individual protein spot quantity was normalized as follows: the raw quantity of each spot in a member gel was divided by the total quantity of the valid spots in the gel, and normalized spot intensities were expressed in ppm. Quantitative analysis was performed using the Student's *t* test between protein gels from the control and GAD-treated group. The significantly differentially expressed protein spots ( $p < 0.05$ ) with 2-fold or more increased or decreased intensity between the control and GAD-treated group were selected and subjected to further identification by MALDI-TOF MS/MS.

Proteins of interest were excised from the gels with an EXQuest spot cutter (Bio-Rad) and placed into a 96-well microtiter plate. MS analysis was performed at the Institutes of Biomedical Sciences, Fudan University, Shanghai, China (16). Briefly gel pieces were destained with a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1:1) for 2 min at room temperature. Then the gel pieces were washed twice with deionized water and shrunk by dehydration in ACN. The samples were then swollen in a digestion buffer containing 25 mM ammonium bicarbonate and 12.5 ng/ $\mu$ l trypsin at 4 °C. After 30-min incubation, the gels were digested for more than 12 h at 37 °C. Peptides were then extracted twice using 0.1% TFA in 50% ACN. The extracts were dried under the protection of  $N_2$ . For MALDI-TOF MS/MS, peptides were mixed with 0.7  $\mu$ l of MALDI matrix (5 mg/ml  $\alpha$ -cyano-4-hydroxycinnamic acid diluted in 0.1% TFA and 50% ACN) and spotted onto the 192-well stainless steel MALDI target plates. MS measurements were carried out on an ABI 4700 Proteomics Analyzer with delayed ion extraction (Applied Biosystems). PMFs and peptide sequence spectra were obtained using the settings presented in supplemental Tables 1 and 2. The first five precursor ions with highest intensity were selected for fragmentation. The accelerated voltage was operated at 20 kV, and the positive ion mass spectra were recorded. MS accuracy was internally calibrated with trypsin-digested peptides of horse myoglobin. Using the individual PMF spectra, peptides exceeding a signal-to-noise ratio of 20 that passed through a mass exclusion filter (supplemental Table 3) were submitted to fragmentation analysis. MS/MS accuracy was calibrated against the MS/MS fragments of *m/z* 1606.85, which is one of the

peaks generated in myoglobin PMF. The parameters for peak matching were: minimum signal-to-noise ratio was 20, mass tolerance was 0.2 Da, minimum peaks to match reference masses was 4, and maximum outlier error was set to 100 ppm. The number of total shots for each PMF spectrum was 2000, whereas for MS/MS the total number of shots was 3000. All PMF and MS/MS peak list data were generated by GPS Explorer software 3.6 with parameter settings as summarized in supplemental Table 4. Data search files were generated according to the settings presented in supplemental Table 5 and submitted for protein homology identification by using the MASCOT 2.1 search engine (Matrix Science) against the *Homo sapiens* (human, 138,060 sequences) subset of the sequences in the National Center for Biotechnology non-redundant (NCBI nr) database (updated on March 17, 2007 with 4,736,044 sequences; 1,634,373,987 residues). Peptide differential modifications allowed during the search were carbamidomethylation of cysteines and oxidation of methionines. The maximum number of missed cleavages was set to 1 with trypsin as the protease. Protein homology identifications of the top hit (first rank) with a relative score exceeding 95% probability and additional hits (second rank or more) with a relative score exceeding 98% probability threshold were retained. The probability-based score, assuming that the observed match is significant ( $p < 0.05$ ), had to be more than 64 when submitting PMF data to the database and be more than 30 for individual peptide ions when submitting peptide sequence spectra. Proteins belonging to a protein family with multiple members were singled out based on the identification of unique and diagnostic peptides.

#### Western Blotting Analysis

As reported before (12), cells were washed three times with cold TBS, harvested using a cell scraper, and lysed in 10 volume of cold lysis buffer (50 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 5  $\mu$ g/ml aprotinin, and 5  $\mu$ g/ml leupeptin) on ice. Lysates were centrifuged, and then the supernatant protein was denatured by mixing with an equal volume of 2 $\times$  sample loading buffer and then boiling at 100 °C for 5 min. An aliquot (containing 50  $\mu$ g protein) of the supernatant was loaded onto a 12% SDS gel, separated electrophoretically, and transferred to a PVDF membrane (Bio-Rad). After the PVDF membrane was incubated with 10 mM TBS with 1.0% Tween 20 and 10% dehydrated skim milk to block nonspecific protein binding, the membrane was incubated with primary antibodies overnight at 4 °C. The primary antibodies used were mouse anti-eIF-5A monoclonal antibody (1:1000; BD Biosciences), rabbit anti-14-3-3E polyclonal antibody (1:1000; Abgent, San Diego, CA), rabbit anti-PRDX3 polyclonal antibody (1:1000; Proteintech Group, Chicago, IL), mouse anti-EB1 monoclonal antibody (1:500; BD Biosciences), and mouse anti-actin monoclonal antibody (1:2000; Sigma). Blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) or horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) for 1 h at room temperature at a 1:5000 dilution and then visualized using chemiluminescence (Pierce).

#### Statistical Analysis

Significances of difference between groups were determined by a non-paired Student's *t* test. For each variable three independent experiments were carried out. Data are given as the mean  $\pm$  S.D.

#### Identification of Potential Protein Targets for GAD

To verify the proteins related to possible GAD targets derived from the experimental results, a flexible ligand-protein inverse docking program, INVDOCK, was adopted that can predict proteins directly binding with a small molecule through an automatic search of every entry in a protein cavity database (17). To save the computing time, a

subset of the cavity database was derived from the 3-D structures of all the experimentally derived proteins beforehand. And this small dataset, instead of the huge cavity database derived from all Protein Data Bank entries, was used to run INVDOCK. Those proteins containing the cavities hit by the GAD molecule were predicted as possible protein targets of GAD.

### SPR Biosensor Analysis

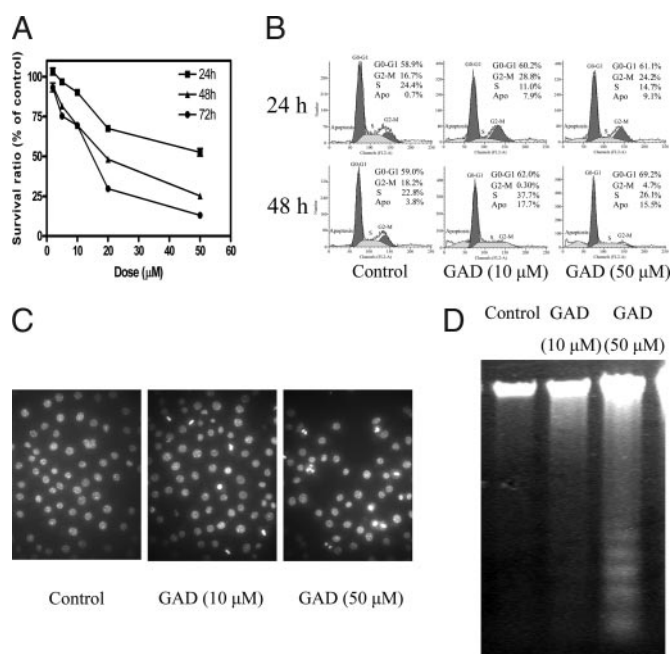
The binding affinity of GAD to 14-3-3  $\zeta$  *in vitro* was assayed by the Drug Discovery and Design Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences using an SPR-based Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) as reported before (18, 19). Human recombinant GST-14-3-3  $\zeta$  protein expressed in *Escherichia coli* (molecular mass, 55 kDa; pI 5.36 in PBS) with a purity of more than 90% was bought from Calbiochem. The manufacturer indicated that it could be used in *in vitro* binding assays. Human recombinant GST expressed in *E. coli* (molecular mass, 27 kDa; pI 8.91 in PBS) was a gift from Prof. Jia Li (Shanghai Institute of Materia Medica, Chinese Academy of Sciences) and used as control in the SPR analysis. Both the GST-14-3-3  $\zeta$  and GST protein were dissolved in coupling buffer (15  $\mu$ g/ml, in 10 mM sodium acetate, pH 4.36) and immobilized onto the same sensor chip but on different flow cells. The GST-14-3-3  $\zeta$  and GST protein were immobilized on a CM5 sensor chip as ligand in 8000 response units (RU) with *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide according to the standard primary amine-coupling procedures, and HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4) was used as the running buffer. Equilibration of the base line was performed by a continuous flow of HBS-EP through the chip surface for 1–2 h. Biacore data were collected at 25 °C with HBS-EP as the running buffer at a constant flow of 30  $\mu$ l/min. GAD was serially diluted into the running buffer to a final DMSO concentration of 0.5%. The samples were injected into the channels at a flow rate of 30  $\mu$ l/min followed by washing with the running buffer. The binding responses were recorded continuously in RU at a frequency of 1 Hz as sensorgrams and presented as a function of time. The association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rate constants and the equilibrium dissociation constant ( $K_D = k_{off}/k_{on}$ ) were determined by analysis of the sensorgram curves obtained at different concentrations of GAD by use of BIA evaluation software version 3.1 (Biacore) and the 1:1 Langmuir binding fitting model. The curve fitting efficiency was evaluated by statistical parameter  $\chi^2$ .

### Network Construction and Simplification for Protein Association

Various on-line databases containing experimental information of protein interactions and associations have been set up with the development of high throughput proteomics technology (20). A PPI network was mapped among those experimentally derived proteins based on the collective information retrieved through exhaustive search from these resources. The direct partners interacting with our experimental proteins were further used as a new query seed to fish out another round of partner proteins. Through this way, the network was expanded step by step until the proteins of interest could be included into the network. Then for better clarification, the network was simplified to a minimum network containing experimentally derived proteins through the Steiner minimal tree algorithm (21).

## RESULTS

**Cytotoxic Effects by GAD Treatment in Carcinoma Cells**—As shown in Fig. 2A, after treatment of the cells with increasing concentrations (1, 5, 10, 20, and 50  $\mu$ M) of GAD for 24, 48, and 72 h, the cell survival rate of cells was reduced in



**Fig. 2. Effect of GAD on HeLa cell viability, cell cycle arrest, and apoptosis.** A, HeLa cells were treated with 1, 5, 10, 20, and 50  $\mu$ M GAD for 24, 48, and 72 h, and cell viability was determined by MTT assay. B, DNA histograms of HeLa cells obtained by flow cytometry analysis. Accumulation in  $G_2/M$  phase was observed in 10 and 50  $\mu$ M GAD-treated cells after 24-h treatment. An increase in the percentage of apoptotic cells was observed in GAD-treated cells after 48-h treatment. C, morphological change induced by 10 and 50  $\mu$ M GAD in HeLa cells after 48-h treatment ( $\times 600$  magnification). Typical apoptotic morphological change in GAD-treated cells was observed. D, DNA fragmentation induced by 10 and 50  $\mu$ M GAD in HeLa cells after 48-h treatment. Typical apoptotic DNA fragmentation (DNA ladder) was observed in HeLa cells treated with 50  $\mu$ M GAD. Shown are representative results of three independent experiments.

a dose- and time-dependent manner. The  $IC_{50}$  value of GAD was  $17.3 \pm 0.3 \mu$ M for 48-h treatment. Furthermore as shown in Fig. 2B, the representative DNA histograms of HeLa cells exposed to GAD showed that GAD at 10 and 50  $\mu$ M both induced  $G_2/M$  phase arrest and apoptosis. For example, cells treated with 10  $\mu$ M GAD displayed a cell cycle profile with an elevated  $G_2/M$  cell population after 24-h treatment (16.7 and 28.8% for control and GAD-treated, respectively). At this time, the apoptosis rates of cells were 0.7 and 7.9% for control and 10  $\mu$ M GAD-treated, respectively. This indicated that some cells were arrested in  $G_2/M$  phase with no significant change in cell viability (about 90%, as shown in Fig. 2A). After 48 h treatment, the  $G_2/M$  cell population was 0.30% in the 10  $\mu$ M GAD-treated group and 18.2% in the control group. At the same time, the apoptosis rate was 17.7% in the 10  $\mu$ M GAD-treated group and 3.8% in the control group. The possible reason for this is that the cells blocked in  $G_2/M$  phase underwent apoptosis and eventually died after extended culture without progressing to mitosis. And the total cell viability also markedly decreased (about 69%, as shown in Fig. 2A) at the time point of 48 h. Apoptosis of cells induced by GAD also

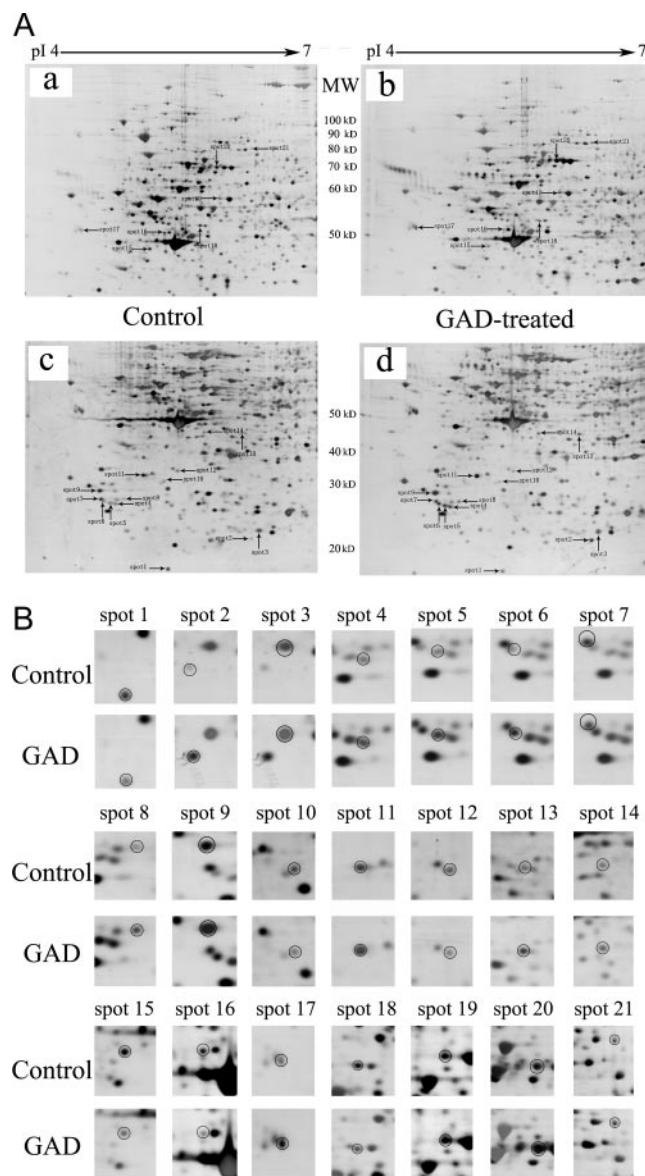
could be characterized by nuclear fragmentation and chromatin condensation. As shown in Fig. 2C, treatment with 10  $\mu\text{M}$  GAD or 50  $\mu\text{M}$  GAD for 48 h induced a morphological change typical of apoptosis in HeLa cells. The result of the DNA ladder assay (Fig. 2D) indicated that treatment with 50  $\mu\text{M}$  GAD for 48 h induced typical apoptosis-related DNA fragmentation (ladder) in HeLa cell genomic DNA.

**2-DE of Control and GAD-treated HeLa Cells**—To further investigate the mechanism of cell toxicity induced by GAD, protein profiles of control and GAD-treated cells were studied by comparative proteomics analysis. Representative two-dimensional gel images of control and GAD-treated cells are shown in Fig. 3A. To identify more protein spots, two kinds of electrophoresis conditions were used. *Panels a* and *b* are the gel images with better separation of higher molecular weight proteins, whereas *panels c* and *d* are the gel images with better separation of lower molecular weight proteins. Each gel resolved up to 700 protein spots. The proteome maps of control and GAD-treated cells were compared with PDQuest software to identify the protein spot variations. After GAD treatment, significantly differentially expressed protein spots ( $p < 0.05$ ) with 2-fold or more increased or decreased intensity as observed in all nine replicate gels were scored. Seven down-regulated protein spots and 14 up-regulated protein spots were found as indicated by the spots marked with arrows in Fig. 3A and by the expanded plots in Fig. 3B. Table I shows the average intensity values and their standard deviations of the spots, the statistical assay results, and the -fold differences between the control and GAD-treated group. The -fold difference is represented by the ratio of the intensity value of the GAD-treated group to the value of the control group.

**Identification of the Differentially Expressed Proteins**—After analyzing the two-dimensional gels, peptides were extracted from each differentially expressed protein spot by in-gel tryptic digestion, and proteins were identified using MS/MS. The results of MS/MS analysis are summarized in Table II. The protein score, coverage, number of identified peptides, and best ion score of each spot are also shown in Table II. The result of MALDI-TOF MS/MS analysis of spot 9 is shown in Fig. 4 as an example.

**Confirmation of Differentially Expressed Proteins by Western Blotting**—Western blotting was used to assess the expression of eukaryotic translation initiation factor 5A (eIF5A), 14-3-3E, thioredoxin-dependent peroxide reductase mitochondrial precursor (PRDX3), and microtubule-associated protein RP/EB family member 1 (EB1) in control and GAD-treated HeLa cells. Consistent with the proteomics results, eIF5A and EB1 were found to be down-regulated whereas 14-3-3E and PRDX3 were found to be up-regulated in GAD-treated HeLa cells (Fig. 5).

**Identification of Potential Protein Targets for GAD**—Among the 21 proteins derived from the experiments, 19 of them (except 26 S proteasome subunit p40.5 and mitofilin) have



**FIG. 3. The proteome maps (2-DE images) of GAD-treated HeLa cells.** A, *panels a* and *b*, are 2-DE images with better separation of higher molecular weight proteins of control and GAD-treated HeLa cells, respectively. *Panels c* and *d* are 2-DE images with better separation of lower molecular weight proteins of control and GAD-treated HeLa cells, respectively. GAD-treated HeLa cells were treated with 10  $\mu\text{M}$  GAD for 48 h. The gel pair is the representative gel of nine replicate gels collected from three independent experiments. Differentially expressed spots are shown by the arrows. B, the expanded region of differentially expressed protein spots. The proteins within the circles are the differentially expressed proteins.

Protein Data Bank structures. Because the premise of searching for GAD targets through the INVDOCK program is the 3-D structure of the protein, we are not able to compute whether they can bind to GAD or not if the proteins do not have 3-D structures. Currently INVDOCK results suggest that eight of the 19 proteins with Protein Data Bank structures can bind with the GAD molecule directly. The ligand-protein interaction

TABLE I  
Summary of differentially expressed proteins in GAD-treated HeLa cells

| Spot | Pairs of gels (n) | Spot volume                  |                                  | -Fold difference | p value |
|------|-------------------|------------------------------|----------------------------------|------------------|---------|
|      |                   | Control<br>(mean $\pm$ S.D.) | GAD-treated<br>(mean $\pm$ S.D.) |                  |         |
|      |                   | ppm                          |                                  |                  |         |
| 1    | 9                 | 2749.6 $\pm$ 184.8           | 1266.3 $\pm$ 260.0               | 0.46             | <0.05   |
| 2    | 9                 | 465.7 $\pm$ 115.3            | 4885.3 $\pm$ 552.6               | 10.49            | <0.05   |
| 3    | 9                 | 2614.6 $\pm$ 735.4           | 8326.3 $\pm$ 1727.1              | 3.18             | <0.05   |
| 4    | 9                 | 1517.3 $\pm$ 491.1           | 4684.3 $\pm$ 1367.8              | 3.09             | <0.05   |
| 5    | 9                 | 1478.0 $\pm$ 483.6           | 5152.1 $\pm$ 1598.4              | 3.49             | <0.05   |
| 6    | 9                 | 1629.2 $\pm$ 642.3           | 5153.0 $\pm$ 1586.8              | 3.16             | <0.05   |
| 7    | 9                 | 3418.4 $\pm$ 481.1           | 1453.7 $\pm$ 343.4               | 0.43             | <0.05   |
| 8    | 9                 | 963.7 $\pm$ 75.3             | 2913.7 $\pm$ 511.4               | 3.02             | <0.05   |
| 9    | 9                 | 3774.1 $\pm$ 1725.8          | 9645.6 $\pm$ 2282.7              | 2.56             | <0.05   |
| 10   | 9                 | 1308.2 $\pm$ 291.9           | 519.3 $\pm$ 162.7                | 0.40             | <0.05   |
| 11   | 9                 | 1842.9 $\pm$ 660.9           | 5574.3 $\pm$ 1569.6              | 3.02             | <0.05   |
| 12   | 9                 | 1189.2 $\pm$ 153.3           | 522.3 $\pm$ 160.3                | 0.44             | <0.05   |
| 13   | 9                 | 423.7 $\pm$ 29.5             | 1722.3 $\pm$ 160.1               | 4.06             | <0.05   |
| 14   | 9                 | 475.7 $\pm$ 159.6            | 1529.7 $\pm$ 217.1               | 3.22             | <0.05   |
| 15   | 9                 | 3262.0 $\pm$ 303.4           | 1456.1 $\pm$ 384.8               | 0.45             | <0.05   |
| 16   | 9                 | 2307.7 $\pm$ 339.7           | 1010.3 $\pm$ 219.8               | 0.44             | <0.05   |
| 17   | 9                 | 2263.7 $\pm$ 300.6           | 4950.3 $\pm$ 646.7               | 2.19             | <0.05   |
| 18   | 9                 | 2726.3 $\pm$ 275.6           | 1267.3 $\pm$ 171.8               | 0.46             | <0.05   |
| 19   | 9                 | 697.7 $\pm$ 33.6             | 1488.7 $\pm$ 135.7               | 2.13             | <0.05   |
| 20   | 9                 | 1531.7 $\pm$ 114.4           | 3520.6 $\pm$ 317.4               | 2.30             | <0.05   |
| 21   | 9                 | 192.7 $\pm$ 64.4             | 711.0 $\pm$ 215.3                | 3.69             | <0.05   |

TABLE II  
The results of protein identifications of differentially expressed proteins using MALDI-TOF MS/MS

| Spot | Target protein                                                      | NCBI<br>accession<br>no. | Theoretical<br>molecular<br>mass<br>(kDa)/pI | Protein<br>score | Sequence<br>coverage | Number of<br>peptides<br>matched/<br>unmatched | Unique<br>peptides | Best<br>ion<br>score |
|------|---------------------------------------------------------------------|--------------------------|----------------------------------------------|------------------|----------------------|------------------------------------------------|--------------------|----------------------|
|      |                                                                     |                          |                                              |                  | %                    |                                                |                    |                      |
| 1    | Translation initiation factor 5A (eIF5A)                            | 54037409                 | 16.7/5.08                                    | 334              | 35                   | 19/11                                          | 3                  | 153                  |
| 2    | Ephrin receptor EphA7 (EphA7)                                       | 4758282                  | 31.8/5.14                                    | 82               | 56                   | 4/11                                           | 2                  | 102                  |
| 3    | PRDX3                                                               | 2507171                  | 27.7/7.67                                    | 171              | 19                   | 18/15                                          | 2                  | 113                  |
| 4    | 14-3-3 $\beta/\alpha$                                               | 1345590                  | 28.1/4.76                                    | 137              | 62                   | 8/7                                            | 3                  | 90                   |
| 5    | 14-3-3 $\zeta/\delta$                                               | 68085578                 | 27.7/4.73                                    | 252              | 46                   | 27/23                                          | 2                  | 84                   |
| 6    | 14-3-3 $\theta$                                                     | 54696890                 | 29.1/5.17                                    | 126              | 27                   | 17/12                                          | 2                  | 39                   |
| 7    | 14-3-3 $\sigma$                                                     | 16306737                 | 24.3/4.77                                    | 78               | 37                   | 6/12                                           | 2                  | 40                   |
| 8    | 14-3-3 $\gamma$                                                     | 5726310                  | 28.3/4.66                                    | 73               | 29                   | 9/31                                           | 2                  | 33                   |
| 9    | 14-3-3 $\epsilon$ (14-3-3E)                                         | 51702210                 | 29.2/4.63                                    | 296              | 40                   | 26/10                                          | 3                  | 88                   |
| 10   | EB1                                                                 | 20138589                 | 29.8/5.02                                    | 93               | 39                   | 15/23                                          | 3                  | 67                   |
| 11   | Annexin A5                                                          | 113960                   | 35.8/4.94                                    | 648              | 61                   | 28/9                                           | 4                  | 204                  |
| 12   | Spermidine synthase                                                 | 134811                   | 33.8/5.30                                    | 139              | 30                   | 21/11                                          | 3                  | 46                   |
| 13   | 26 S proteasome subunit p40.5                                       | 3618343                  | 42.9/5.53                                    | 183              | 27                   | 16/16                                          | 3                  | 43                   |
| 14   | AHA1                                                                | 6912280                  | 38.2/5.41                                    | 137              | 28                   | 10/7                                           | 2                  | 59                   |
| 15   | Cytokeratin 19                                                      | 24234699                 | 44.1/5.04                                    | 96               | 34                   | 19/24                                          | 4                  | 64                   |
| 16   | Cytokeratin 1                                                       | 1346343                  | 66.0/8.16                                    | 94               | 49                   | 9/31                                           | 2                  | 31                   |
| 17   | Calumenin                                                           | 2809324                  | 37.0/4.47                                    | 196              | 34                   | 9/11                                           | 3                  | 36                   |
| 18   | Ubiquinol-cytochrome c reductase core I<br>protein (Core I protein) | 515634                   | 52.6/5.94                                    | 109              | 15                   | 11/26                                          | 2                  | 43                   |
| 19   | PDI                                                                 | 860986                   | 56.6/6.10                                    | 98               | 22                   | 12/13                                          | 2                  | 30                   |
| 20   | Aminopeptidase B                                                    | 40316915                 | 72.5/5.51                                    | 80               | 15                   | 17/38                                          | 2                  | 42                   |
| 21   | Mitofilin                                                           | 8131894                  | 83.6/6.08                                    | 124              | 41                   | 16/2                                           | 2                  | 33                   |

energy values of binding between GAD and the eight proteins are listed in Table III. Interestingly six of them (14-3-3  $\zeta/\delta$ , 14-3-3  $\beta/\alpha$ , 14-3-3  $\theta$ , 14-3-3  $\sigma$ , 14-3-3  $\gamma$ , and 14-3-3  $\epsilon$ ) belong to the same 14-3-3 family. The other two binding proteins are

annexin A5 and aminopeptidase B. The conformation of the GAD molecule binding with 14-3-3  $\zeta$  (Protein Data Bank code 1qja) is shown in Fig. 6 as an illustration. In fact, GAD was predicted to accept three cavities in 14-3-3 proteins theoret-

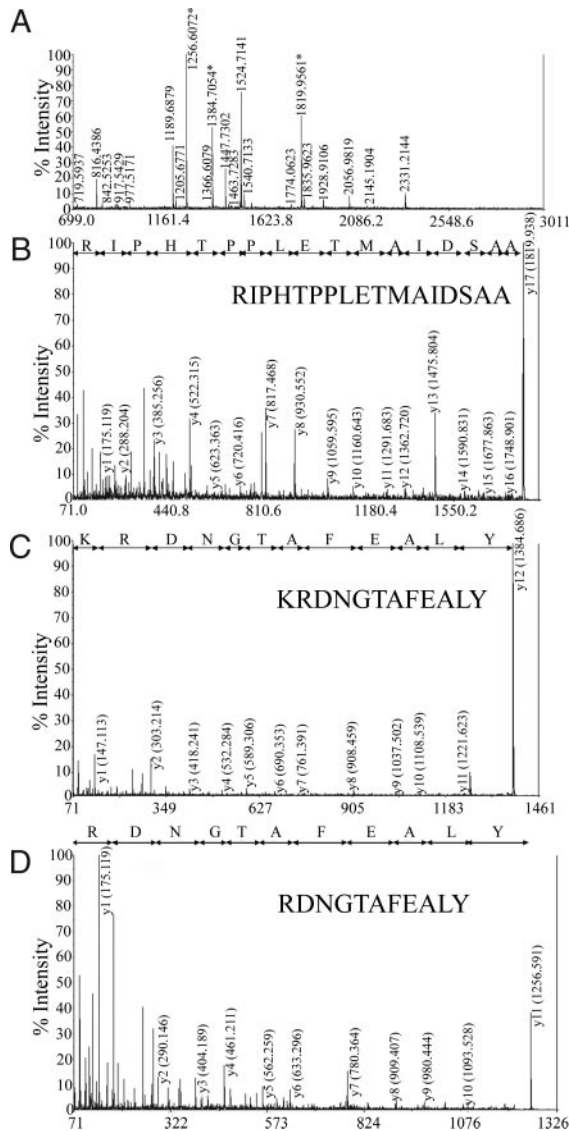


FIG. 4. The result of the MALDI-TOF MS/MS analysis of protein that marked as spot 9 in Fig. 1. The protein was identified to be human 14-3-3E by protein database search. A, peptide mass fingerprint of the tryptic digest of spot 9. \* indicates unique peptides further identified by MS/MS. B, MS/MS profile of the peptide with a mass of 1819.95 Da. C, MS/MS profile of the peptide with a mass of 1384.70 Da. D, MS/MS profile of the peptide with a mass of 1256.61 Da. y-ions resulting from fragmentation of the peptides and amino acids they represent are indicated.

ically, including the cavity between the homodimers and the two symmetrical phosphopeptide binding sites in the two monomers. But INVDOCK does not compare and choose which cavity is the best to accommodate a chemical molecule among several putative cavities in a protein structure. In reality, there is the possibility for the GAD molecule to choose only one type of cavity to attach. According to a previous report (22), the phosphopeptide binding sites of binding sites of proteins belong to 14-3-3 family are the common sites that could be occupied by client proteins. So in Fig. 6, only the

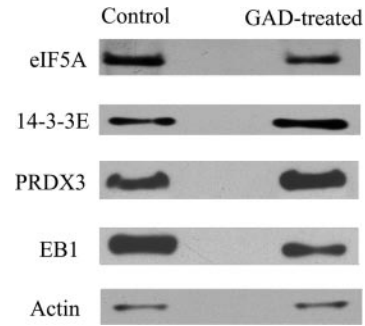


FIG. 5. Western blotting of eIF5A, 14-3-3E, PRDX3, and EB1. "Control" and "GAD-treated" above the panel represent the control cells and HeLa cells treated with 10  $\mu$ M GAD for 48 h, respectively. Each blot is the representative result of three independent experiments.

TABLE III  
The INVDOCK-predicted binding between GAD and proteins

| Number | Protein               | Protein Data Bank identification number | Ligand-protein interaction energy value |
|--------|-----------------------|-----------------------------------------|-----------------------------------------|
| 1      | 14-3-3 $\zeta$        | 1qja                                    | -48.6                                   |
| 2      | 14-3-3 $\beta/\alpha$ | 2c23                                    | -37.4                                   |
| 3      | 14-3-3 $\theta$       | 2btp                                    | -48.5                                   |
| 4      | 14-3-3 $\sigma$       | 1ywt                                    | -56.1                                   |
| 5      | 14-3-3 $\gamma$       | 2b05                                    | -41.5                                   |
| 6      | 14-3-3 $\epsilon$     | 2br9                                    | -45.2                                   |
| 7      | Aminopeptidase B      | 1hs6                                    | -49.3                                   |
| 8      | Annexin A5            | 1sav                                    | -39.2                                   |

docking model of GAD with the phosphopeptide binding sites in the two monomers is shown.

**The Binding Affinity of GAD Toward 14-3-3  $\zeta$  Estimated by SPR Biosensor Analysis**—To verify the prediction from INVDOCK analysis that GAD could bind directly to 14-3-3 proteins, the binding affinity of GAD toward 14-3-3  $\zeta$  was determined by using SPR biosensor technology. The binding ability of GAD toward 14-3-3  $\zeta$  was reflected by RU values recorded directly by the Biacore 3000 instrument. As shown in Fig. 7A, RU increased with increasing GAD concentration, indicating that GAD was able to bind to 14-3-3  $\zeta$  in a dose-dependent manner. The association ( $k_{on}$ ), dissociation ( $k_{off}$ ), and equilibrium dissociation ( $K_D$ ) constants of GAD binding to the immobilized GST-14-3-3  $\zeta$  were  $(5.02 \pm 0.24) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ,  $(2.03 \pm 0.17) \times 10^{-2} \text{ s}^{-1}$ , and  $(4.04 \pm 0.32) \times 10^{-6} \text{ M}$ , respectively. The curve fitting efficiency was evaluated by statistical parameter  $\chi^2$ , a statistical parameter in the SPR assay. The  $\chi^2$  value was calculated to be 0.32. In a control study, GAD was injected over the immobilization GST surface, and the result exhibited weak nonspecific binding affinity as shown in Fig. 7B. The  $k_{on}$ ,  $k_{off}$ ,  $K_D$ , and  $\chi^2$  values of GAD binding to the GST were  $(3.62 \pm 0.15) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ,  $(2.92 \pm 0.21) \times 10^{-2} \text{ s}^{-1}$ ,  $(8.06 \pm 0.37) \times 10^{-6} \text{ M}$ , and 0.15, respectively. The nonspecific interaction between GAD and GST protein might be partly caused by the attraction between

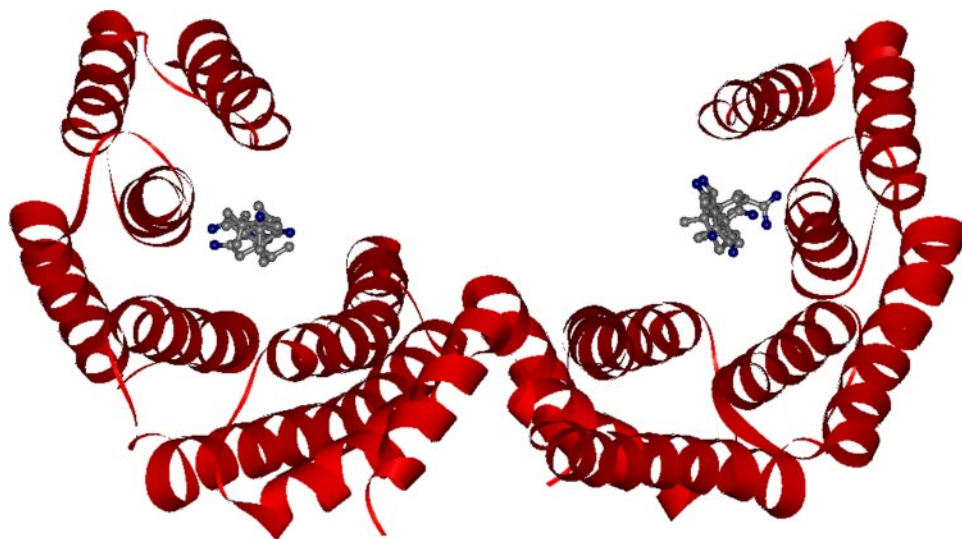


FIG. 6. Illustration of GAD molecule docked into 14-3-3  $\zeta$  protein by INVDOCK program. The Protein Data Bank code for 14-3-3  $\zeta$  protein is 1qja. The GAD molecule is displayed in ball and stick model; the protein is displayed in ribbon model.

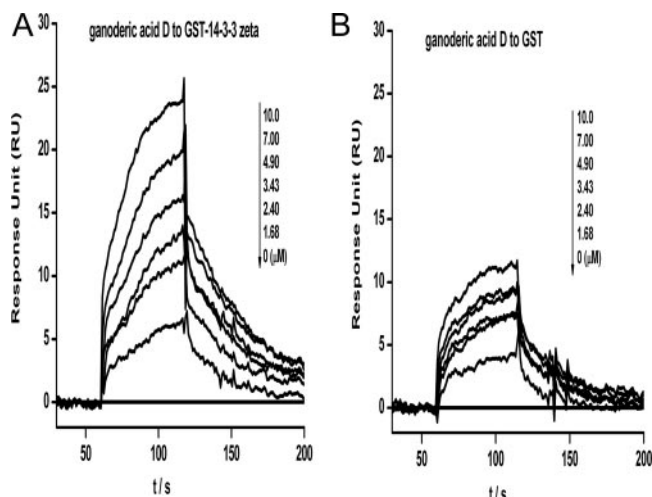


FIG. 7. Binding affinity of GAD to the GST-14-3-3  $\zeta$  protein and GST protein (control) determined by SPR. Real time binding affinity measurements of GAD to the GST-14-3-3  $\zeta$  protein (A) and the GST protein (B) using Biacore 3000 were carried out. Representative sensorgrams were obtained from injections of GAD at concentrations of 10, 7, 4.9, 3.43, 2.40, and 1.68  $\mu\text{M}$  (curves from top to bottom) over the immobilized GST-14-3-3  $\zeta$  or GST (control) surface. The GAD was injected for 60 s, and dissociation was monitored for more than 120 s.

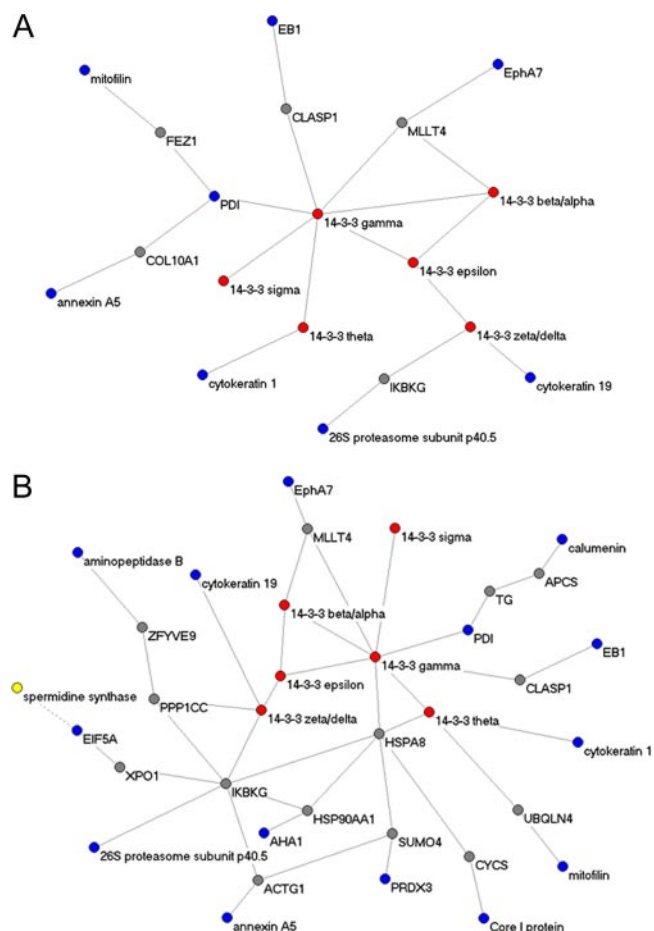
negatively charged GAD and positively charged GST protein with pI 8.91 in SPR running buffer at pH 7.4. For GST-14-3-3  $\zeta$  protein, the situation might be different because its pI was 5.36. Furthermore at the same concentration, binding between GAD and GST-14-3-3  $\zeta$  showed higher RU values than that of GAD and GST (Fig. 7). The results indicated that, although there might be nonspecific binding between GAD and GST-14-3-3  $\zeta$ , GAD had specific binding affinity toward 14-3-3  $\zeta$ .

**Protein Association Network among the Identified 21 Proteins**—Among the 21 proteins, 14 of them can link together

into one network through direct interaction or only one intermediate partner at the PPI level as shown in Fig. 8A. When this network is expanded one step further, 20 proteins can fall into one large network that suggests the inherent correlation among all of them. According to database searching, the spermidine synthase protein could not be covered automatically in the network. However, according to the published research studies, it is well known that the product of spermidine synthase, spermidine, is indispensable to the synthesis of eIF5A protein (23). By adding this association manually, the minimum protein-protein interaction network among all 21 proteins presents a picture as shown in Fig. 8B. The full names of the intermediate partners in the network are shown in Table IV.

#### DISCUSSION

*G. lucidum* (Lingzhi) is a popular Asian mushroom that has been used for more than 2 millennia for the general promotion of health and was therefore called the “mushroom of immortality.” Recently the dried powder of *G. lucidum* was recommended as a complementary cancer therapy agent in traditional Chinese medicine. This investigation found that GAD, one of the major components in *Ganoderma* triterpenes, could impede the proliferation of HeLa human cervical carcinoma cells. In addition, the results of the flow cytometry assay and nuclear DNA fragmentation assay indicated that GAD might induce cell cycle arrest at G<sub>2</sub>/M phase and trigger apoptosis in HeLa cells. Similarly previous studies (24–26) also showed that *G. lucidum* extract could induce apoptosis in cancer cells. As to the cell cycle arrest, our results were consistent with the previous reports (Muller *et al.* (24), Lu *et al.* (27), Jiang *et al.* (25), and Lin *et al.* (6)) that the cell cycle was arrested at G<sub>2</sub>/M phase. However, Jiang *et al.* (28) and Yang (7) showed that there was a G<sub>0</sub>/G<sub>1</sub> phase arrest after *G. lucidum* treatment. One possible reason for the difference is



**FIG. 8. The constructed minimum protein-protein interaction network.** The red dots illustrate 14-3-3 proteins; the blue dots are other proteins identified from experiments. Proteins in the network are interacting with each other via intermediate partners (shown in gray) from known PPI information. A, the network constructed by 14 identified proteins. The 14 proteins can link together into one network through direct interaction or only one intermediate partner. B, the expanded network constructed by 21 identified proteins. 20 of the proteins can link together into one network through no more than two intermediate partners. Compared with A, this network was expanded one step further. The yellow dot is the protein (spermidine synthase) manually added according to the reported relation with protein eIF5A. Symbols and full names of the intermediate partners in the network are shown in Table IV.

the use of different cell lines. For example, Jiang *et al.* (28) found that *G. lucidum* induced G<sub>0</sub>/G<sub>1</sub> phase arrest in MDA-MB-231 breast cancer cells but induced G<sub>2</sub>/M phase arrest in PC-3 prostate cancer cells (25).

Mechanistically the cytotoxic effects of *G. lucidum* have been implicated in the (i) down-regulation of Akt/NF- $\kappa$ B signaling and thus the expression of NF- $\kappa$ B-regulated cyclin D1 (28), (ii) up-regulation of expression of p21 and Bax (25, 26), and (iii) suppression of protein kinase C and activation of mitogen-activated protein kinases (6). However, the molecular mechanism in which *G. lucidum*, especially *Ganoderma* triterpenes, induces proliferation inhibition, apoptosis, and cell cycle arrest is still not clear.

This study implemented the proteomics scheme to search globally for the differentially expressed proteins in HeLa cells affected by GAD, a purified *Ganoderma* triterpene.

In the present study, 21 proteins whose expressions were significantly changed under GAD treatment were identified. Among the 19 proteins with three-dimensional structures, eight of them were predicted by INVDOCK analysis to possess the ability of binding to GAD. The proteins identified in the proteomics study might include both direct targets and downstream regulated proteins. Proteins that can directly bind to GAD might be considered as possible direct targets of GAD. Among the eight proteins that were predicted by INVDOCK to be able to bind directly to GAD, the most interesting proteins were the six members of the 14-3-3 family, *i.e.* 14-3-3  $\zeta/\delta$ , 14-3-3  $\beta/\alpha$ , 14-3-3  $\theta$ , 14-3-3  $\sigma$ , 14-3-3  $\gamma$ , and 14-3-3  $\epsilon$ . The 14-3-3 protein family is a family of highly conserved dimeric phosphoserine-binding proteins. In mammals, there are nine homologous members including 14-3-3  $\beta/\alpha$ , 14-3-3  $\gamma$ , 14-3-3  $\epsilon$ , 14-3-3  $\eta$ , 14-3-3  $\sigma$ , 14-3-3  $\theta$ , and 14-3-3  $\zeta/\delta$ . The 14-3-3  $\alpha$  and  $\delta$  are the phosphorylated forms of 14-3-3  $\beta$  and  $\zeta$ , respectively. The binding affinity of GAD toward 14-3-3  $\zeta$  was confirmed in the present study using SPR biosensor analysis. Interestingly the results of network construction also suggested the central role of 14-3-3 proteins in all proteins identified in the proteomics study. The results suggested that 14-3-3 proteins might play important roles in the cytotoxicity mechanism of GAD. The prediction that 14-3-3 proteins are possible direct targets of GAD also supports the previous study results (6, 25, 26, 28) about the cytotoxicity mechanism of *G. lucidum*. It is well known that 14-3-3 proteins are involved in many different cellular processes, including mitogenesis, cell cycle control, and apoptosis. Several models for how 14-3-3 proteins function have been recently proposed: 1) 14-3-3 proteins can modulate the biochemical activity of certain ligands like Raf-1, protein kinase C, and tryptophan hydroxylase; 2) 14-3-3 can affect the activity of ligands such as BAD, FKHRL1, and Cdc25 by altering their intracellular localization; and 3) 14-3-3 proteins might function as novel adapters or scaffold molecules (29–31). So by regulating 14-3-3 proteins, GAD might suppress protein kinase C (14-3-3 proteins were previously called “protein kinase C inhibitor protein 1”), consistent with the reported suppression of protein kinase C by *G. lucidum* (6). And by regulating 14-3-3 proteins, GAD might adjust some apoptosis-related proteins and some cell cycle-related proteins, consistent with the report of Hu *et al.* (26) and Jiang *et al.* (28), respectively.

In the present study, GAD treatment also caused the regulation of 15 other proteins besides 14-3-3 proteins. Briefly based on their biological functions, these 15 proteins could be generally classified into one of the following four categories: 1) cell survival and proliferation, 2) cell death and protein degradation, 3) metabolism, and 4) cytoskeleton structure. Note that some proteins may have multiple functions and play roles in more than one pathway.

TABLE IV  
Symbols and full names of the intermediate partners in the network shown in Fig. 8

| Number | Symbol   | Full name                                                                                             |
|--------|----------|-------------------------------------------------------------------------------------------------------|
| 1      | ACTG1    | Actin, $\gamma$ 1                                                                                     |
| 2      | APCS     | Amyloid P component, serum                                                                            |
| 3      | CLASP1   | Cytoplasmic linker-associated protein 1                                                               |
| 4      | COL10A1  | Collagen, type X, $\alpha$ 1 (Schmid metaphyseal chondrodysplasia)                                    |
| 5      | CYCS     | Cytochrome c, somatic                                                                                 |
| 6      | FEZ1     | Fasciculation and elongation protein $\zeta$ 1 (zyglin I)                                             |
| 7      | HSPA8    | Heat shock 70-kDa protein 8                                                                           |
| 8      | HSP90AA1 | Heat shock protein 90-kDa $\alpha$ , class A member 1                                                 |
| 9      | IKBKG    | Inhibitor of $\kappa$ light polypeptide gene enhancer in B-cells, kinase $\gamma$                     |
| 10     | MLLT4    | Myeloid/lymphoid or mixed lineage leukemia (trithorax homolog, <i>Drosophila</i> ); translocated to 4 |
| 11     | PPP1CC   | Protein phosphatase 1, catalytic subunit, $\gamma$ isoform                                            |
| 12     | SUMO4    | SMT3 suppressor of mif two 3 homolog 4 ( <i>Saccharomyces cerevisiae</i> )                            |
| 13     | TG       | Thyroglobulin                                                                                         |
| 14     | UBQLN4   | Ubiquilin 4                                                                                           |
| 15     | XPO1     | Exportin 1 (CRM1 homolog, yeast)                                                                      |
| 16     | ZFYVE9   | Zinc finger, FYVE domain-containing 9                                                                 |

Proteins including eIF5A and spermidine synthase play important roles in cell growth and proliferation. eIF5A is a small (16–18-kDa) abundant protein that is highly conserved in eukaryotes, and it is fundamental to cell survival and proliferation (32, 33). Spermidine synthase, also known as putrescine aminopropyltransferase, is the enzyme responsible for the synthesis of spermidine. Protein eIF5A is the only known cellular protein that undergoes an unusual post-translational modification on a specific lysine residue to form hypusine. The unique hypusine modification in mammalian cells occurs by a two-step pathway that involves the attachment of an aminobutyl group from spermidine to the  $\epsilon$ -amine group of lysine 50 followed by hydroxylation on carbon 2 of the butyl group to form hypusine. Thus, the essential nature of spermidine for hypusine modification of eIF5A is well established. And in addition to the indispensable role of spermidine for hypusine modification in eIF5A, polyamines are also required for optimal growth of mammalian cells (34). Many studies have indicated that down-regulation or inhibition of hypusine synthesis impedes cancer cell growth, and eIF-5A can be considered as a target of anticancer strategies (35–37). Thus, the decreased expression of eIF5A and spermidine synthase after GAD treatment may contribute to the cell growth inhibition induced by GAD in HeLa cells.

Proteins including annexin A5 and 26 S proteasome subunit p40.5 play important roles in cell death and protein degradation. Annexin A5 is a calcium-binding protein that belongs to the annexin family, a superfamily of ubiquitous proteins characterized by their calcium-dependent ability to bind to biological membranes. The involvement of annexins in several physiological processes, such as membrane trafficking, calcium signaling, cell motility, proliferation and differentiation, and apoptosis has been proposed. Importantly annexins have been implicated in the pathogenesis of benign and malignant neoplasms of different origins (38–40). As to annexin A5, its expression exhibits different regulation tendency in carcinoma

development of different organs. For example, the loss of annexin A5 was identified as a marker for cutaneous squamous cell carcinoma (41), whereas annexin A5 protein expression was augmented in growth hormone-secreting carcinoma (42). Importantly the expression of annexin A5 was markedly suppressed in both cervical and endometrial carcinoma cells when compared with their normal counterparts (43). Because the HeLa cell line used in the present study is a type of human cervical carcinoma cell line, the increased expression of annexin A5 in GAD-treated HeLa cells may contribute to growth inhibition induced by GAD. Similarly an increase of annexin A5 levels was also observed in butyrate-treated colon adenocarcinoma cell lines, whereas butyrate induced cell differentiation and growth arrest in these cells (44). Note that GAD could directly bind to annexin A5 according to the INVDOCK analysis. The role of annexin A5 in the cytotoxicity of GAD deserves further study. 26 S proteasome subunit p40.5 is an important subunit of proteasomes, which are eukaryotic ring-shaped or cylindrical particles with multicatalytic protease activities. The increase of 26 S proteasome subunit p40.5 found in the present study may contribute to the possible protein degradation of HeLa cells induced by GAD treatment.

Proteins including ephrin receptor EphA7, thioredoxin-dependent peroxide reductase mitochondrial precursor, activator of heat shock 90-kDa protein ATPase homolog 1, ubiquinol-cytochrome c reductase core I protein, protein-disulfide isomerase, aminopeptidase B, and mitofilin are enzymes or regulators of enzymes that play important roles in cell metabolism. The regulation of these proteins by GAD might cause a change of metabolism in HeLa cells. Moreover some proteins were also involved in pathways like cell proliferation/cell death and play important roles in carcinogenesis. Ephrin receptor EphA7 (receptor protein-tyrosine kinase, EC 2.7.10.1) is a receptor for members of the ephrin-A family and can catalyze the reaction of “ATP + a L-tyrosine in protein = ADP + a

L-tyrosine phosphate in protein." A previous report showed that a significant reduction of EphA7 expression is found in human colorectal cancers (45). In the present study, GAD treatment increased the expression of EphA7 in HeLa cells. PRDX3, a type of peroxiredoxin (EC 1.11.1.15), was found to be up-regulated in HeLa cells by GAD. Peroxiredoxins are a family of peroxidases that reduce hydrogen peroxide ( $H_2O_2$ ) and alkyl hydroperoxides to water and alcohol, respectively. The major role of peroxiredoxins is to control the constitutive level of  $H_2O_2$  in the cell and thus protect cell against reactive oxygen species-induced damage. PRDX3 expression is thought to play a role in the antioxidant defense system and homeostasis within the mitochondria. It was reported that PRDX3 overexpression led to decreased cell growth (46). So the increase in PRDX3 expression may be related to the growth inhibition caused by GAD treatment in HeLa cells. Activator of heat shock 90-kDa protein ATPase homolog 1 (AHA1) could act as cochaperone that stimulates HSP90 ATPase activity by influencing the conformational state of the "ATP lid" and consequent N-terminal dimerization (47). Ubiquinol-cytochrome c reductase core I protein (EC 1.10.2.2) is a component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome  $bc_1$  complex), which is part of the mitochondrial respiratory chain. Protein-disulfide isomerase (PDI; EC 5.3.4.1) catalyzes the rearrangement of -S-S- bonds in proteins. It was reported that resistance to the apoptosis-inducing agent Aplidin in HeLa cells was related to the down-regulation of PDI expression (48). In GAD-treated HeLa cells, the expression of PDI was increased; this may contribute to sensitivity of HeLa cells to apoptosis. Aminopeptidase B (EC 3.4.11.6) is an exopeptidase that selectively removes arginine and/or lysine residues from the N terminus of several peptide substrates including Arg-Leu-enkephalin, Arg-Met-enkephalin, and Arg-Lys-somatostatin-14. Note that aminopeptidase B was predicted to be able to bind directly with GAD in the INVDOCK analysis of present study. And it was reported that the aminopeptidase B activity was decreased in human renal cell carcinoma samples compared with non-tumor tissues (49). The increase of aminopeptidase B protein expression in GAD-treated HeLa cells may play an important role in the cytotoxicity of GAD. Mitofilin is a trans-membrane protein of the inner mitochondrial membrane and may be involved in catabolic pathways (50, 51). The contribution of mitofilin to the cytotoxicity of GAD is unknown.

Proteins including microtubule-associated protein RP/EB family member 1, cytokeratin 19, cytokeratin 1, and calumenin are generally cytoskeleton-related proteins. And these proteins were also reported to participate in pathways such as cell cycle control and apoptosis. For example, the plus ends of microtubules are important binding sites for proteins that regulate microtubules. EB1 is one of the best characterized "plus end-binding proteins." Properly regulating the dynamic properties of microtubules is critical for ensuring the accurate segregation of chromosomes in mitosis. The function of EB1

as an "antipausing" factor is well conserved, and inhibition of EB1 in a number of systems results in nondynamic microtubules that spend the majority of time in a paused state (52–54). Thus, it can be anticipated that the decrease of EB1 expression in GAD-treated HeLa cells will contribute to the cell cycle arrest induced by GAD. Cytokeratin 19 and cytokeratin 1 are intermediate filament proteins associated with the integrity of cell structure. According to previous reports, keratin expression may be related to carcinogenesis (55). Interestingly in cervical cancer cells like HeLa cells, the functional role of cytokeratin 19 was shown to be associated with the apoptosis prevention and drug resistance of cells. Cytokeratin 19 expression was found to be higher in cervical carcinoma cell lines compared with control cell lines, and the elevation of the cytokeratin 19 level was associated with clinical cervical cancer staging. The reduction of the cytokeratin 19 level by specific antibody caused apoptosis in a cervical carcinoma HeLa cell line (56, 57). So the decrease of cytokeratin 19 expression in GAD-treated HeLa cells may contribute to apoptosis induced by GAD. Calumenin is a multiple EF-hand  $Ca^{2+}$ -binding protein located in endo/sarcoplasmic reticulum of mammalian tissues (58). It was suggested in the previous reports to be related to the organization of cytoskeleton and carcinoma metastasis. The expression of calumenin was found to be decreased in carcinoma cell strains with higher metastasis potential (59, 60). The increase of calumenin in GAD-treated HeLa cells may also play a role in the cytotoxicity of GAD.

To date, this study is the first to use a proteomics technique to search globally for the proteins influenced in cancer cells by a purified *G. lucidum* component. We found 21 proteins that might be target-related proteins of GAD. By using computer-automated analysis, we tried to predict the possible targets and network of GAD. Most interestingly, our results suggested the important role of 14-3-3 proteins in the cytotoxicity mechanism of GAD. The results of the present study shed light on the anticancer mechanism of *G. lucidum* from a molecular perspective. Furthermore we are continuing to find new triterpenes from *G. lucidum* (61) and other herbs. It is possible that we can obtain promising triterpenes for cancer therapy either by isolating them from herbs or by modifying the structure of natural triterpenes. Understanding of the cytotoxicity mechanism of GAD will be helpful to the study and the use of likely promising triterpenes.

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## REFERENCES

- Yun, T. K. (1999) Update from Asia. Asian studies on cancer chemoprevention. *Ann. N. Y. Acad. Sci.* **889**, 157–192
- Sliva, D. (2004) Cellular and physiological effects of Ganoderma lucidum (Reishi). *Mini-Rev. Med. Chem.* **4**, 873–879
- Yuen, J. W., and Gohel, M. D. (2005) Anticancer effects of Ganoderma lucidum: a review of scientific evidence. *Nutr. Cancer* **53**, 11–17
- Sliva, D. (2006) Ganoderma lucidum in cancer research. *Leuk. Res.* **30**, 767–768
- Yeung, W. H., Lu, Q. L., Zhang, Q., and Go, V. L. W. (2004) Chemical and biochemical basis of the potential anti-tumor properties of Ganoderma lucidum. *Curr. Top. Nutraceutical Res.* **2**, 67–77
- Lin, S. B., Li, C. H., Lee, S. S., and Kan, L. S. (2003) Triterpene-enriched extracts from Ganoderma lucidum inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. *Life Sci.* **72**, 2381–2390
- Yang, H. L. (2005) Ganoderic acid produced from submerged culture of Ganoderma lucidum induces cell cycle arrest and cytotoxicity in human hepatoma cell line BEL7402. *Biotechnol. Lett.* **27**, 835–838
- Kimura, Y., Taniguchi, M., and Baba, K. (2002) Antitumor and antimetastatic effects on liver triterpenoid fractions of Ganoderma lucidum: mechanism of action and isolation of active substance. *Anticancer Res.* **22**, 3309–3318
- Min, B. S., Gao, J. J., Nakamura, N., and Hattori, M. (2000) Triterpenes from the spores of Ganoderma lucidum and their cytotoxicity against meth-A and LLC tumor cells. *Chem. Pharm. Bull.* **48**, 1026–1033
- Wang, X. M., Yang, M., Guan, S. H., Liu, R. X., Xia, J. M., Bi, K. S., and Guo, D. A. (2006) Quantitative determination of six major triterpenoids in Ganoderma lucidum and related species by high performance liquid chromatography. *J. Pharm. Biomed. Anal.* **41**, 838–844
- Komoda, Y., Nakamura, H., Ishihara, S., Uchida, M., Kohda, H., and Yamasaki, K. (1985) Structures of new terpenoid constituents of Ganoderma lucidum (Fr.) Karst (Polyporaceae). *Chem. Pharm. Bull.* **33**, 4829–4835
- Liu, X., Fan, X. L., Zhao, Y., Luo, G. R., Li, X. P., Li, R., and Le, W. D. (2005) Estrogen provides neuroprotection against activated microglia-induced dopaminergic neuronal injury through both estrogen receptor- $\alpha$  and estrogen receptor- $\beta$  in microglia. *J. Neurosci. Res.* **81**, 653–665
- Liu, X., and Zhu, X. Z. (1999) Roles of p53, c-Myc, Bcl-2, Bax and caspases in glutamate-induced neuronal apoptosis and the possible neuroprotective mechanism of basic fibroblast growth factor. *Brain Res. Mol. Brain Res.* **71**, 210–216
- Roberts, K., Bhatia, K., Stanton, P., and Lord, R. (2004) Proteomic analysis of selected prognostic factors of breast cancer. *Proteomics* **4**, 784–792
- Garfin, D., and Heerdt, L. (2001) *2-D Electrophoresis for Proteomics: a Methods and Product Manual*, pp. 25–27, Bio-Rad Laboratories, Richmond, CA
- Shen, H., Cheng, G., Fan, H., Zhang, J., Zhang, X., Lu, H., Liu, C., Sun, F., Jin, H., Xu, X., Xu, G., Wang, S., Fang, C., Bao, H., Wang, Y., Wang, J., Zhong, H., Yu, Z., Liu, Y., Tang, Z., and Yang, P. (2006) Expressed proteome analysis of human hepatocellular carcinoma in nude mice (LCI-D20) with high metastasis potential. *Proteomics* **6**, 528–537
- Chen, Y. Z., and Zhi, D. G. (2001) Ligand-protein inverse docking and its potential use in the computer search of protein targets of a small molecule. *Proteins* **43**, 217–226
- Ye, F., Zhang, Z. S., Luo, H. B., Shen, J. H., Chen, K. X., Shen, X., and Jiang, H. L. (2006) The dipeptide H-Trp-Glu-OH shows highly antagonistic activity against PPAR $\gamma$ : bioassay with molecular modeling simulation. *Chembiochem* **7**, 74–82
- Chen, S., Chen, L. L., Tan, H. Z., Chen, J., Du, L., Sun, T., Shen, J. H., Chen, K. X., Jiang, H. L., and Shen, X. (2005) Severe acute respiratory syndrome coronavirus 3C-like proteinase N terminus is indispensable for proteolytic activity but not for enzyme dimerization. *J. Biol. Chem.* **280**, 164–173
- Chaurasia, G., Iqbal, Y., Hanig, C., Herzel, H., Wanker, E. E., and Futschik, M. E. (2007) UniHI: an entry gate to the human protein interactome. *Nucleic Acids Res.* **35**, D590–D594
- Klein, P., and Ravi, R. A. (1995) Nearly best-possible approximation algorithm for node-weighted Steiner trees. *J. Algorithms* **19**, 104–115
- Gardino, A. K., Smerdon, S. J., and Yaffe, M. B. (2006) Structural determinants of 14-3-3 binding specificities and regulation of subcellular localization of 14-3-3-ligand complexes: A comparison of the X-ray crystal structures of all human 14-3-3 isoforms. *Semin. Cancer Biol.* **16**, 173–182
- Park, M. H. (2006) The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). *J. Biochem. (Tokyo)* **139**, 161–169
- Muller, C. I., Kumagai, T., O'Kelly, J., Seeram, N. P., Heber, D., and Koeffler, H. P. (2006) Ganoderma lucidum causes apoptosis in leukemia, lymphoma and multiple myeloma cells. *Leuk. Res.* **30**, 841–848
- Jiang, J., Slivova, V., Valachovicova, T., Harvey, K., and Sliva, D. (2004) Ganoderma lucidum inhibits proliferation and induces apoptosis in human prostate cancer cells PC-3. *Int. J. Oncol.* **24**, 1093–1100
- Hu, H., Ahn, N. S., Yang, X., Lee, Y. S., and Kang, K. S. (2002) Ganoderma lucidum extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. *Int. J. Cancer* **102**, 250–253
- Lu, Q. Y., Jin, Y. S., Zhang, Q., Zhang, Z., Heber, D., Go, V. L., Li, F. P., and Rao, J. Y. (2004) Ganoderma lucidum extracts inhibit growth and induce actin polymerization in bladder cancer cells in vitro. *Cancer Lett.* **216**, 9–20
- Jiang, J., Slivova, V., Harvey, K., Valachovicova, T., and Sliva, D. (2004) Ganoderma lucidum suppresses growth of breast cancer cells through the inhibition of Akt/NF- $\kappa$ B signaling. *Nutr. Cancer* **49**, 209–216
- Tzivion, G., Gupta, V. S., Kaplun, L., and Balan, V. (2006) 14-3-3 proteins as potential oncogenes. *Semin. Cancer Biol.* **16**, 203–213
- Hermeking, H., and Benzinger, A. (2006) 14-3-3 proteins in cell cycle regulation. *Semin. Cancer Biol.* **16**, 183–192
- Masters, S. C., and Fu, H. (2001) 14-3-3 proteins mediate an essential anti-apoptotic signal. *J. Biol. Chem.* **276**, 45193–45200
- Jao, D. L., and Chen, K. Y. (2002) Subcellular localization of the hypusine-containing eukaryotic initiation factor 5A by immunofluorescent staining and green fluorescent protein tagging. *J. Cell. Biochem.* **86**, 590–600
- Park, M. H., Lee, Y. B., and Joe, Y. A. (1997) Hypusine is essential for eukaryotic cell proliferation. *Biol. Signals* **6**, 115–123
- Nishimura, K., Murozumi, K., Shirahata, K., Park, M. H., Kashiwagi, K., and Igarashi, K. (2005) Independent roles of eIF5A and polyamines in cell proliferation. *Biochem. J.* **385**, 779–785
- Caraglia, M., Budillon, A., Vitale, G., Lupoli, G., Tagliaferri, P., and Abbruzzese, A. (2000) Modulation of molecular mechanisms involved in protein synthesis machinery as a new tool for the control of cell proliferation. *Eur. J. Biochem.* **267**, 3919–3936
- Caraglia, M., Marra, M., Giuberti, G., D'Alessandro, A. M., Budillon, A., del Prete, S., Lentini, A., Beninati, S., and Abbruzzese, A. (2001) The role of eukaryotic initiation factor 5A in the control of cell proliferation and apoptosis. *Amino Acids* **20**, 91–104
- Kang, H. A., and Hershey, J. W. B. (1994) Effect of initiation factor eIF-5A depletion on protein synthesis and proliferation of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**, 3934–3940
- Kenis, H., van Genderen, H., Bennaghmouch, A., Rinia, H. A., Frederik, P., Narula, J., Hofstra, L., and Reutelingsperger, C. P. (2004) Cell surface-expressed phosphatidylserine and annexin A5 open a novel portal of cell entry. *J. Biol. Chem.* **279**, 52623–52629
- Hayes, M. J., and Moss, S. E. (2004) Annexins and disease. *Biochem. Biophys. Res. Commun.* **322**, 1166–1170
- Bastian, B. C. (1997) Annexins in cancer and autoimmune diseases. *CMLS Cell. Mol. Life Sci.* **53**, 554–556
- Dooley, T. P., Reddy, S. P., Wilborn, T. W., and Davis, R. L. (2003) Biomarkers of human cutaneous squamous cell carcinoma from tissues and cell lines identified by DNA microarrays and qRT-PCR. *Biochem. Biophys. Res. Commun.* **306**, 1026–1036
- Mulla, A., Christian, H. C., Solito, E., Mendoza, N., Morris, J. F., and Buckingham, J. C. (2004) Expression, subcellular localization and phosphorylation status of annexins 1 and 5 in human pituitary adenomas and a growth hormone-secreting carcinoma. *Clin. Endocrinol. (Oxf.)* **60**, 107–119

43. Karube, A., Shidara, Y., Hayasaka, K., Maki, M., and Tanaka, T. (1995) Suppression of calphobindin I (CPB I) production in carcinoma of uterine cervix and endometrium. *Gynecol. Oncol.* **58**, 295–300
44. Guzman-Aranguez, A., Olma, N., Turnay, J., Lecona, E., Perez-Ramos, P., Lopez de Silanes, I., and Lizarbe, M. A. (2005) Differentiation of human colon adenocarcinoma cells alters the expression and intracellular localization of annexins A1, A2, and A5. *J. Cell. Biochem.* **94**, 178–193
45. Wang, J., Kataoka, H., Suzuki, M., Sato, N., Nakamura, R., Tao, H., Maruyama, K., Isogaki, J., Kanaoka, S., Ihara, M., Tanaka, M., Kanamori, M., Nakamura, I., Shinmura, K., and Sugimura, H. (2005) Downregulation of EphA7 by hypermethylation in colorectal cancer. *Oncogene* **24**, 5637–5647
46. Nonn, L., Berggren, M., and Powis, G. (2003) Increased expression of mitochondrial peroxiredoxin-3 (thioredoxin peroxidase-2) protects cancer cells against hypoxia and drug-induced hydrogen peroxide-dependent apoptosis. *Mol. Cancer Res.* **1**, 682–689
47. Siligardi, G., Hu, B., Panaretou, B., Piper, P. W., Piper, P. W., Pearl, L. H., and Prodromou, C. (2004) Co-chaperone regulation of conformational switching in the Hsp90 ATPase cycle. *J. Biol. Chem.* **279**, 51989–51998
48. Gonzalez-Santiago, L., Alfonso, P., Suarez, Y., Nunez, A., Garcia-Fernandez, L. F., Alvarez, E., Munoz, A., and Casal, J. I. (2007) Proteomic analysis of the resistance to Aplidin in human cancer cells. *J. Proteome Res.* **6**, 1286–1294
49. Varona, A., Blanco, L., Lopez, J. I., Gil, J., Agirregoitia, E., Irazusta, J., and Larrinaga, G. (2007) Altered levels of acid, basic, and neutral peptidase activity and expression in human clear cell renal cell carcinoma. *Am. J. Physiol.* **292**, F780–F788
50. Odgren, P. R., Toukatly, G., Bangs, P. L., Gilmore, R., and Fey, E. G. (1996) Molecular characterization of mitofilin (HMP), a mitochondria-associated protein with predicted coiled coil and intermembrane space targeting domains. *J. Cell Sci.* **109**, 2253–2264
51. Navet, R., Mathy, G., Douette, P., Dobson, R. L., Leprince, P., De Pauw, E., Sluse-Goffart, C., and Sluse, F. E. (2007) Mitoproteome plasticity of rat brown adipocytes in response to cold acclimation. *J. Proteome Res.* **6**, 25–33
52. Green, R. A., Wollman, R., and Kaplan, K. B. (2005) APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. *Mol. Biol. Cell* **16**, 4609–4622
53. Kline-Smith, S. L., and Walczak, C. E. (2004) Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics. *Mol. Cell* **15**, 317–327
54. Tirnauer, J. S., Grego, S., Salmon, E. D., and Mitchison, T. J. (2002) EB1-microtubule interactions in *Xenopus* egg extracts: role of EB1 in microtubule stabilization and mechanisms of targeting to microtubules. *Mol. Biol. Cell* **13**, 3614–3626
55. Chu, P. G., and Weiss, L. M. (2002) Keratin expression in human tissues and neoplasms. *Histopathology* **41**, 365–366
56. Yuan, C. C., Huang, T. S., Ng, H. T., Liu, R. S., Hung, M. W., and Tsai, L. C. (1998) Elevated cytokeratin-19 expression associated with apoptotic resistance and malignant progression of human cervical carcinoma. *Apoptosis* **3**, 161–169
57. Yuan, C. C., Huang, H. C., Tsai, L. C., Ng, H. T., and Huang, T. S. (1997) Cytokeratin-19 associated with apoptosis and chemosensitivity in human cervical cancer cells. *Apoptosis* **2**, 101–105
58. Jung, D. H., Mo, S. H., and Kim, D. H. (2006) Calumenin, a multiple EF-hands  $\text{Ca}^{2+}$ -binding protein, interacts with ryanodine receptor-1 in rabbit skeletal sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* **343**, 34–42
59. Ding, S. J., Li, Y., Shao, X. X., Zhou, H., Zeng, R., Tang, Z. Y., and Xia, Q. C. (2004) Proteome analysis of hepatocellular carcinoma cell strains, MHCC97-H and MHCC97-L, with different metastasis potentials. *Proteomics* **4**, 982–994
60. Wu, W., Tang, X., Hu, W., Lotan, R., Hong, W. K., and Mao, L. (2002) Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clin. Exp. Metastasis* **19**, 319–326
61. Guan, S. H., Yang, M., Liu, X., Xia, J. M., Wang, X. M., Jin, H., and Guo, D. A. (2006) Two new lanostanoid triterpenes from the fruit body of *Ganoderma lucidum*—the major component of SunRecome®. *Nat. Prod. Commun.* **1**, 177–181