Binding of Lactoferrin to IGBP1 Triggers Apoptosis in a Lung Adenocarcinoma Cell Line

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Abstract. *Background: Lactoferrin (Lf), an iron-binding protein present in mammalian secretions, plays important roles in cancer prevention by inducing apoptosis. Materials and Methods: PC-14 lung adenocarcinoma cells were exposed to bovine Lf (bLf) protein and the expression of caspase-3 and apoptosis protease-activating factor-1 (APAF-1) was assessed. To investigate the molecular mechanism of apoptosis induced by bLf, a major Lf-binding protein was screened using a protein microarray with bLf protein as the probe. Protein interaction was demonstrated by co-immunoprecipitation, immunofluorescence and phosphatase activity assay. Results: Lf directly suppressed the proliferation of the PC-14 cells by triggering their apoptosis. Lf was shown to bind specifically with a 36-kDa protein, immunoglobulin (CD79A)-binding protein 1 (IGBP1). The binding complex interacted with the catalytic subunit of protein phosphatase 2A (PP2A), thus reducing the phosphatase activity of PP2A and triggering apoptosis. Conclusion: Lf binds IGBP1 and promotes the acceleration of cellular apoptosis.*

Lactoferrin (Lf), a member of the transferrin gene family, is an 80-kDa iron-binding glycoprotein expressed in the mucosal epithelium and present in milk, seminal fluid, bronchial gland secretions and neutrophils (1). It is considered to be a key component of host first-line defence, being expressed in response to a variety of physiological and environmental changes (2). Lf has been reported to participate in host defense mechanisms against various infections and cancer. Several previous reports have described the physiological anti-tumor functions of Lf both *in vivo* and *in vitro*. In rat models, Lf treatment significantly reduced the incidence of azoxymethane-induced colonic carcinogenesis (3). Lf exerted an antiproliferative effect on endothelial cells *in vitro*, and significantly inhibited the vascular endothelial growth factor-mediated angiogenic response *in vivo* (4). In addition, it has been found that bovine Lf (bLf) induced apoptotic cell death through the production of reactive oxygen species (ROS) in human leukemia cells (5) and recently, Lf has been found to alter apoptosis-related gene expression in colonic mucosa (6). These findings suggest that the antitumor action of Lf is based on the triggering of apoptosis. Furthermore, some other studies have demonstrated that treatment of human breast cancer cells with Lf resulted in growth arrest at the G_1 to S transition of the cell cycle and an increase in the level of cyclin-dependent kinase (CDK) inhibitor $P21^{cip1}$ protein through a p53-independent mechanism (7). Although various mechanisms for the antiproliferative effects of Lf have been proposed, none have been conclusively proven, and the antitumor effects of Lf appear to be mediated by multiple mechanisms.

In the present study, the molecular mechanisms involved in the direct antitumor effect of Lf were investigated using a protein microarray, an attempt was made to globally clarify specific Lf-binding proteins using bLf protein, which shows 69% amino acid identity with human Lf (8).

Materials and Methods

Cell culture and proliferation assay. bLf protein powder (purity, 99.8% of total protein by high performance liquid chromatography; endotoxin, 0.063 μg/g bLf; iron content, 0.113 mg/g bLf) was kindly supplied by Morinaga Milk Industry Co. Ltd (Tokyo, Japan). PC-14 lung adenocarcinoma cells (Riken Cell Bank, Tsukuba, Japan) were propagated at 1×10^4 cells/well in 12 wells of 96-well plates in RPMI-1640 (Invitrogen Corp., Carlsbad, CA, USA) with bLf protein (0, 0.125 0.625, 1.25, 2.5, 5 or 10 μM), and cultured for 72 h at 37˚C in a 5% CO_2 humidified air atmosphere. Relative cell

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numbers were determined by WST-1 ((4-[3-(4-iodophenyl)-2*H*-5-tetrazolio])-1, 3-benzene-disulfonate) assay (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

Reverse transcription-PCR and quantitative real-time reverse transcription-PCR. For reverse transcription-PCR (RT-PCR), the cells were plated on 10-cm dishes and grown to 80% confluence. Equal quantities of cells were then treated or untreated with bLf (1.25 μM) for 72 h. The RNA was extracted using an RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions and samples were stored at –70˚C until use. The quality of the RNA was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Liberty Lake, WA, USA). A 1-μg sample of RNA was used for cDNA synthesis with a TaKaRa Exscript® RT Reagent Kit (Perfect Real Time; Takara Bio Inc., Shiga, Japan). Expression of the apoptosis protease-activating factor-1 (*APAF-1*) gene was evaluated by real-time RT-PCR based on the SYBR Green I method and a Gene Amp 7300 real-time PCR system (Life Technologies Corporation, Carlsbad, CA, USA). The PCR primers were purchased from Takara Bio and the primer sequences were: *APAF-1* F, GTCATCATCTTCTCTTAGCC; *APAF-1* R, CCTTGCACCATCAGCAGACC. The numbers of target cDNA molecules were normalized to those of glyceraldehyde-3-phosphate dehydrogenase cDNA molecules as a control.

Protein microarray. A protoarray (ProtoArray® Human Protein Microarray v5.0 (Invitrogen Corp.) service provided by Filgen Inc. (Nagoya, Japan) was used for analyzing the *in vitro* interaction of human proteins and Lf. This microarray allows rapid and efficient detection of protein–protein interactions using a protein probe containing a suitable tag. The bLf protein was biotinylated with Biotin-XX sulfosuccinimidyl ester and 30 μg of biotinylated bLf was applied to the protoarray, which contained 8,295 types of human protein, incubated for 2 h and then scanned with a GenePix 4000B scanner (Molecular Device, Inc. Sunnyvale, CA, USA). After scanning, the array was analyzed using the Array-Pro Analyzer® Ver.4.5 software package (Media Cybernetics, Inc., Bethesda, MD, USA) to determine the fluorescence intensity of every spot.

Immunoprecipitation. The PC-14 cells were treated with bLf at a concentration of 1.25 μM. After 72 h of propagation, the cells were lysed in immunoprecipitation buffer (Sigma-Aldrich Co., St. Louis, MO, USA). After sonication, the lysates were clarified by centrifugation at 10,000 × *g* for 10 min. Immunoprecipitation assays were performed using a ProFound Mammalian Co-Immunoprecipitation kit (Thermo Fisher Scientific, Rockford, IL, USA). Briefly, the clarified lysates were precleared by incubation with the control gel component overnight at 4˚C, then incubated with gel-immobilized anti-Lf polyclonal antibody (Thermo Fisher Scientific) or anti-protein phosphatase 2Ac (anti-PP2Ac) subunit monoclonal antibody (Millipore Corp., Billerica, MA, USA) for 4 h at room temperature. The immunoprecipitates were washed four times with the immunoprecipitation buffer and once with reduced-salt immunoprecipitation buffer (125 mM NaCl) before elution. The purified protein was analyzed by Western blotting.

Western blotting. The PC-14 cells were lysed in Immunoprecipitation Lysis/Wash Buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Sigma-Aldrich Co.). Equal quantities of cell lysate protein or co-immunoprecipitation-purified protein were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Invitrogen Corp.). After blocking, the membranes were probed with primary antibodies against Lf (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), caspase-3/cleaved caspase-3 (Cell Signaling Technology Inc., Beverly, MA, USA), immunoglobulin (CD79A)-binding protein 1 (IGBP1) (Sigma-Aldrich Co.) or PP2Ac subunit (Millipore Corp.). After incubation with anti-mouse or anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific), the antigen-antibody complexes were visualized using SuperSignal West Dura or Femot Extended Duration Substrate (Thermo Fisher Scientific).

Immunofluorescence. The PC-14 cells were plated on collagen-coated chamber slides (Iwaki Biosciences, Tokyo, Japan) and fixed in methanol. For immunofluoresence analysis, the PC-14 cells were incubated with anti-Lf (1:50) plus anti-IGBP1 (1:500) antibodies or anti-Lf (1:50) plus anti-PP2Ac antibodies overnight at 4˚C. After incubation with Alexa Fluor 488-labeled secondary antibody (1:1,000; Santa Cruz Biotechnology Inc.), the nuclei were counterstained with 4, 6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA, USA) and analyzed using a fluorescence microscope (Biorevo BZ-9000, Keyence, Osaka, Japan).

Serine/threonine phosphatase activity assay. A serine/threonine phosphatase assay system (Promega Corp., Madison, WI, USA) was used to analyze PP2A activity at various concentrations of bLf. One gram of PC-14 cells were homogenized in phosphatase storage buffer and centrifuged at 100,000 × *g* at 4˚C for 1 h to remove particulate matter. After removing endogenous phosphate in the cell lysates using a Sephadex® G-25 Spin Column (Promega), the mix of enzyme samples, phosphatase substrate (100 μM Ser/Thr phosphopeptide), PPase-2A 5× reaction buffer and various concentrations of bLf protein or PP2A inhibitor (Okadaic acid, Merck KGaA, Darmstadt, Germany) was incubated for 30 min at 30˚C. The reaction was then stopped with Molybdate Dye and the optical density of the samples was read by a plate reader with a 600-nm filter.

Results

By WST-1 absorbance, PC-14 cell proliferation was found to be reduced significantly with increasing bLf concentration (from 0.625 to 10 μM) in comparison with the untreated cells (Figure 1A). As shown by real-time RT-PCR, the rate of APAF-1 expression in the bLf-treated PC-14 cells was significantly increased (Figure 1B, left panel). Western blot analysis detected an inactive form of caspase-3 in the PC-14 cells and then cleaved caspase-3 appeared in the cells after exposure to bLf (Figure 1B, right panel).

By the protein microarray method, 258 human proteins were identified as binding to bLf (Table I), and among them, IGBP1 had the highest Lf protein-binding affinity. In order to determine the interactions between Lf, IGBP1 and PP2Ac, co-immunoprecipitation for affinity purification of the bLf-binding proteins was performed in the bLf-treated cells before Western blotting analysis with anti-Lf, anti-IGBP1 and anti-PP2Ac antibodies. As shown in Figure 2A (left panel), in the whole-cell lysate of the bLf-treated cells, IGBP1 protein expression increased with bLf concentration, but the expression of PP2Ac showed no noticeable change.

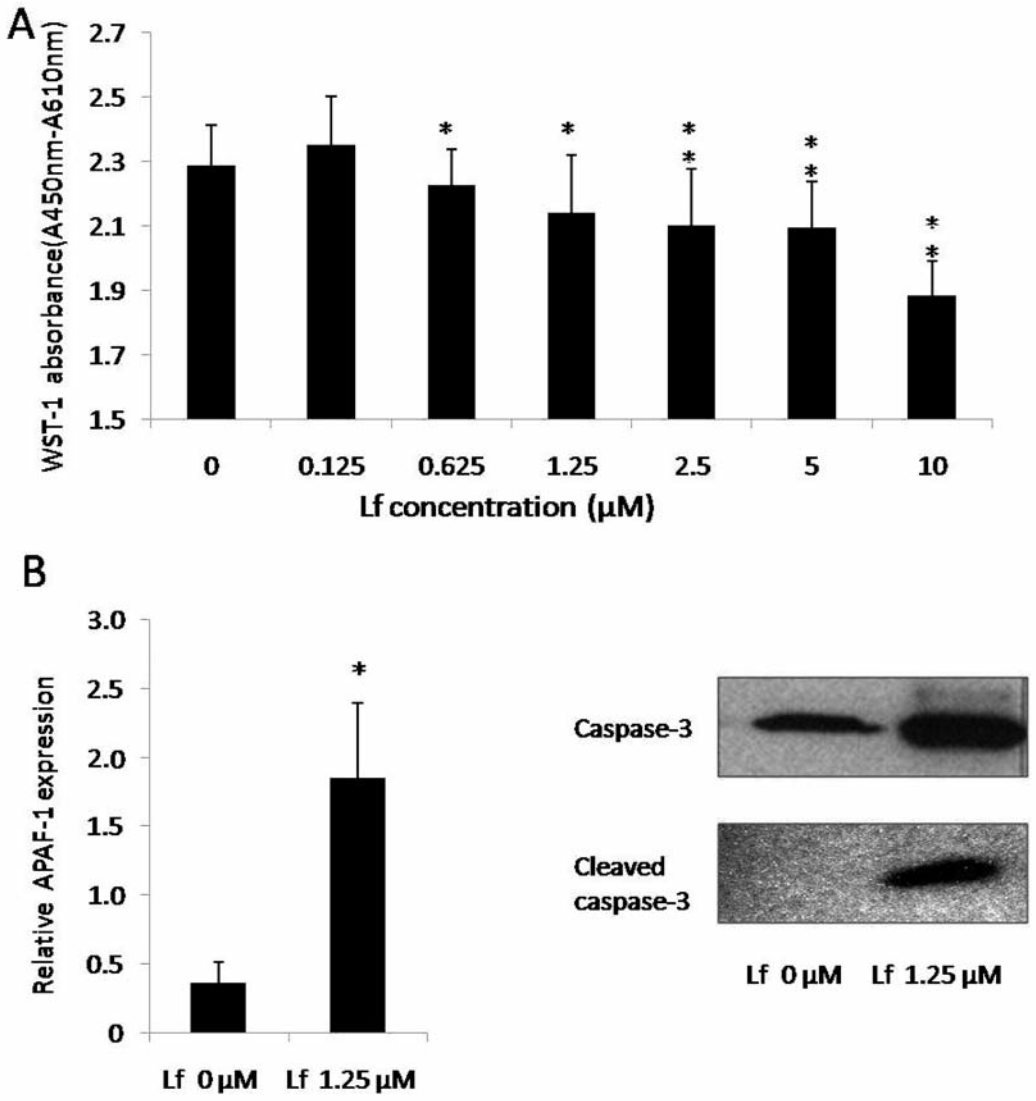

Figure 1. *Effect of exogenous bovine lactoferrin (bLf) on the proliferation and apoptosis of PC-14 cancer cells. A) Cell proliferation after 72 h treatment expressed by absorbance of WST-1. *p<0.05, **p<0.001, as compared with the control (0 μM bLf). B) Left panel, APAF-1 expression analyzed by real-time RT-PCR. Columns represent the means of duplicate measurements; bars, SD.*p<0.05. Right panel, Western blot of bLf-treated and untreated cells.*

After immunoprecipitation with anti-Lf antibody, bLf-binding protein in the lysate of the bLf-treated PC-14 cells was blotted and examined after addition of anti-Lf, anti-IGBP1 and anti-PP2Ac antibodies. As shown in Figure 2A (middle panel), both IGBP1 and PP2Ac were found to bind with the bLf protein. To examine the affinity and binding specificity between bLf, IGBP1 and PP2Ac, co-immunoprecipitation with anti-PP2Ac antibody was also performed. As shown in Figure 2A (right panel), no Lf binding to PP2Ac was evident, but a large quantity of PP2Ac was bound to IGBP1. These data suggested that the Lf-IGBP1 protein complex might regulate the activity of PP2A phosphatase (Figure 2A middle panel), but that a considerable quantity of the IGBP1–PP2Ac complex did not bind to bLf, in comparison with the Lf–IGBP1–PP2Ac complex (Figure 2A right panel).

As shown by serine/threonine phosphatase activity assay (Figure 2B), PP2A activity was significantly inhibited by bLf at a concentration of 250 and 500 μM ($p<0.001$).

Immunofluorescence microscopy analysis was used to determine the location and association of bLf, IGBP1 and PP2Ac in the bLf-treated PC-14 cells. Figure 3 (Column I) shows images filtered for detection of only green fluorescence, indicating the localization of exogenously applied bLf protein.

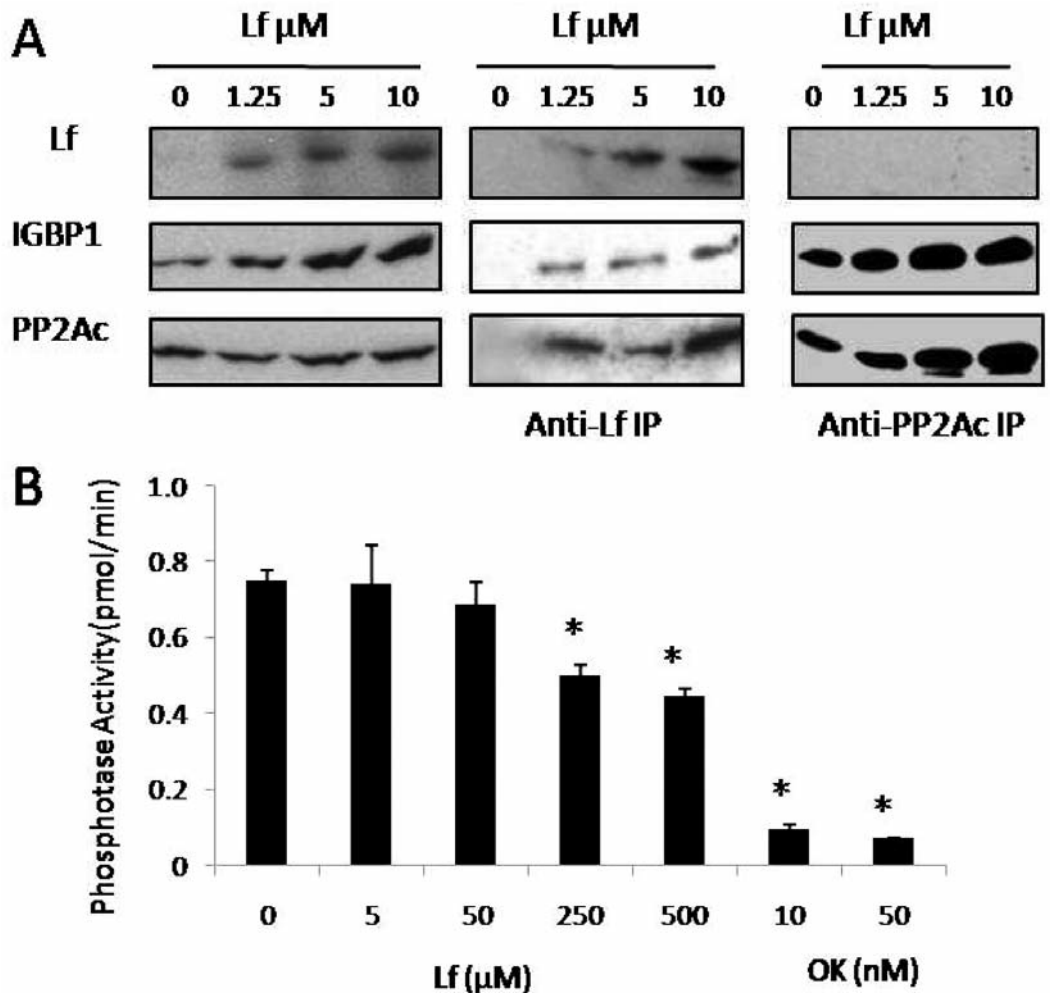

Figure 2. *Interactions between Lf, IGBP1 and PP2Ac in PC-14 cells. A) Western blot analysis of bLf, IGBP1 and PP2Ac levels after bLf treatment (left panel); immunoprecipitation with Lf-specific antibody (middle panel) and with PP2Ac-specific antibody (right panel); B) Protein phosphatase activity assay of cell lysate (250 μl) treated with substrate, bLf protein or okadaic acid (OK). *p<0.001, compared with the untreated control.*

Table I. *Identification of Lf-binding proteins using a protein microarray.*

Accession no.	Description	Net intensity (mean)
NM_001551.1	Immunoglobulin (CD79A) binding protein 1 (IGBP1)	65223.2542
NM_017583.2	Tripartite motif-containing 44 (TRIM44)	64619.34275
BC014991.1	*N*-Methylpurine-DNA glycosylase (MPG)	25754.27542
NM_000282.1	Propionyl coenzyme A carboxylase, alpha polypeptide (PCCA)	23488.73859
NM_032345.1	Within bgcn homolog (Drosophila) (WIBG)	22801.12879
NM_006713.2	SUB1 homolog (*S. cerevisiae*) (SUB1)	14273.37187
NM_015933.1	Coiled-coil domain containing 72 (CCDC72)	12896.78695
NM_144659.1	T-complex 10 (mouse)-like (TCP10L)	12436.62794
NM_004123.1	Gastric inhibitory polypeptide (GIP)	12205.08387
BC033088.1	Lamin A/C (LMNA)	11826.73457
NM_001219.2	Calumenin (CALU)	1991.993238
BC014928.1	MYC-induced nuclear antigen (MINA)	1991.921465
NM_002754.3	Mitogen-activated protein kinase 13 (MAPK13)	1981.816554

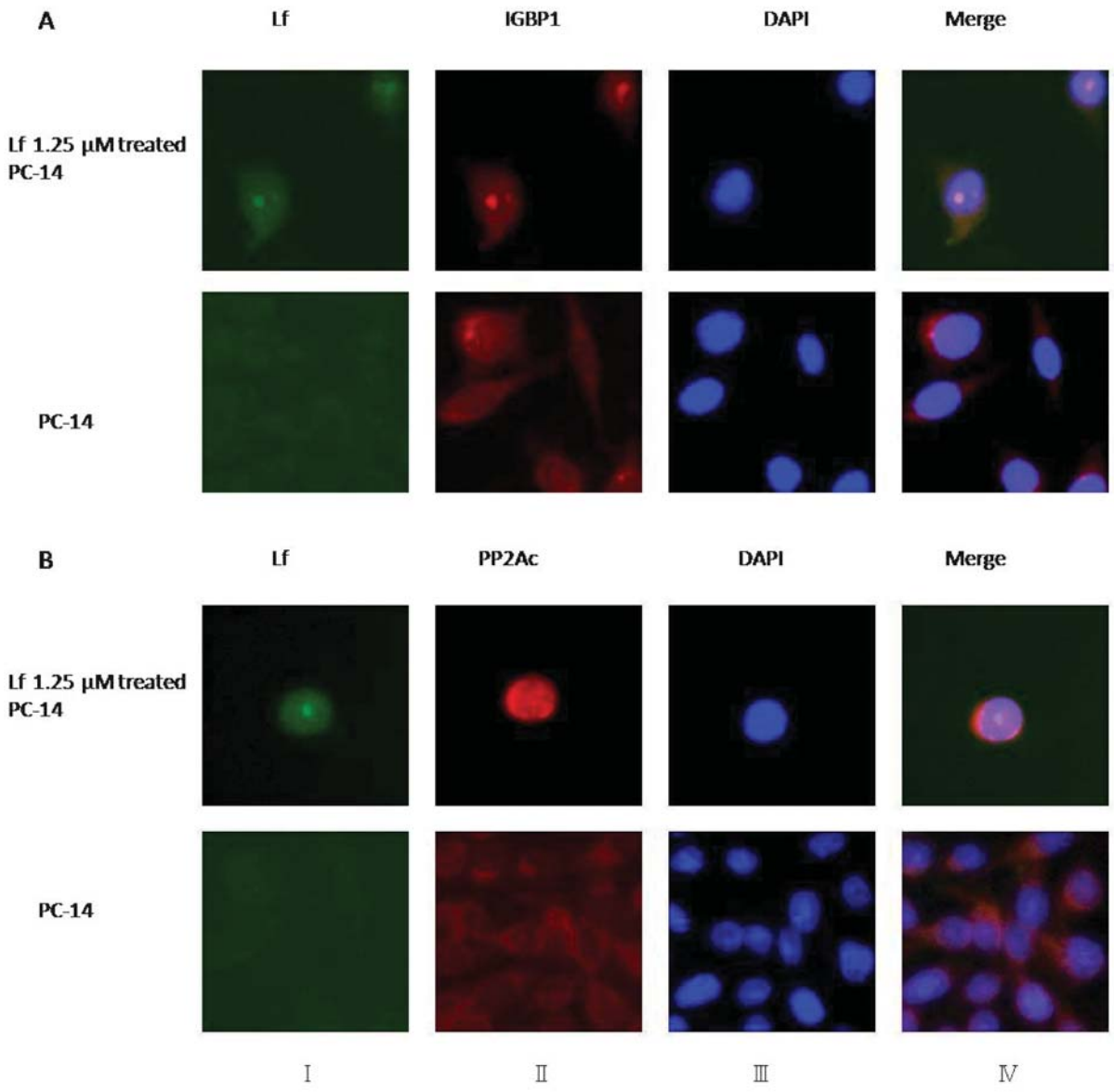

Figure 3. *Localization and association of bLf, IGBP1 and PP2Ac in the PC-14 cells after 72 h of bLf treatment by immunofluorescence staining using antibodies specific for Lf and IGBP1 (A) and PP2Ac (B). Green fluorescence indicates localization of exogenously applied Lf protein. Red fluorescence indicates localization of IGBP1 (A) or PP2Ac (B). Nuclei visualized by DAPI staining.*

Fluorescence was clearly detected on the bLf-treated but not on the control PC-14 cells. Column II shows images filtered for only red fluorescence, indicating localization of endogenous IGBP1 (A) and PP2Ac (B). Column III shows images of the nucleus, and column IV shows a merged image of images in columns I, II and III. Merged signals for bLf and IGBP1 were detected in both the cytoplasm and the nucleus (Figure 3A merged). For PP2Ac, red fluorescence was detected in the cytoplasm of both control cells and Lf-treated cells (Figure 3B, column II), and there was little expression in the nucleus of the bLf-treated cells, similar to the situation in the control cells (Figure 3B merged). These data suggested that Lf-IGBP1 protein was located in not only the cytoplasm, but also the nucleus after Lf treatment, and that the PP2Ac–IGBP1 complex was interrupted by Lf (Figure 3B, merged).

Discussion

Animal studies have shown that oral administration of Lf resulted in T-cell-dependent inhibition of head and neck squamous cell carcinoma (9), and an *in vivo* study has demonstrated that Lf induced apoptosis and arrested tumor

growth (10). In the present study, bLf suppressed the proliferation of a lung cancer cell line in accordance with its concentration and induced apoptosis as shown by APAF-1 and cleavage of caspase-3. APAF-1 is an initiator of apoptosis (11) and cleavage of caspase-3 is a crucial mediator of apoptosis. In many cases of apoptotic cell death, the 32-kDa precursor of caspase-3 is cleaved into activated fragments of approximately 20, 19, and 17 kDa (12).

Once Lf had entered the PC-14 cells, it became bound to the IGBP1 protein, which was shown by the protoarray to have the highest bLf-binding affinity. IGBP1 has been shown to bind to the catalytic subunit of various protein phosphatases (PP2A, PP4 and PP6) in mammals (13, 14). Through its action as a binding protein for the catalytic subunit of serine/threonine PP2Ac (15), IGBP1 is an essential regulator of PP2A (15). PP2A is a major cellular phosphatase that plays key regulatory roles in growth, differentiation and apoptosis (16). In the present study, Lf bound to IGBP1 and Lf appeared to induce disruption of the PP2Ac–IGBP1 complex in the cytoplasm and suppress the phosphatase activity of PP2A (Figure 2). It has been reported that treatment with rapamycin, an immunosuppressant, disrupts the association of PP2Ac–IGBP1 in parallel with an inhibitory effect on lymphoid cell proliferation (17) and induces apoptosis (18). The function of bLf reported here is similar to that of rapamycin. However, as Figure 2A (right panel) indicates, there was still a considerable quantity of IGBP1–PP2Ac complex that did not bind to exogenous bLf. These results suggested that treatment with a higher concentration of Lf might enhance the apoptotic effect. If future studies are able to reveal the domain by which Lf binds to IGBP1, more effective treatment with Lf might become possible. This speculation is supported by several reports focusing on lactoferricin, which is a peptide released from Lf, and which not only retains many of the activities of intact Lf, but in some cases can exert more potent activity (19).

In conclusion, for the first time an Lf-binding protein, IGBP1, has been isolated which is involved in the induction of apoptosis by Lf in a lung adenocarcinoma cell line, indicating that Lf has potential application for the therapy or chemoprevention of lung adenocarcinoma.

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