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Importance of nuclear localization for the apoptosis-induced activity of a fungal galectin AAL (*Agrocybe aegerita* lectin)

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ABSTRACT

Agrocybe aegerita lectin (AAL) was identified previously in our group as a novel galectin from medicinal fungi *Agrocybe aegerita*, and has been shown to effectively induce cancer cell cycle arrest and apoptosis *in vitro* and tumor regression *in vivo*. Here, AAL was observed to translocate into the HeLa cell nucleus and induce cell apoptosis when it was predominantly in the nucleus. The N-terminus and C-terminus of AAL were required for nuclear localization. Site mutated proteins were generated based on AAL structure. Dimer interface mutant I25G, carbohydrate recognition domain (CRD) mutant R63H, and loop region mutant L33A could not enter the nucleus and lost the ability to induce apoptosis. CRD mutant H59Q and loop region mutant I144G maintained nuclear localization activity, and H59Q retained residual bioability but I144G had no activity, indicating that nuclear localization is important but not sufficient for AAL to become apoptotically active. Our findings provide a novel antitumor mechanism of fungal galectin.

Introduction

Lectins are di- or multivalent carbohydrate binding proteins or glycoproteins and found in organisms ranging from viruses and plants to humans and mediate different biological events [1,2]. An increasing number of lectins have been described to have possible promising bioscientific and biomedical uses, especially for cancer therapy, and some have been employed as clinical drugs, such as mistletoe lectins [3–6].

Mushrooms have a long history of medicinal use in traditional oriental therapy and the bioactive compounds effective against cancer have captured the attention of many investigators [7]. In recent years, a variety of proteins from macrofungi with potential activity have been reported, in which lectin is the largest family [3,5]. Some mushroom lectins display immunomodulatory and/or antitumor/cytotoxic activities. For example, *Grifola frondosa* lectin (GFL), an *N*-acetylgalactosamine-specific glycoprotein lectin, is cytotoxic to HeLa cells [8]. *Agaricus bisporus* lectin (ABL), the Galβ1-3GalNAcα (TF antigen)-binding lectin, has a potent anti-proliferative effect on HT29, Caco-2 colon cancer cells, MCF-7 breast cancer cells and Rama-27 rat mammary fibroblasts [9]. *Volvariella volvacea* lectin (VVL) can remarkably inhibit the proliferation of cultured tumor cell lines [10]. *Kurokawa* lectin inhibits the proliferation of human monoblastic leukemia U937 cell due to the apoptosis induction [11]. *Clitocybe nebularis* lectin (CNL), a ricin B-like lectin, displays the antiproliferation effect on leukemic T cell lines Mo-T and Jurkat [12]. The investigation on hundreds of fungi containing haemagglutinins by Pemberton [13] has shown that mushroom lectin is a valuable resource for antitumor drug discovery and worth more consideration.

However, apart from ABL with the antiproliferation mechanism well defined [14,15], the information about the antitumor mechanism of the mushroom lectins available is still limited. *Agrocybe aegerita* lectin (AAL), purified previously in our group, shows apoptosis inducing activity on cultured cell lines and S180 bearing mice [16]. We have cloned AAL cDNA and identified that AAL is the 4th fungal galectin reported [17] after *Coprinus cinereus* galectins (CGL I and CGL II) [18] and *Agrocybe cylindracea* galectin (ACG) [19]. Here, we continue the story about AAL and present the study about the internalization of AAL and nuclear sequestration resulting in cell apoptosis. Using mutagenesis we further investigate the relationship between AAL nuclear localization and its apoptosis-induced activity. These data is the first report that a foreign lectin enters the cell nucleus and exerts the bioactivity, and provides a novel antitumor mechanism of lectins.

Materials and methods

Construction of plasmids expressing recombinant full-length and mutant AAL. The coding region of AAL was PCR amplified by using

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pGEM-T-easy-AAL as a template and the appropriate primer set with Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). To clone full-length AAL into pEGFP-C1 vector (BD Biosciences Clontech, Palo Alto, CA), the primers were: forward 5′-GGAAGATCTC AGGGCGTCAAC-3′, reverse 5′-CGCGGATCCCGCCAAACCCGTGTAT-3′. The truncation mutants were cloned into the pEGFP-C1 vector. Primers for pEGFP-C1-AAL ΔN: forward 5′-GGAAGATCTACCAC TGGCGAC-3′, reverse 5′-CGCGGATCCCGCCAAACCCGTGTAT-3′. Primers for pEGFP-C1-AAL ΔC: forward 5′-GGAAGATCTCAGGGCGT CAAC-3′, reverse 5′-CGCGGATCCCGAGATTTGCTTCG-3′. Primers for pEGFP-C1-AAL ΔN ΔC: forward 5′-GGAAGATCTCAGGGCGTCAAC-3′, reverse 5′-CGCGGATCCCGAGATTTGCTTCG-3′.

The plasmids expression rAAL and site mutants was performed using a previously reported protocol [20,21]. Mutagenic primers for H59Q: forward 5′-TCCTCCAAATCGCCTTCCGCC-3′, reverse 5′-GCGATTTGGAGGAGGTATGCG-3′.

Purification of native AAL, recombinant AAL and the site mutants. Native AAL (nAAL) was purified as reported previously [16]. Recombinant AAL (rAAL) and site mutant proteins with C-terminal 6 × His-tag were expressed in *Escherichia coli* BL21 (DE3) cells and purified following described previously [21]. Purified rAAL and site mutant proteins were analyzed with rabbit AAL antisera by Western blot.

Immunofluorescence staining. Full-length AAL or mutant proteins were applied to HeLa cell cultures at a concentration of 3.1 μM (50 μg/mL) and incubated for 36 h. Then the cells were washed ten times by PBS at room temperature and fixed in Methanol/Acetone (v/v: 1/1) for 30 s. The fixed cells were washed by PBS and blocked in 10% BSA for 1 h at 4 °C. After washing in PBS, the cells were incubated with rabbit AAL antisera (1:100) for 45 min and then finally washed in PBS. To obviate nonspecific binding of AAL primary antisera, negative control (cells untreated with AAL) was performed in each experiment. FITC-conjugated Goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL, USA) was added to the sample and incubated for 30 min followed by washing ten times with PBS. The cells were then stained with the Propidium Iodide (PI, 5 μg/mL) for 15 min, the images of FITC and PI fluorescence were acquired by Leica confocal laser scanning microscope (Leica TCS SP2, DM RXE, Leica-Microsystems, Rijswijk, the Netherlands). The captured images were processed using Leica Confocal Software and Adobe Photoshop CS.

The migration of AAL (3.1 μM) from outside the cell to the nucleus at different stages (membrane, cytoplasm, around nucleus, nucleus) was counted at different time points (6, 12, 24, 36 h).

The plasmid pEGFP-C1-AAL and truncation mutants were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) by following the manufacturer's instructions. After transfection, the cells were incubated for 36 h and the localization was detected by confocal microscopy.

Annexin V staining and FACS analysis. HeLa cells were exposed to rAAL or mutant proteins at different concentrations (3.1, 6.2, 12.5 μM) for different time. The cells were trypsinized, washed twice with ice-cold PBS (pH 7.4), and assayed for apoptosis using the Annexin V/PI apoptosis kit (MultiSciences Biotech Co., Ltd, Hangzhou, Zhejiang, China). The cells were resuspended in binding buffer at a cell density of 1×10^5 cells/mL, and 5 μL of Annexin V-FITC and 10 μL PI were added. The samples were gently mixed and incubated in the dark at room temperature for 5 min, and was analyzed by flow cytometry (BECKMAN, EPICS ALTRA) within one hour.

Results

AAL migrates into the nucleus of HeLa cells from the culture medium and induces cell apoptosis

Various papers have suggested that lectins trigger signaling pathways through binding their receptors at the cell surface [22,23]. To study the cell surface binding capacity of AAL, immunofluorescence and confocal microscopy assays were used. Strikingly, 36 h after being added into cell cultures, AAL was observed to have localized in the cell nucleus (Fig. 1A).

Fig. 1B and C shows the cellular localization of AAL in HeLa cells over a time course. The internalization process of AAL could be divided into 4 stages: attachment at the cell membrane (M), dispersion in cytoplasm (C), aggregation around the nucleus (AN) and localization in the nucleus (N). Six hours after exogenously applying AAL to cell culture, 61% cells were in M stage, 17.33% in C stage, 15.66% in AN stage, and few cells were in N stage (6%). At 12 h, no cells were in M stage and most cells (69%) were in C stage. At 24 h, 18.66% cells were in C stage, 47.66% cells in AN stage, and 33.66% in N stage. After 36 h, 80.66% cells were in N stage and 17.66% in AN stage, and no cells in M or C stage.

The apoptosis-induced effect of AAL on cells was reported previously [16]. Here, we further detected the apoptotic activity of AAL during the internalization (Fig. 1D). At early stages of migration (6, 12 h), AAL was mainly localized in the cell membrane or in the cytoplasm and did not induce apoptosis compared to the control group. At 24 h, 33.66% of cells were observed to be in N stage, with 6.65% apoptotic activity. We have previously shown that AAL could induce HeLa cell apoptosis after being applied exogenously to cell cultures [16]. When Hela cells were exposed to AAL and incubated for 36 h, 80.66% cells were in the N stage, while the percentage of Annexin V positive cells increased to about 14.15%, which was far lower than that of HeLa cell with AAL in the nucleus at that time. This indicates that AAL entering the nucleus is not the result but before the onset of cell apoptosis. AAL apoptotic activity might be linked with nucleus localization.

N-terminus and C-terminus of AAL is important for its nuclear localization

Analysis of the AAL amino acid sequence shows that there is no typical nuclear localization sequence (Arg/Lys rich) in mediating AAL nuclear import. To delineate the elements involved in AAL nuclear localization, we made EGFP truncation AAL mutants and determined their localization.

Three truncated pEGFP-C1-AAL plasmids were constructed: one without the N-terminal 20 residues (pEGFP-C1-AAL ΔN), one without the C-terminal 30 residues (pEGFP-C1-AAL ΔC) and one with neither (pEGFP-C1-AAL ΔN ΔC) (Fig. 2A). Thirty-six hours after the plasmids were transfected into HeLa cells, AAL localization was detected by confocal microscopy. As shown in Fig. 2B, pEGFP-C1-AAL is dispersed throughout the whole cell, while both N-terminal and C-terminal truncation mutants do not localize in the nucleus. This data shows that both of N- and C-terminus are required for efficient nuclear targeting.

The cellular localization and apoptotic activity of purified AAL and mutant proteins

To further investigate the relationship between AAL cellular localization and apoptotic activity, the localization and apoptotic activity of site mutant proteins was examined.

AAL has been crystallized and the overall structure could be divided into 3 parts: S, F and loop region [21]. The S region contains the CRD domain and is responsible for carbohydrate/the sugar binding specificity, the F region is responsible for dimer formation, and the loop region contributes to sugar binding specificity and other activities. Site mutants were designed in all three regions of AAL: two CRD mutants H59Q and R63H, dimer interface mutants I25G, and two loop region mutants L33A and I144G (Fig. 3A). The binding of rabbit AAL antisera with purified proteins was tested by Western blot (Fig. 3B).

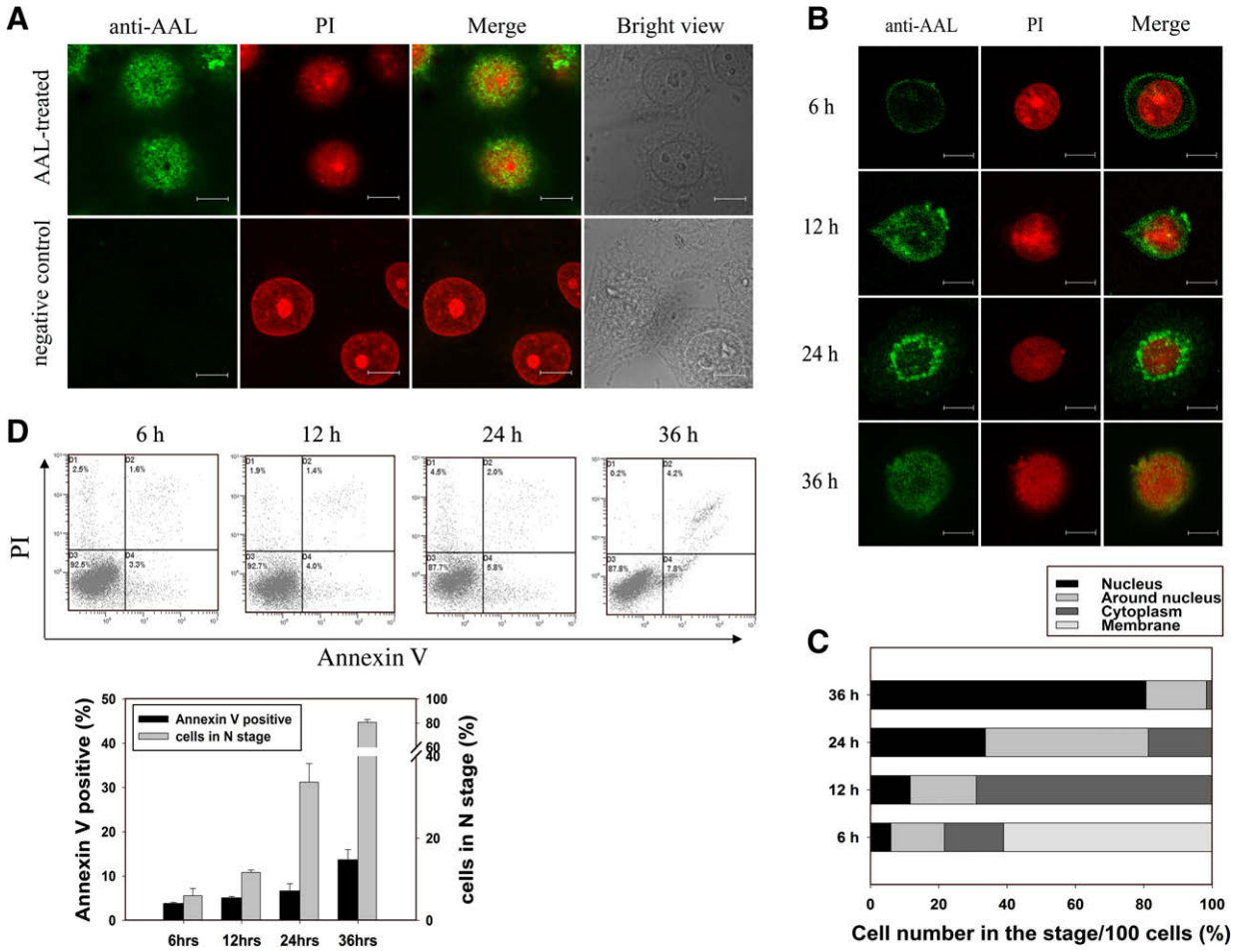

Fig. 1. Exogenously applied nAAL to HeLa cell cultures results in nuclear localization. (A) Exogenous nAAL was applied to Hela cell cultures for 36 h, and immunofluorescence was performed to detect the localization of AAL. Nuclei were stained for by PI. The green fluorescence represents AAL. To obviate nonspecific binding of AAL primary antisera, negative control (cell untreated with nAAL) was performed. Scale bar = 7 μm. (B) nAAL was added to the HeLa cell cultures and representative data of localization was detected at different time points (6, 12, 24, and 36 h) by confocal microscope. Scale bar = 7 μm. (C) The quantification of (A). Each sample was repeated in triplicate and 100 cells were counted each time. The cells at each stage (membrane, cytoplasm, around nucleus, nucleus) were counted. (D) The apoptotic activity of AAL in HeLa cells was assayed by the Annexin V staining at 6, 12, 24, and 36 h. The data was repeated three times, and the lower panel represents the statistical analysis. The error bars represent the standard deviations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The cellular localization of rAAL and mutant proteins was detected by immunofluorescence assay, and the apoptotic activity was determined by Annexin V staining and flow cytometry. In Fig. 3C and D, dimer interface mutant I25G, was mostly anchored to the cell membrane and was unable to induce HeLa cell apoptosis. L33A, the loop region mutant, is able to pass through membrane but loses the ability to enter the nucleus. The apoptotic activity was very low even at high concentration (12.5 μM) of recombinant protein. No fluorescence signal was observed with R63H indicating that mutation of the conserved CRD residue Arg63 could completely abort the adherence activity to membrane, resulting in loss of apoptosis-induced activity. The two mutants H59Q and I144G were distributed throughout the whole cells and maintain nuclear localization. I144G lost the apoptosis activity, and the activity of H59Q was reduced to about 40% of 'normal' AAL activity at the concentrations of 6.2 and 12.5 μM.

Overall, it was observed that mutant proteins I25G, L33A and R63H were mainly localized in regions other than the nucleus and lost apoptosis-induced ability. H59Q and I144G entered the nucleus, but only H59Q retained some bioactivity. Taken together, the nuclear import of AAL from the culture medium is important but not sufficient for AAL apoptotic activity.

Discussion

The novel antitumor mechanism for lectins

To our knowledge, no foreign lectins has been reported to be translocated to nucleus from outside cells and be able to functions. ABL entered and accumulated in perinuclear region of HT29 color cancer cell, interfering with the nuclear pore complex [14]. *Xerocomus chrysenteron* lectin (XCL) with toxic activity towards insects can be endocytosed in SF9, NIH3T3, and HeLa cells by clathrin-dependent pathway, but the relationship between the cellular sorting and the biological activity still remains unclear [24]. Immunotoxin ricin could be uptaken and located in the cytosol, where it exerted the cytotoxic effects [25]. As the 4th fungal galectin reported, AAL is the first one reported to have the antitumor activity. Here, we report that AAL is the first one to be internalized and localized in the nucleus of HeLa cells. Moreover, the apoptosis-induced activity is closely related to the nuclear localization. These results supply a novel explanation of lectins' activity.

Current popular belief is that galectins or lectins perform functions by binding to glycoconjugates at the cell membrane [22,23], but our data differ. The mutant protein I25G, almost anchored to the cell membrane, and lost its bioactivity completely. At early

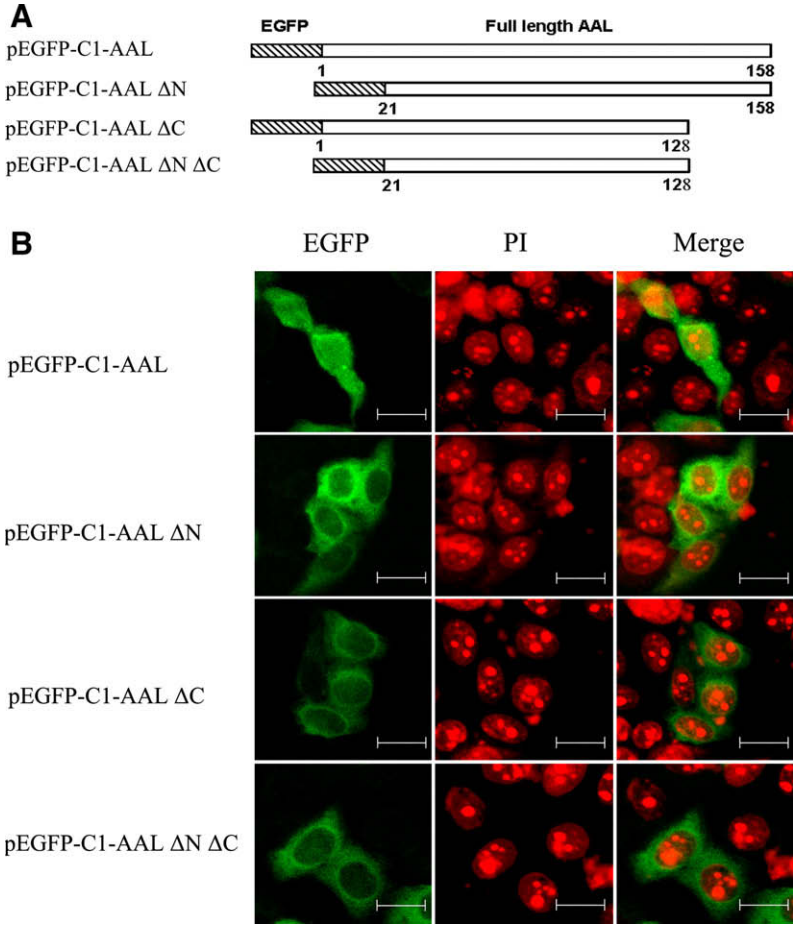

Fig. 2. N- or C-terminal truncation in AAL leads to loss of nuclear localization. (A) The construction of the pEGFP-truncated AAL mutants. (B) The localization of truncated AAL mutant proteins. pEGFP-truncated AALs were transfected into HeLa cells. After 36 h, the localization was detected by confocal microscopy. Nuclei were stained by PI. Green fluorescence represents truncated AAL or full-length AAL, red fluorescence represents the nucleus. Scale bar = 20 μm in panel (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

times/stages, AAL bind to the membrane of 61% cells, and the apoptotic activity is only 3.87% as low as the control group (Fig. 1D).

In previous study [21], we investigated the structural basis for the antitumor property of AAL: dimerization was a prerequisite, CRD and hydrophobic pocket were essential for the apoptosis-induced activity. Here, we provided a further explanation to the structural basis for the activity. CRD mutant R63H could not bind to the cell surface; anchorage of I25G on cell membrane implied that dimer interface may be required for transport from membrane to cytoplasm; the retention of L33A in cytoplasm indicated that the loop region was involved in regulating migrating into the nucleus. CRD mutant H59Q and loop region mutant I144G were distributed in the nucleus and cytoplasm, indicating that they maintained the ability to adhere to cell membranes and transport through nucleus membrane. We propose that AAL migration is determined by a 3 step process: (a) binding to the cell membrane; (b) translocation from cell membrane to cytoplasm; (c) transport into the nucleus. The complex process depends on spatial structure rather than certain nuclear localization sequences, which also explains that N-terminal or C-terminal truncation will destroy the nuclear localization activity.

The differences in the internalization process between AAL and mammalian galectins

Knowledge of galectins is mainly obtained from numerous investigations about mammalian galectins. Fifteen mammalian galectins have been identified and analyzed to play important roles in cancer biology, immunomodulation [26]. Many of them (galectin 1, 3, 8, 9 etc.) are explored deeply in structure and function, while the studies on fungal galectins are limited. This research, therefore, helps to a statement of differences between AAL and mammalian galectins.

The internalization of galectin 1 (Gal-1) was detected as early as 1 min after its addition to Jurkat cells [27], and galectin 8 (Gal-8) was internalized in CHO cells after 30 min [28]. Even the internalization of ABL and XCL can be observed after 1 h and 30 min incubation, respectively [14,24]. In our research, the kinetics of AAL migration to the nucleus seemed to be slow. At 6 h, AAL was found in the cytoplasm of only 17% cells (Fig. 1B and C). This suggests that AAL internalization is distinct from Gal-1, Gal-8 and other mushroom lectins.

Of all the galectins, the nuclear import mechanism of galectin 3 (Gal-3) shuttling between cytoplasm and nucleus has been studied in much detail [29–32]. In our research, the localization of truncated AAL mutants shows that the N-terminus and C-terminus are both important for nuclear import. Mutants I25G, L33A, and R63H lose nuclear entry ability and apoptotic activity suggesting that the internalization of AAL is a multi-step process that is determined by different spatial structures in the cell.

The regulation of AAL localization in the nucleus seems to be more complex than first thought. Impairment of nuclear localization leading to the bioactivity loss points to the hypothesis that there are some nuclear partners playing an essential role on the apoptosis activity. Even though the nuclear pathway appears to

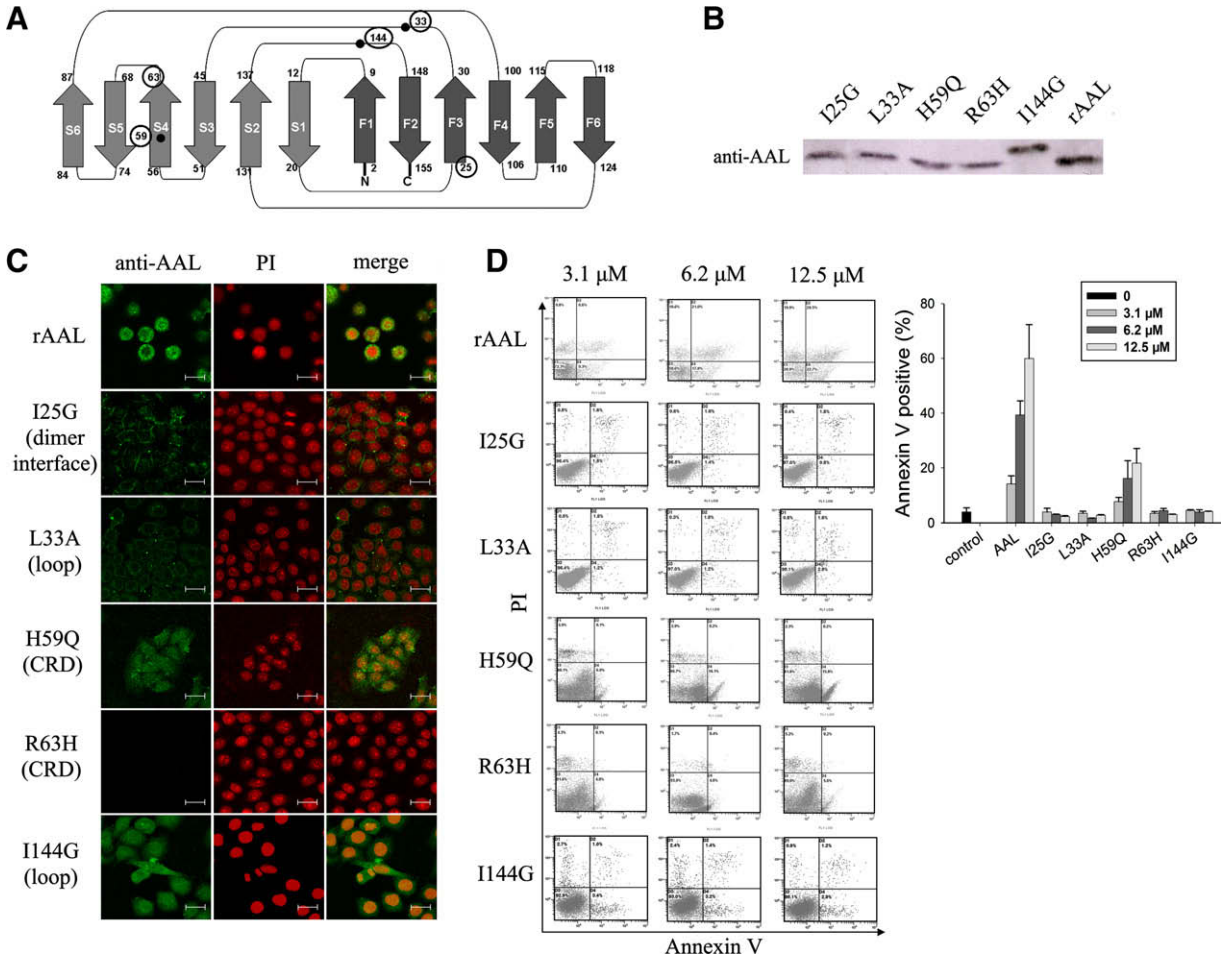

Fig. 3. The localization and apoptosis-induced activity of rAAL and mutant proteins in HeLa cells. (A) The position of site mutants on AAL secondary structure: CRD mutants (H59Q, R63H), dimer interface mutant (I25G) and loop region mutants (L33A, I144G). (B) Represents the recognition of the antisera to purified rAAL and mutant proteins by Western blot. (C) After incubating HeLa cell cultures with proteins for 36 h, the localization was detected by immunofluorescence. Scale bar = 25 μm. (D) The apoptotic activity of proteins at different concentrations was assayed by the Annexin V staining. The data was representative of one experiment. The right panel indicates statistic analysis.

be the most dominant, alternative cytoplasmic pathways for AAL induced cell death should not be excluded. Several interacting partners of Gal-3 have been identified in the nucleus, and may be important in regulating Gal-3 activity [33]. An N-terminal truncated form of Orp150 is identified to be a cytoplasmic ligand for anti-proliferative ABL [15]. It will be of interest to determine which factors or events, such as the effect of different AAL binding partners or modifications enable AAL to be apoptotic. AAL is the first fungal galectin reported to have antitumor and nuclear localization activity and merits further detailed investigation.

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