Edible Mushroom (Agaricus bisporus) Lectin, Which Reversibly Inhibits Epithelial Cell Proliferation, Blocks Nuclear Localization Sequence-dependent Nuclear Protein Import*

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The Galβ1–3GalNAc (TF antigen)-binding lectin (ABL) from the common edible mushroom (Agaricus bisporus) has a potent anti-proliferative effect without any apparent cytotoxicity. This unusual combination of properties prompted investigation of its mechanism of action. In contrast to soluble lectin, agarose-immobilized, and hence noninternalizable ABL had no effect on proliferation of HT29 colon cancer cells. Electron microscopy of HT29 cells incubated with fluorescein- and gold-conjugated ABL showed internalization of the lectin into endocytotic vesicles and multivesicular bodies. Confocal microscopy showed perinuclear accumulation of fluorescein isothiocyanate-conjugated lectin, which also inhibits HT29 cell proliferation, raising the possibility that the lectin might interfere with nuclear pore function. Transport of heat shock protein 70 into the nucleus in response to heat shock was blocked by pre-incubation of HT29 cells for 6 h with 40 μg/ml ABL. In digitonin-permeabilized cells, nuclear uptake of bovine albumin conjugated to a nuclear localization sequence (NLS)-containing peptide was also inhibited by a 15-min preincubation with 40–100 μg/ml ABL. In contrast, serum-stimulated nuclear translocation of mitogen-activated protein kinase, which is NLS-independent, was not affected by pretreatment of cells with the lectin. These results suggest that the anti-proliferative effect of ABL is likely to be a consequence of the lectin trafficking to the nuclear periphery, where it blocks NLS-dependent protein uptake into the nucleus.

Many lectins affect the proliferation of cells to which they bind; classic examples include concanavalin A and phytohaemagglutinin as well as more surprising mitogens such as solubilized hepatocyte asialoglycoprotein receptor (1). Some lectins affect cell proliferation by cross-linking of cell surface glycosconjugates probably without any need for internalization (2, 3), whereas ricin is internalized and translocated to the cytosol, where its associated toxin exerts its cytotoxic effects (4). In many cases, however, the mechanism for the effects of lectins on proliferation is unclear.

Increased cell surface expression of the oncofetal carbohydrate antigen, Thomsen-Friedenreich (TF,1 Galβ1–3GalNAcα), is a common feature of malignant and premalignant epithelia (5–7). In normal epithelia this structure is usually concealed (7), probably by sialylation (8) or sulfation (9). We have previously shown that nontoxic dietary lectins, which bind the TF antigen, are capable of having marked proliferative and anti-proliferative effects both in vitro and in vivo on normal and cancerous human intestinal epithelial cells (10–14). Thus, stimulation of proliferation has been shown with the TF-binding lectin, peanut (Arachis hypogea) agglutinin (PNA) (10), as well as with anti-TF monoclonal antibodies (15). In contrast, the lectin from the common edible mushroom Agaricus bisporus (ABL), which unlike PNA, can also bind to the 2–3 sialylated TF antigen, has been shown to inhibit proliferation in a reversible and nontoxic fashion in a wide range of epithelial cell types, blocking the growth-stimulatory effects of epidermal growth factor, serum, and insulin (14). Because many lectins are tightly globular proteins that are highly resistant to heat and digestion (16) and can be detected in active form in feces (10), their presence in foods is likely to be of considerable importance in the functional relationship between diet and intestinal proliferation and hence for intestinal cancer development (17). Because ABL has a powerful anti proliferative effect on malignant colon cells without any apparent cytotoxicity, we have investigated the mechanism by which this lectin elicits its biological effects.

EXPERIMENTAL PROCEDURES

Cell Proliferation, RNA, and Protein Synthesis

The HT29 cell line, established from an adenocarcinoma from a 44-year-old female Caucasian, was obtained from the European Cell Culture Collection (Public Health Laboratory Service, Porton Down Wiltshire, UK) and cultured as described previously (14). Subconfluent HT29 cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) (v/v) in 24-well plates were washed twice with PBS, and serum-free DMEM containing 250 μg/ml bovine serum albumin (BSA) was added. After incubation at

1 The abbreviations used are: TF antigen, Thomsen-Friedenreich antigen (Galβ1–3GalNAcα); ABL, A. bisporus lectin; BSA, bovine serum albumin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Hsp70, heat shock protein 70; MAPP (ERK-1 and ERK-2), mitogen-activated protein kinase; NLS, nuclear localization sequence; NTF2, nuclear transport factor 2; PNA, A. hypogea lectin; WGA, wheat germ agglutinin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

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37 °C for 24 h, ABL (Sigma), agarose immobilized-ABL (EY Laboratories Inc., San Mateo, CA), 10-nm colloidal gold-conjugated ABL (EY Laboratories) or FITC-conjugated ABL (EY Laboratories) was added for 24 h before a 1-h pulse with 0.8 µCi/well [methyl-³H]thymidine. The cell-associated radioactivity was determined as described previously (15).

To assess the effect of ABL on DNA, RNA, and protein synthesis in subconfluent cells, the culture medium was replaced by serum-free DMEM containing 250 µg/ml BSA, and 20 µg/ml ABL was introduced. The cells were pulsed with 1 µCi/ml [methyl-³H]thymidine, [³H]uridine, or [³H]leucine for 1 h at the time indicated in the figure legends, and the radioactivity associated with macromolecules was determined as described previously (15).

**Internalization Assessed by Electron Microscopy**

Gold-ABL—HT29 cells were cultured in DMEM containing 5% FCS (v/v) in 24-well plates until 80% confluent. The culture medium was replaced by serum-free DMEM containing 250 µg/ml BSA and 5 µg/ml 10-nm colloidal gold-conjugated ABL. In control wells, the medium was replaced with either serum-free DMEM containing 250 µg/ml BSA or 5 µg/ml 10-nm colloidal gold (Sigma). The cells were incubated at 37 °C for 1, 2, 4, 6, 8, 12, or 24 h. After three washes with filtered PBS, the cells were released from the plates by trypsinization. Cells were pelleted by centrifugation at 1000 × g for 5 min and then fixed in 2.5% glutaraldehyde (v/v) (electron microscopy grade, Agar Scientific Ltd., Stansted, UK) in Sorensen’s buffer, pH 7.4. The samples were processed for conventional electron microscopy using a standard 3-day processing protocol, stained with uranyl acetate and Reynolds’ lead citrate, and viewed on a Philips CM-10 electron microscope.

FITC-ABL—HT29 cells were cultured as above in the presence of 0 to 40 µg/ml FITC-ABL for 1 or 24 h. The cells were released, washed, and pelleted as above before fixation in 2% paraformaldehyde and 0.1% glutaraldehyde in Sorensen’s buffer. Cell pellets were dehydrated through an ethanol series before embedding in LR White (London Resin Co., UK). Sections were incubated with rabbit anti-fluorescein antibody (DAKO Ltd., Cambridge, UK) followed by gold (10 nm)-conjugated goat anti-rabbit antibody (Sigma). On some sections the gold labeling was silver-enhanced (British Biocell International, UK) before staining with uranyl acetate and Reynolds’ lead citrate. Control sections included untreated cells and omission of primary antifluorescein antibody.

**Intracellular Distribution of FITC-ABL and Tetramethylrhodamine Isothiocyanate (TRITC)-Dextran Analyzed by Confocal Microscopy**

Subconfluent HT29 cells cultured in glass-bottomed dishes (MatTek Corp. Ashland, MA) were incubated at 37 °C with 20 µg/ml FITC-ABL in serum-free DMEM containing 250 µg/ml BSA. After 1 h, unbound lectin was removed by three washes with PBS, and the cells were cultured in fresh serum-free DMEM containing 250 µg/ml BSA at 37 °C in a CO₂ chamber attached to a LSM510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany). The FITC distribution was then monitored every h for 24 h.

In dual-label experiments, the cells were incubated for 30 min with 0.15 µg/ml TRITC-dextran (155 kDa, Sigma) followed by the addition of 2 µg/ml FITC-ABL. The subcellular distributions of FITC and TRITC were monitored simultaneously using the 488-nm and 543-nm laser lines every h for 10 h.

**Effect of ABL on Nuclear Import of Heat Shock Protein**

HT29 cells were cultured on glass coverslips in 24-well plates in DMEM containing 5% FCS (v/v) at 37 °C for 5 days. The cells were washed once with PBS and cultured in DMEM containing 1% FCS (v/v) for a further 24 h at 37 °C. ABL (0–80 µg/ml) or FNA (40 µg/ml) was added, the same volume of diluent (PBS) was added to control cells, and the cells were then cultured at 37 °C for 6 h before being placed into a 43 °C incubator (5% CO₂,95% air) for 1 or 3 h of heat stress. The time course of the ABL effect was also assessed by varying the length of incubation of HT29 cells with 80 µg/ml ABL from 0–24 h before heat shock. The cells were then washed 3 times with ice-cold PBS and fixed over night in 2.5% paraformaldehyde (Sigma) and 0.1% Triton-100 (v/v) in PBS at 4 °C (18). The fixed cells were washed 3 times with ice-cold PBS and then washed with PBS containing 1% BSA (w/v) and 1% Triton X-100 (v/v) for 10 min at room temperature. Nonspecific binding was blocked by incubation with 5% normal rabbit serum (v/v) for 1 h at room temperature. Mouse IgG monoclonal antibody, which recognizes Hsp73 (Sigma) (1.50 dilution with 1% BSA (w/v) in PBS) or mouse IgG monoclonal antibody to inducible Hsp72 (Amersham Pharmacia Biotech) (1:200 dilution with 1% BSA (w/v) in PBS), was applied and incubated for 90 min at room temperature. After four washes with PBS, anti-mouse IgG conjugated with FITC (Sigma) (1:50 dilution with 1% BSA (w/v) in PBS) was then applied and reacted in the dark for 1 h at room temperature. The cells were then washed 3 times with PBS, 2 times with 1% BSA (w/v), 1% Triton X-100 (v/v), and PBS, 3 times and again with PBS. The glass coverslips were mounted (Vectashield mounting medium for fluorescence H-1000, Vector, Burlingame, CA), and photographs were taken with incident illumination in a fluorescence microscope (Polyvar, Reichert-Jung, Austria).

**Conjugation of NLS Peptide with BSA**

A peptide containing the SV40 large T antigen wild type NLS CGGG-PKKKKKVKED with the N-terminal C providing a thiol group for conjugation and the GGG acting as a spacer was synthesized using Fmoc
(N-9-fluorenyl)methoxycarbonyl) chemistry and pentafluorophenyl ester on a PerSeptive 9050 synthesizer (PerSeptive Biosystems, Hertford, UK) using standard protocols. Before conjugation, BSA (5 mg/ml) was activated by incubation for 90 min at room temperature in 100 mM HEPES-NaOH, pH 7.3, containing a 100-fold molar excess of sulfo-succinimidyl 4-[N-maleimidomethyl] cyclohexane 1-carboxylate (Pierce and Warriner, Chester, UK) (19). Excess cross-linker was removed by gel filtration on a PD10 column containing 9.1 ml of Sephadex-G25 (Amersham Pharmacia Biotech). The activated BSA solution was added to a 30-fold molar excess of NLS peptide. The pH was adjusted to 7.5, and the reaction was allowed to proceed for 2 h at 37 °C. Uncoupled peptide was removed by gel filtration on a PD10 column containing 150 mM NaCl. The molar ratio of coupling was 20–30 peptides/BSA molecule as estimated from the electrophoretic mobility. For fluorescein conjugation, carboxyfluorescein-N-hydroxysuccinimide ester (Boehringer Mannheim) was dissolved in Me2SO and then added to peptide-BSA solution in 0.2 M NaHCO3 at a 2:1 molar ratio to BSA. After overnight incubation at 4 °C, free fluorescein was removed by gel filtration on a PD10 column. The resulting conjugates were freeze-dried, dissolved in 150 mM NaCl, and kept at −80 °C.

**FIG. 2. Intracellular trafficking of gold-ABL and FITC-ABL.**

A, 10-nm colloidal gold-conjugated ABL internalized in clathrin-coated vesicles (thin arrow) and in pinocytic vesicles (thick arrow) after a 2-h incubation with HT29 cells. B, gold-ABL seen in multivesicular bodies (arrow) after a 6-h incubation. C, FITC-ABL identified after a 1-h incubation using anti-fluorescein antibody and gold-conjugated anti-rabbit antibody with silver enhancement. Labeling is intense on the cell surface, and there is also evidence of internalization within vesicles. The fixation protocol (2% paraformaldehyde and 0.1% glutaraldehyde) did not allow identification of clathrin coating. D, FITC-ABL identified within multivesicular bodies (24-h incubation). Some FITC-ABL is apparently in adjacent cytoplasm. E, FITC-ABL around the periphery of two large organelles containing heterogeneous inclusions in close proximity to the nuclear membrane (24-h incubation). HT29 cells were cultured in serum-free DMEM containing 250 μg/ml BSA with 5 μg/ml 10-nm colloidal gold ABL or 40 μg/ml FITC-ABL for up to 24 h, and then the cells were fixed, and 90-nm sections were cut and examined by transmission electron microscopy. Bar, 0.15 μm.
Preparation of Cytosol Fractions for Nuclear Protein Import

Subconfluent HT29 cells (10^5) were collected by scraping with a rubber policeman and washed twice with ice-cold PBS followed by centrifugation for 5 min at 600 × g. The cells were then washed once with 10 mM HEPES-HCl buffer (10 mM HEPES, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, and 2 mM dithiothreitol). The cell pellet was gently resuspended in 1.5 volumes of 5 mM Hepes-HCl buffer (5 mM Hepes, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 20 mM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride, and 1 µM each of aprotinin and leupeptin) and kept for 15 min on ice before homogenization by 30 strokes in a No. 2 bulb homogenizer (Jencons Scientific Ltd, Leighton Buzzard, UK). The resulting homogenate was centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was dialyzed extensively against transport buffer (20 mM Hepes-HCl, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol) and stored at −80 °C. Protein concentration was 10–20 mg/ml.

Cell Permeabilization and in Vitro Nuclear Protein Import—Cell permeabilization and nuclear protein import was carried out by a modification of the method described by Adam et al. (20). HT29 cells cultured in glass chamber slides (Nunc, Kamstrup, Denmark) were first rinsed in cold transport buffer and then permeabilized in 40 µ/ml digitonin (Sigma) in transport buffer for 5 min at 37 °C. After extensive washes with ice-cold transport buffer, transport mixture (final volume 140 ml) was applied (20% cytosol fraction, 1 mM ATP, 5 mM creatine phosphate, 20 units/ml creatine phosphokinase, 20 µM BSA-NEFluorescein in transport buffer). The chamber slides were maintained on ice during the procedure. The protein transport reaction was started by transferring the chamber slides into a 30 °C incubator and, after 30 min, was stopped by the addition of 1 ml of ice-cold transport buffer.

For the ABL inhibition experiment, before the addition of the NLS-BSA-fluorescein, the permeabilized cells were first incubated with ABL (0–100 µg/ml) for 15 min at room temperature in the presence of cytosol fraction and ATP-regenerating system as above. Substitution of wheat germ agglutinin (WGA, 100 µg/ml) for ABL acted as a positive control for this experiment. The cells were then washed extensively with transport buffer, and the slides were mounted and observed by fluorescence microscopy (Polyvar, Reichert-Jung, Austria) with a 25× objective.

Effect of ABL on Mitogen-activated Protein Kinase (MAPK (ERK-1 and ERK-2)) Nuclear Translocation

HT29 cells were cultured in 8-well glass chamber slides (Nunc) for 24 h. After 1 wash with PBS, the cells were cultured in serum-free DMEM containing 250 µg/ml BSA for a further 24 h before the addition of 40 µg/ml ABL (the same volume of PBS was added to control wells). Six h later, 20% FCS (v/v) was added to each well for 20 min. The cells were then washed once with PBS and fixed in 1% formaldehyde (w/v) in PBS for 15 min at room temperature followed by permeabilization with 100% methanol for 10 min at −20 °C. The cells were blocked with 1% BSA (w/v) in PBS for 1 h and then incubated with 1.5 µg/ml rabbit polyclonal antibody to phosphorylated MAPK (Promega Ltd, Southampton, UK) in 1% BSA (w/v), 0.25% Nonidet P-40 (v/v), PBS for 4 h (the same volume of 1% BSA (w/v), 0.25% Nonidet P-40 (v/v), PBS was added to control wells but without anti-MAPK antibody). After 6 washes with PBS, biotin-conjugated anti-rabbit IgG (1:250 dilution in 1% BSA (w/v) in PBS) (Sigma) was applied for 1 h followed by incubation of the cells with avidin-FITC conjugate (1:200) (Sigma) for 1 h in the dark. After washing with PBS, the cells were visualized and photographed using a fluorescent microscope (Polyvar, Reichert-Jung, Austria) with a 25× objective.

Quantification of Relative Cytoplasmic and Nuclear Fluorescence by Confocal Microscopy

Quantification of apparent nuclear and cytoplasmic fluorescent ratios was achieved using Kinetic Imaging Lucida software (Kinetic Imaging, Liverpool, UK). From each cell in randomly chosen fields, a 2-µm-thick confocal slice centered on the nucleus was obtained. The

**Fig. 3. Intracellular trafficking of FITC-ABL and TRITC-dextran in HT29 cells.** Fluorescence image of the cells assessed by confocal microscopy after incubation of the cells with 20 µg/ml FITC-ABL for 1 (A), 3 (B), and 6 (C) h. After a 1-h incubation (A), ABL fluorescein is mainly seen in association with cell membranes but with some internalization. After 3 (B) and 6 (C) h, it shows perinuclear accumulation. D shows phase contrast image. E shows a dual image of FITC-ABL and TRITC-dextran 4 h after addition to the cells at 2 µg/ml and 0.15 µg/ml, respectively. The staining patterns are clearly distinct.

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Quantification of apparent nuclear and cytoplasmic fluorescent ratios was achieved using Kinetic Imaging Lucida software (Kinetic Imaging, Liverpool, UK). From each cell in randomly chosen fields, a 2-µm-thick confocal slice centered on the nucleus was obtained. The
nucleus and whole cell sections were carefully traced using the “lasso” function of the kinetic imaging software. At least 20 cells were analyzed for each datum point.

RESULTS
Comparison of Effects of Free and Conjugated ABL on HT29 Cell Proliferation

Twenty μg/ml free ABL produced 81% inhibition of thymidine incorporation into DNA in HT29 colon cancer cells (Fig. 1). When ABL was immobilized by conjugation to agarose, no significant inhibitory effect was seen at concentration up to 100 μg/ml ABL (Fig. 1), suggesting that the lectin has to be internalized to have its inhibitory effect on proliferation. Minimum concentrations of ABL conjugates capable of agglutinating type O human red blood cells were free ABL (2 μg/ml) and FITC-ABL (4 μg/ml).

As a prelude to electron and confocal microscopic studies of intracellular trafficking of the lectin, FITC- and gold-conjugated ABL were also assessed for their effects on agglutination and proliferation. Colloidal gold (10 nm)-conjugated ABL (20 μg/ml ABL) had no significant effect on HT29 cell proliferation (Fig. 1), but at this concentration, the gold-ABL conjugate had only weak agglutinating ability (see below). Higher concentrations of gold-ABL were not attainable. In contrast, FITC-conjugated ABL (20 μg/ml ABL) produced 49% inhibition of HT29 cell proliferation (Fig. 1). The minimum concentration of these conjugates capable of agglutinating red blood cells was 16 μg/ml for both gold- and agarose-ABL. Neither 10-nm colloidal gold alone nor FITC-BSA (20 μg/ml BSA) had any effect on HT29 cell proliferation. We next examined the intracellular fate of gold-ABL and FITC-ABL.

Intracellular Trafficking of ABL

Electron Microscopy Studies—Internalization of ABL by HT29 cells was demonstrated by electron microscopy in 10 separate experiments using 10-nm gold-conjugated ABL and also using fluorescein-conjugated ABL in view of the ability of the latter conjugate to inhibit proliferation. After incubation with HT29 cells for 1 h, gold-ABL was bound to the cell surface, and internalization had begun. The gold-ABL was aggregated in clathrin-coated pits and immediately beneath the plasma membrane in clathrin-coated vesicles (Fig. 2A). The cellular entry of gold-ABL also involved clathrin-independent pathways, because noncoated pits and pinocytic vesicles also contained gold particles (Fig. 2A). A third mechanism of entry was macropinocytosis, recognized by association of the gold label with surface folds or villi and subsequent incorporation into vacuoles of 0.5 μm or more (result not shown). The gold-ABL extended beyond the system of early endosomes and was identified frequently within the endosomal carrier vesicles, often called multivesicular bodies (Fig. 2, B and D), which traffic to the late endosomal system (21). Over a 12-h incubation, more lectin became internalized, but it remained within membrane-bound vesicles that were often found in close proximity to the nuclear envelope. In control cells incubated with 10-nm colloidal gold, no gold was found inside the cells (results not shown). A further series of experiments with both 5- and 10-nm colloidal gold-conjugated ABL confirmed localization within the lumen of vesicles that were often close to the nucleus, but for both particle sizes, there was no clear evidence of lectin outside vesicles. Studies of the fluorescein-conjugated lectin (Fig. 2, C, D, and E) yielded almost identical results, with prominent accumulation within multivesicular bodies, although there was possibly some fluorescein-conjugated lectin identified within cytoplasm close to these bodies.

Confocal Microscopy—After a 1-h incubation of the cells with 20 μg/ml FITC-ABL, strong cell surface and intracellular fluorescence was observed (Fig. 3A). By 3 h, accumulation of fluorescence around the nucleus was seen in the majority of cells in each of 12 independent experiments (Fig. 3, B–D). In all cells, punctate intracellular FITC-ABL fluorescence was seen, but in addition, a ring of perinuclear fluorescence was observed. We then compared the intracellular trafficking of TRITC-dextran, a marker of fluid phase endocytosis (22), with that of FITC-ABL. Both conjugates were clearly internalized. However, the TRITC-dextran fluorescence was always punctate and showed no overlap with the distribution of FITC-ABL (Fig. 3E). These
results suggested that FITC-ABL internalization is receptor-mediated rather than occurring via fluid phase endocytosis.

**Effect of ABL on the Nuclear Import of Heat Shock Protein 70 in Live Cells**—The perinuclear accumulation of FITC-ABL raised the possibility that the inhibitory effect of ABL on cell proliferation might be the consequence of interaction with the protein import machinery of the nuclear pores. To test this hypothesis, the effect of ABL on nuclear import of heat shock protein 70 (Hsp70) was studied. Hsp70 is produced by prokaryotic and eukaryotic cells in response to environmental changes such as heat stress. There are two forms of Hsp70, one (Hsp70) is constitutively expressed, whereas the synthesis of the other (Hsp72) is induced by heat shock from existing mRNAs (23). During heat stress, both forms of the Hsp70 are imported into the nucleus, whereas a small quantity remains cytoplasmic (18, 23). During recovery from heat stress, Hsp70 leaves the nucleus and becomes distributed throughout the cytoplasm (18). As expected, Hsp73 became concentrated in the nuclei of the control cells after 1 h of heat shock at 43 °C (Fig. 4A, a). However, when the cells were pretreated with 40 μg/ml ABL

**Fig. 5.** Preincubation with ABL prevents nuclear import of Hsp72 in response to heat shock. HT29 cells were incubated with or without 40 μg/ml ABL or PNA for 6 h at 37 °C before being subjected to heat stress at 43 °C for 3 h. The cells were either fixed and Hsp72 identified using the monoclonal antibody to Hsp72 followed by fluorescein-conjugated second antibody (A) or lysed followed by Western blotting with the same monoclonal antibody (B). Quantification of Hsp72 intracellular localization was carried out using confocal microscopy on the cells treated with 0–80 μg/ml ABL for 6 h or with 80 μg/ml ABL for 6 h in the presence of 100 μg/ml asialo bovine mucin (ASM) (D) or 80 μg/ml ABL for various times before heat stress (E). Before heat stress, Hsp72 was only observed in the cytoplasm (A, a). After heat stress, the control (A, b) and PNA-treated (A, d) cells showed concentrations of Hsp72, identified by uniform fluorescence within the nucleus, whereas ABL-pretreated cells (A, c) showed Hsp72 retained within the cytoplasm. The bar in d represents 15 μm. Western blotting showed an increased expression of Hsp72 (arrow) upon heat shock that was not affected by pretreatment with ABL (B). Inhibition by ABL of Hsp72 nuclear import was both dose- (C) and time-dependent (E) and was abolished by the presence of TF-expressing ASM (D).
NLS-BSA fluorescein in permeabilized cells.

HT29 cells grown on glass chamber slides were permeabilized with 40 μg/ml WGA for 15 min before the application of NLS-BSA fluorescein. For 6 h, Hsp73 remained in the cytoplasm after the same heat shock (Fig. 4A, b). To rule out the possibility that this effect might be because of the inhibition of Hsp73 synthesis by ABL, a parallel experiment was performed in which the cells were cultured in the presence or absence of ABL and lysed after 1 h of heat shock at 43 °C, and immunoblots were performed with anti-Hsp73. This demonstrated that ABL had no effect on the cellular content of Hsp73 (Fig. 4B). Pretreatment of the cells with the growth stimulatory TF-binding peanut lectin (PNA), did not have any effect on Hsp73 nuclear translocation (Fig. 4A, c).

Further experiments were performed with an anti-Hsp70 monoclonal antibody specific for the heat-inducible form of Hsp70 (Hsp72). Using this antibody, Hsp72 in non-heat-shocked cells was found only in the cell cytoplasm (Fig. 5A, a). After heat treatment at 43 °C for 3 h, Hsp72 accumulated inside the nucleus (Fig. 5A, b). Pretreatment of the HT29 cells with 40 μg/ml ABL for 6 h again excluded the entry of Hsp72 into the nucleus (Fig. 5A, c), whereas 40 μg/ml PNA pretreatment of the cells for 6 h again did not affect Hsp72 subcellular distribution (Fig. 5A, d). A similar increase of Hsp72 expression after heat treatment occurred in both the ABL-treated and untreated cells, suggesting that ABL has no effect on the induction of Hsp72 by heat shock (Fig. 5B). Quantification of the cellular distribution of Hsp72 using confocal microscopy shows that the inhibition of Hsp72 nuclear import by ABL is both dose- and time-dependent (Fig. 5, C and E). The maximal effect produced by 80 μg/ml ABL occurred after a 6-h incubation with the cells. This effect of ABL on Hsp72 nuclear import was abolished by the presence of 100 μg/ml TF-expressing asialo bovine mucin (Fig. 5D).

**Effect of ABL on Nuclear Import of NLS-BSA**

**Fluorescein in Digitonin-permeabilized Cells**

The observation that ABL inhibits the nuclear import of Hsp70 suggested that this lectin may have a more general effect on protein transport into the nucleus. For many large proteins, import into the nucleus requires the presence in the cytoplasmic protein, importin α (25). To assess the effect of ABL on NLS-dependent nuclear protein import, a synthetic peptide containing the SV40 large T antigen wild type NLS (CGGGPKKKRKVED) (24, 26) was directionally conjugated through the thiol group of the N-terminal C to BSA. The conjugate was labeled with fluorescein and employed to assess nuclear import in digitonin-permeabilized cells, as described by Adam et al. (20). In the absence of lectin, NLS-BSA fluorescein was transported into the cell nucleus after 30 min (Fig. 6a). In three separate experiments, when permeabilized cells were pretreated for 15 min with 100 μg/ml ABL, a marked inhibition of nuclear import of the NLS-BSA fluorescein was observed (Fig. 6c). Substitution of WGA (100 μg/ml) for ABL acted as a positive control for this experiment (Fig. 6b). No direct binding of ABL to NLS-BSA fluorescein could be demonstrated in a control experiment in which the NLS-BSA (30 μg/ml) c, cells pretreated with 100 μg/ml ABL for 15 min before the application of NLS-BSA fluorescein. d, cells pretreated with 0–100 μg/ml ABL for 15 min in the presence or absence of 100 μg/ml asialo bovine mucin (ASM) and the intracellular distribution of fluorescein was analyzed by confocal microscopy. In the control cells (a) NLS-BSA fluorescein is seen to have entered the nuclei, whereas with WGA (b) and ABL (c) pretreatment, the NLS-BSA fluorescein is retained in the cytoplasm where it appears as a fluorescent ring. Bar, 15 μm. Inhibition by ABL of NLS-BSA fluorescein (FLUOS) nuclear import was dose-dependent and could be abolished by the presence of TF-expressing ASM (d).
NLS-specific blockade of nuclear protein import by ABL does not affect the cytotoxic inhibition of cell growth by ABL and confirms that the continued ability of HT29 cells to synthesize RNA and protein reflects the non-cytotoxic effect of ABL on DNA, RNA, and protein synthesis in HT29 cells was almost completely inhibited after 24 h of incubation with 20 μg/ml ABL, the inhibition of RNA synthesis and protein synthesis was much less marked (Fig. 8). This continued ability of HT29 cells to synthesize RNA and protein reflects the non-cytotoxic inhibition of cell growth by ABL and confirms that NLS-specific blockade of nuclear protein import by ABL does not result in cell death.

**DISCUSSION**

*A. bisporus* lectin ABL inhibits cell proliferation in a wide range of cells without cytotoxicity (14), suggesting that this lectin might affect a cellular process fundamental to cell division. In the present study, we found that agarose-conjugated ABL is not anti-proliferative, indicating that ABL has to be internalized to inhibit HT29 cell proliferation. Internalization of the lectin was directly demonstrated with both gold- and FITC-conjugated ABL. Both gold-ABL and FITC-ABL were predominantly associated with the lumen of vesicles and were both present within multivesicular bodies, many of which were in close proximity to the nuclear membrane (Figs. 2 and 3). Some FITC-ABL but not gold-ABL was apparently in cytoplasm around the multivesicular bodies, but it is not clear to what extent this difference between the lectin conjugates might have been the consequence of the different fixation processes used for the electron microscopy sections. The FITC-ABL but not the gold-ABL was shown to inhibit cell proliferation.

The demonstration by confocal microscopy that internalized FITC-ABL accumulated around the nucleus (Fig. 3) led us to speculate that the lectin might interfere with nuclear pores, perhaps blocking nuclear protein import. In live HT29 cells, preincubation with ABL inhibited nuclear import of Hsp70 in response to heat shock (Figs. 4 and 5). Hsp70 has itself been shown to be an essential component of the mechanism for NLS-dependent nuclear protein import (29–32), possibly as a molecular chaperone to promote the formation and stability of the NLS cargo complex (32). Inhibition by ABL of nuclear import of synthetic NLS peptide in permeabilized HT29 cells (Fig. 6) provides further evidence that ABL inhibits the NLS-dependent nuclear protein import mechanism. Inhibition of nuclear protein transport has previously been demonstrated with WGA and *Sambucus nigra* lectins but only in digitonin-permeabilized cells or following direct injection of the lectin into cells or isolated nuclei (33–35).

Molecular exchanges between the nucleus and cytoplasm occurring via the nuclear pore complexes (36, 37) are fundamentally important for cell growth. The nuclear pore complexes accommodate both passive diffusion of ions and globular proteins of less than 20 kDa (38, 39) and active transport of larger macromolecules (36, 37) through a gated channel by sequence-dependent (40, 41), and energy-dependent mechanisms (42, 43). Protein transport into the nucleus is multisteped and includes the binding of the transport ligand by NLS receptors and movement of the transport ligand-NLS receptors to the nuclear pores (25). Cytosolic factors such as the NLS receptor/
importins (44, 45), p97 (46), the GTPase Ran/TC4 (47, 48), Hsp70 (29, 30), and NTF2/B-2 (49) have been shown to be essential for this process. After GTP hydrolysis by Ran at the nuclear pore complex binding site, the receptor-ligand-Ran.GDP complex is translocated into the nucleus. This involves interactions of the complex with the O-GlcNAc glycosylated glycoprotein p62 localized on both the nucleoplasmic and cytoplasmic side of the nuclear pore complexes (50–52). Binding to p62 by monoclonal antibody (53), WGA (GlcNAc binding) (33–35), and S. nigra agglutinin (sialic acid 2–6 Gal/GalNAc binding) (35) all inhibit protein transport into the nucleus. Several pieces of evidence suggest, however, that ABL is unlikely to be acting via direct interaction with p62. ABL has been shown not to bind N-acetyl-glucosamine (54), and the absence of staining of the nuclear periphery by FITC-conjugated peanut lectin (33) implies the absence of unsialylated Gal\(^1\)–3GalNAc\(^a\)– at this site. Moreover, although sialic acid has recently been demonstrated on p62, its enzymatic removal does not reveal the peanut lectin receptor (55), suggesting that sialyl 2–3 Gal\(^1\)–3GalNAc\(^a\), the alternative ligand for ABL, is also absent from p62.

Alternatively, ABL may bind to a cytosolic component, which is essential for nuclear protein import, e.g., p97, importin \(\alpha\) or \(\beta\), RanBP2, or NTF2. This also raises the possibility that the ligand for ABL may also be a ligand for one of the naturally occurring intracellular galactose-binding lectins (galectins) (56). However, no intracellular \(\beta\)-galactoside-containing glycoproteins have been identified so far, and it is also possible that ABL may interact with its ligand by protein-protein interaction, as it has been shown that peptides may mimic galactosides and interact with the carbohydrate recognition domain of galactose-binding lectins (57). We do not yet have clear evidence that the lectin gains access to the cytosol, and an alternative possibility is that it is exerting its effect by interaction with the luminal side of a transmembrane glycoprotein.

It is now apparent that there are at least three different mechanisms for nuclear import of large proteins; first, the NLS-dependent mechanism, which has been well characterized (43, 52); second, a WGA-inhibitable but non-NLS-dependent mechanism described for nuclear import of heterogeneous nuclear ribonucleoprotein via a 38-amino acid M9 domain (58) interacting with transportin (27); and third, the non-WGA-inhibitable non-NLS-dependent mechanism described for nuclear import of MAPK (28). It seems likely that the inhibition of NLS-dependent nuclear import by ABL is relevant to its effect on proliferation, whereas its lack of effect on the MAPK import pathway (Fig. 7) may explain why the cells can still survive after inhibition of the classical NLS-dependent pathway. Previous studies have shown that intracytoplasmic injection of anti-Hsp73 antibody also blocks NLS-dependent nuclear protein import without causing cell death (29).

The present study suggests that inhibition of cell proliferation by ABL is a consequence of the specific trafficking of the lectin to the nuclear periphery where it blocks NLS-dependent protein uptake into the nucleus. Further studies are in progress to characterize the intracellular ABL-binding glycoproteins and identify the molecular target of ABL in the NLS-dependent nuclear transport machinery.

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FIG. 8. Time response of the effect of ABL on DNA, RNA, and protein synthesis in HT29 cells. HT29 cells in serum-free DMEM in the absence or presence of 20 \(\mu\)g/ml ABL were pulsed with 1 \(\mu\)Ci/ml [methyl-\(^3\)H]thymidine, \([\text{H}]\)uridine, or \([\text{H}]\)leucine for 1 h at different times after the addition of 20 \(\mu\)g/ml ABL. Significant inhibition of protein synthesis by ABL occurred only after 31 h, whereas significant inhibitory effects on DNA and RNA synthesis were seen after 6 to 8 h.

\(*\), \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.005\) (two-tailed \(t\) test).
