Fungal lectin, XCL, is internalized via clathrin-dependent endocytosis and facilitates uptake of other molecules

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Fungal lectin – clathrin – facilitation of endocytosis

The lectin isolated from Xerocomus chrysenteron (XCL) displays a toxic activity towards insects. In order to assess its possible mode of action and to gather useful data for its potential use in insect-resistant transgenic plants, we investigated the effects of XCL at the cellular level. Immunofluorescence microscopy studies revealed that XCL is rapidly internalized into small endocytic vesicles that further coalesce in the perinuclear region. We show that XCL is endocytosed by the clathrin-dependent pathway, and is delivered to late endosome/lysosome compartments. The internalization of XCL seems to be general since it occurs in different cell types such as insect (SP9) or mammalian (NIH-3T3 and Hela) cell lines. In the presence of XCL, the uptake of GFP and BSA is greatly enhanced, demonstrating that XCL facilitates endocytosis. Thus, XCL could serve as a delivery agent to facilitate the endocytosis of proteins that do not enter the cell alone.

Abbreviations. ABL Agaricus bisporus lectin. – AOL Anthrobathyrs oligospora lectin. – AF-2 Adaptor protein complex 2. – BSA Bovine serum albumin. – GFP Green fluorescent protein. – PBS 10 mM phosphate buffer, pH 7.2, 150 mM NaCl. – XCL Xerocomus chrysenteron lectin.

Introduction

Lectins are carbohydrate-binding proteins that bind glycanics, glycolipids or polysaccharides with high affinity (Goldstein et al., 1980). Although over 100 plant lectins have been isolated and characterized, their physiological role within the plant remains speculative. However, their most likely function is believed to be defence against different kinds of plant-eating organisms (Peumans and Van Damme, 1995). Numerous plant lectins present toxic properties toward different insect species (Janzen et al., 1976; Osborn et al., 1988; Gatehouse et al., 1996; Sauvion et al., 1996) but the mechanisms by which these proteins exert their toxic activity is poorly known. Although a binding between lectins and glycoproteins of the epithelial cells of the midgut appears to be necessary, it is not sufficient to explain the disruption of cellular functions interfering with insect growth and survival (Harper et al., 1995; Powell et al., 1998).

Unlike their plant counterparts, lectins from higher fungi are less documented (Guillot and Konska, 1997). We have purified a lectin from a mushroom with insecticidal properties: Xerocomus chrysenteron. This lectin (XCL) belongs to a group of lectins firstly described for AOL (Anthrobathyrs oligospora Lectin) and ABL (Agaricus bisporus Lectin), which are specific for N-acetyl-galactosamine and galactose (Rosén et al., 1992). XCL induces high mortality rates in several insect species from different orders (Wang et al., 2002; Trigueros et al., 2003).

It is generally accepted that intoxication of cells requires first endocytosis of the toxin, intracellular transport and translocation to the cytosol from intracellular compartments such as the endosomes, the endoplasmic reticulum and the Golgi apparatus (Sandvig et al., 2002; Falnes and Sandvig, 2000; Vetterlein et al., 2002). Different mechanisms of uptake into cells have been identified: receptor-mediated endocytosis via clathrin-coated vesicles, uptake by caveolae, macropinocytosis, and clathrin- and caveolae-independent mechanisms (Pelkmans et al., 2001; Torgersen et al., 2001).

In the present study, we have investigated whether XCL is internalized by distinct cell types, and to what cell compartment the lectin is targeted. We examine the effect of chlorpromazine and intracellular potassium depletion, which inhibit clathrin-
mediated endocytosis, and the effect of filipin, an inhibitor of caveolae, to determine the mechanism of XCL-internalization.

We show that XCL is rapidly internalized into the cells via carbohydrate interactions, and that it facilitates the endocytosis of poorly internalized substrates such as GPP and BSA, making this new lectin a potential delivery agent.

Materials and methods

Materials

A cDNA encoding a lectin from Xenopus laevis was previously obtained by PCR from primers deduced from peptide sequences (Trigueros et al., 2003). This cDNA was expressed in E. coli with an N-terminal histidine tag. Purification of the protein (XCL) was achieved by affinity chromatography using nickel as ligand. Purified extracts were found to be devoid of any contaminant as assessed by overloaded SDS-PAGE.

Primary antibodies: Polyclonal antibodies directed against the XCL-native protein were obtained by inoculation of rabbit erythrocytes with the lectin, followed by intravenous injection into the animal. These antibodies recognize only the native form of XCL. Monoclonal mouse anti-human cathepsin D antibody was purchased from Dako S.A., France. Monoclonal mouse anti α-adaptin (a subunit of adaptor protein complex AP-2) antibody was purchased from Santa Cruz Biotechnology.

Secondary antibodies: FITC- and rhodamine-labeled goat anti-rabbit and anti-mouse antibodies were purchased from Molecular Probes Europe BV.

FITC labeled BSA, filipin and chlorpromazine were purchased from Sigma-Aldrich, France. GFP was purchased from Clontech; the lectin GS-II from Griffonia simplicifolia used for staining the intermediate-totrans Golgi was purchased from Molecular Probes Europe BV. Fluorescein-conjugated transferrin from human serum and dextran-tetramethylrhodamine conjugate (70000 MW) were purchased from Molecular Probes Europe BV.

Cell culture

SP9 cells were grown at 28°C in SF900 medium. HeLa and NIH-3T3 cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a 5% CO₂ incubator at 37°C.

Immunofluorescence labeling

Cells (SP9, Hela or NIH-3T3) were grown in the appropriate medium on glass coverslips and were incubated for different durations in the presence of XCL. Control cells were grown in the absence of lectin.

Labeling of XCL: Cells were washed three times with PBS (10 mM phosphate buffer, pH 7.2, 150 mM NaCl), fixed in PBS/3.6% formaldehyde for 30 min at room temperature and washed three times in PBS Cell permeation was performed in PBS/1% Triton X-100/1% BSA for 10 min at room temperature. After three washes in PBS, non-specific sites were saturated in PBS/3% BSA (w/v) for 1 h at room temperature and the cells were then incubated for 1 h at room temperature with the anti-XCL antibody (dilution 1:400 in PBS/1% BSA). The coverslips were washed twice in PBS/0.05% Tween 20 and once in PBS before being incubated with FITC-labeled anti-rabbit antibody (dilution 1:500) for 1 h at room temperature. The coverslips were washed three times in PBS before being incubated for 5 min at room temperature with 0.2 μg/ml DAPI in PBS, rinsed quickly 5 times, air-dried, and mounted on glass slides with Mowiol (Calbiochem).

Co-labeling of XCL and cathepsin D: Hela cells grown on glass coverslips were treated for 16 h with 17 μg/ml XCL. The cells were then washed in PBS and fixed in 1.85% formaldehyde for 30 min at room temperature. The cells were permeabilized in methanol at -20°C for 10 min before the immuno-labeling procedures. Simple and double labeling of XCL and cathepsin D were performed as described above.

The dilution of the anti-human cathepsin D antibody was 1:25.

Labeling of AP-2: Once treated or not by chlorpromazine or and XCL, the cells were fixed in 3.6% formaldehyde for 30 min at room temperature and permeabilized in PBS/1% Triton X-100/1% BSA for 6 min at room temperature. The immuno-labeling procedure was performed as described using the anti-AP2 antibody at the dilution 1:20.

Inhibition of XCL-internalization by lactose

The lectin (30 μg/ml) was pre-incubated 20 min at 25°C in serum-free medium containing either 2%, 4% or 8% lactose. Then, these solutions containing [XCL + lactose] were added onto Hela cells, and after 90 min of incubation at 37°C, the internalization of XCL was assessed by immunofluorescence labeling as described above.

Treatment with chlorpromazine and filipin

In order to assess potential pathways of XCL endocytosis, Hela cells grown on glass coverslips were pre-incubated 30 min in serum-free medium containing either filipin (5 μg/ml) or chlorpromazine (10 μg/ml) before the addition of XCL (30 μg/ml). The cells were then incubated for 90 min at 37°C in the presence of the drugs before the immunofluorescence labeling of XCL was performed as described above. Control experiments were done using transferrin and dextran as respective markers for clathrin-mediated endocytosis and fluid-phase endocytosis. Lysine-fixable dextran-tetramethylrhodamine conjugate (70 K) (1 mg/ml) was added to the cells for 3 h at 37°C in PBS (with Ca²⁺ and Mg²⁺).

Transferrin uptake

Hela cells were serum starved for 30 min, incubated with 50 μg of FITC-transferrin/ml for 20 min at 4°C for binding, washed, and transferred to 37°C for 15 min. Cells were washed with 0.1 M glycine-0.1 M NaCl, pH 3, to remove any uninternalized ligand, washed in PBS, fixed in 3.7% formaldehyde for 10 min, and mounted on glass slides with Mowiol.

Depletion of intracellular potassium

Potassium depletion was carried out as described previously (Larkin et al., 1983). Briefly, Hela cells were shocked in a hypotonic medium (DMEM/water, 1:1). After 5 min at 37°C, the hypotonic medium was removed, and an isotonic K⁺-free medium (α-MEM with 50 mM NaCl, HEPES, 100 mM NaCl, pH 7.4), was added. XCL or transferrin uptake assays were performed in the presence of isotonic K⁺-free medium.

GFP and BSA–FITC internalization

Hela cells were grown at 37°C on glass coverslips in the appropriate medium and were incubated in serum-free medium for one hour in the presence of GFP or FITC-BSA, or both GFP and XCL or FITC-BSA and XCL. The concentrations of proteins used were 25 μg/ml GFP, 250 μg/ml FITC-BSA, and 35 μg/ml XCL. After the fixation/permeabilization of the cells and DAPI staining, green fluorescence corresponding to FITC-BSA or GFP was directly observed. The immunofluorescence-labeling of XCL was also performed to verify the internalization of the lectin.

Results

Internalization of the fungal lectin (XCL) in three cell lines

Since XCL presented toxic properties toward several insect species (Trigueros et al., 2000), we wondered if XCL was internalized by insect cell lines. SF9 cells were incubated for 16 h with the lectin, and then treated for the immunofluorescence labeling. As shown in Figure 1D, large amounts of XCL were internalized by SF9 cells. SF9 are small cells with reduced volume of cytosol around the nucleus, therefore we turned to the common cell lines Hela and NIH-3T3 for easier microscopic observations. We also observed internalization (Fig. 1E, F). In the three cell lines, fluorescence signals were
associated with vesicles that were mostly concentrated in the perinuclear region. Fluorescent structures with different sizes were present, probably due to fusion of vesicles. The specificity of XCL labeling was controlled with non lectin-treated cells for which no staining was observed.

We hypothesized that the cellular uptake of the lectin was possible thanks to the interactions of its sugar-binding sites with the glycoconjugates of the cell surface. Our previous studies have shown that the hemagglutination activity of XCL, obtained with group B human erythrocytes, was inhibited by D-galactose, lactose and N-acetyl-D-galactosamine (Trigueros et al., 2003). To test our hypothesis, Hela cells were incubated with XCL in serum-free medium containing 2%, 4% or 8% lactose. We still observed endocytosis of the lectin in the presence of 2% and 4% lactose, but its internalization was totally inhibited in the presence of 8% lactose (Fig. 2A, B). In the control experiment, the receptor-mediated endocytosis of transferrin still occurred in the presence of 8% lactose (Fig. 2C). This result suggests that the uptake of XCL occurs via ligand-binding mechanisms mediated through lectin-carbohydrate interactions.

**Intracellular targeting of XCL**

We examined the lectin labeling of Hela cells incubated for different times with XCL. After 15 min, lectin labeling was mostly on the cell surface. Occurrence of XCL-labeled vesicles localized in the cytoplasm was visible after 30 min (Fig. 3A). After 2 hours, XCL-labeled vesicles began to coalesce around the nucleus, and an intense polarized staining close to the nucleus was observed after 4 hours (Fig. 3B). 4 days later, the lectin was still detected in vesicles accumulated around the nucleus.

Once internalized, macromolecules are forwarded to early endosomes, from where they are either recycled to the plasma membrane, sorted to the late recycling compartment, or delivered to late endosomes (the prelysosomal compartment) (Kirchhausen, 2000; Sorkin, 2000). Numerous studies have previously shown that once internalized, lectins are transported to endosomes and lysosomes as well as to the Golgi apparatus (Wu et al., 1999; Schwarz et al., 1982; Balin and Broadwell, 1987; Pavelka et al., 1998; Yu et al., 1999; Olsnes and Kozlov, 2001). To determine in which compartments XCL was localized, Hela cells incubated for 16 h with the lectin were double-labeled with anti-XCL antibodies and either Golgi or lysosomal probes. The lectin GSII from *Griffonia simplicifolia*, which specifically binds to α- and β-N-acetyl-D-glucosaminyl resi-
Endocytic mechanism responsible for the uptake of XCL

The uptake of XCL could presumably occur via any of the endocytic mechanism operating in the cell: clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin- and caveolae-independent endocytosis (Comer and Schmid, 2003; Lamaze et al., 2001; van Deurs et al., 1989). The invagination of caveolae has been investigated by incubating the Hela cells with XCL in the presence of filipin. Filipin is known to binding cholesterol in the plasma membrane and impair the invagination of caveolae, which are highly enriched in cholesterol (Simons and Gruenberg, 2000; Orlandi and Fishman, 1998; Ippoliti et al., 2000). The treatment with filipin did not induce any significant decrease in XCL uptake or redistribution of the XCL-labeled vesicles (Fig. 4A, B), nor did it affect the uptake of FITC-transferrin, a well-established marker of the coated pit pathway (Fig. 4E, F). Clathrin-mediated endocytosis can be inhibited by amphiphilic drugs such as chlorpromazine, which prevent assembly of coated pits at the plasma membrane by disrupting the recycling of AP-2 from endosomes (Wang et al., 1993; Subtil et al., 1994; Shogomori and Futerman, 2001). We analyzed the effect of chlorpromazine on the endocytosis of XCL and of transferrin in Hela cells. As expected, chlorpromazine inhibited transferrin internalization (Fig. 4G). In the same way, the uptake of XCL was inhibited in chlorpromazine-treated cells (Fig. 4C). In contrast, the uptake of tetramethylrhodamine-dextran, a marker for bulk flow-fluid phase endocytosis, was comparable in chlorpromazine-treated and non-treated cells (Fig. 4I, K). As an alternative to chlorpromazine, potassium depletion combined with hypotonic shock has been used as a possibly more specific and less toxic mean of inhibiting clathrin-mediated endocytosis. Such treatment has been well established to reversibly arrest clathrin-coated pit formation (Larkin et al., 1983). Hela cells were subjected to hypotonic shock and then incubated in K⁺-free medium with XCL or FITC-transferrin. As shown in Figure 4D and 4H, this treatment inhibited both transferrin and XCL internalization. Taken together, these experiments indicate a major involvement of clathrin-coated pits in the endocytosis of the lectin.

The adaptor protein complex AP-2 mediates the clathrin-coated vesicle formation at the plasma membrane. Clathrin and AP-2 are rapidly recycled by dissociating from the endosome membrane. We used anti-AP-2 antibodies to determine by immunofluorescence if XCL induced modifications of clathrin-coated pit staining in Hela cells. After 2 h of incubation with the lectin, no significant changes of the AP-2 staining pattern were observed: AP-2 was localized in small vesicles on the cell surface and in the cytoplasm (Fig. 5A, C). We also verified the effect of chlorpromazine on the distribution of AP-2 in the presence of XCL. The drug caused the relocation of AP-2 to numerous vesicles deep within the cytoplasm of the cell (Fig. 5B, D). When the cells were treated with chlorpromazine and XCL, the prominent endosomal staining of AP-2 was still observed. Thus, the lectin did not induce any significant modification of the cellular distribution of AP-2.

Facilitation of endocytosis

We hypothesized that the binding of XCL to glycoconjugates of the cell surface could enhance the endocytic processes and thus, stimulate the uptake of poorly internalized substrates such as GFP or BSA.
Fig. 4. Effect of filipin (B, F, J), chlorpromazine (C, G, K) and K⁺-depletion (D, H) on the internalization of XCL (A–D), transferrin (E–H) and dextran (I–K). Hela cells were treated for 30 min at 37°C with 5 μg/ml filipin or 10 μg/ml chlorpromazine or were K⁺ depleted before adding XCL, FITC-transferrin or dextran. Control cells are shown in (A, E, I). The uptake assays were performed as described in Materials and methods. The internalized substrates were observed by immunofluorescence microscopy. Bar 10 μm.

To test this hypothesis, Hela cells were incubated for 60 min either with XCL and GFP or GFP alone. No significant fluorescent signal was detected with GFP alone (Fig. 6A); on the contrary, numerous green fluorescent vesicles were present in the cytoplasm of the cells incubated with both GFP and XCL (Fig. 6B). Furthermore, an overlapping of the XCL-labeling and GFP staining was observed (Fig. 7). The same experiments were performed using BSA-FITC and XCL. BSA is internalized by the cells and is known to be further targeted to endosomes and lysosomes. When the cells were incubated with BSA-FITC, some labeled vesicles were observed (Fig. 6C). Co-incubation with XCL greatly enhanced the amount of FITC-labeled vesicles and their intensity of fluorescence (Fig. 6D). We also observed the co-localization of XCL and BSA, which confirmed the accumulation of the lectin in endosomes and lysosomes. In the presence of chlorpromazine, the stimulatory effect exerted by XCL on BSA uptake was abolished, but the basal level of BSA internalization was still observed (Fig. 6F).

Discussion

The lectin from Xerocomus chrysenteron (XCL) was isolated following a screening for new insecticidal molecules (Mier et al., 1996). The aim of the present work was to characterize some of the biological properties of this new lectin at the cellular level. In the first part of this study, we used immunofluorescence microscopy to examine the internalization of XCL by different cell lines, its intracellular fate, and the endocytic mechanism involved in the uptake of the lectin. XCL was internalized rapidly (after 30 min) and in large amounts in the
Fig. 5. Effect of chlorpromazine and XCL on AP-2 localization. Hela cells were either not treated (A) or incubated for 2 h in the presence of chlorpromazine (10 μg/ml) (B) or in the presence of XCL (35 μg/ml) (C), or both chlorpromazine and XCL (D). The cells were fixed and processed for indirect immunofluorescence as described. Bar 10 μm.

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Fig. 6. Effect of XCL on GFP and BSA internalization. Hela cells were incubated in serum-free medium for one hour in the presence of GFP (A) or FITC-BSA (C), or both GFP and XCL (B) or FITC-BSA and XCL (D) as described in Materials and methods. The cells were fixed, and green fluorescence corresponding to GFP or BSA-FITC was directly observed. E, F. Effect of chlorpromazine on BSA internalization. Hela cells were treated with chlorpromazine, and BSA uptake was performed in the absence (E) or presence (F) of XCL. Bar 10 μm.
insect cell line SF9, in the mouse fibroblast cell line NIH3T3, and in the tumour cell line Hela. The fact that XCL is endocytosed in such different cell lines suggests that the lectin can enter many other cell types, thanks to its sugar-binding property. Indeed, the inhibition of XCL-uptake by lactose indicates that the binding of XCL on cell-surface glycoproteins is necessary for its internalization. In the three cell lines, the vesicles containing XCL were mostly accumulated around the nucleus. Perinuclear aggregations of internalized lectins have already been described in other studies (Aubin et al., 1980; Schwarz et al., 1982; Yu et al., 1999).

Co-labeling experiments with anti-cathepsin D antibodies allowed us to determine that once internalized, XCL was targeted to the endosome/lysosome system. Identical observations were made with other lectins such as concanavalin A and ABL, which have been shown to accumulate in perinuclear endosomes (Schwarz et al., 1982; Yu et al., 1999).

We have investigated the implication of clathrin-coated pits and caveolae in endocytosis of XCL because these pathways are responsible for the uptake of toxins in many cell types (Schnitzer et al., 1996; Mallard et al., 1998; Sandvig and van Deurs, 2000; Torgersen et al., 2001). We found that the internalization of XCL was inhibited when the clathrin-dependent pathway was blocked by chlorpromazine or by hypotonic shock/K⁺ depletion. On the contrary, the caveolar inhibitor, filipin, did not affect endocytosis of XCL. Thus, the clathrin-mediated endocytosis seems to be the major pathway involved in the uptake of the lectin in Hela cells. Wheat germ agglutinin was shown to react with numerous binding sites on the membrane of hepatoma cells and was also internalized in large amounts by receptor-mediated endocytosis via clathrin-coated pits (Vetterlein et al., 2002). The cellular entry of ABL involved clathrin-coated pits, but also clathrin-independent pathways and macropinocytosis (Yu et al., 1999). It seems probable that XCL enters the cell by binding to multiple glycosylated receptors of the cell surface that are internalized in a constitutive way, like the transferrin or LDL (low density lipoprotein) receptors (Benmerah and Lamaze, 2002). We can also hypothesize that XCL is endocytosed via ligand-induced receptor internalization. The lectin, having at least two sugar-binding sites, could mimic a ligand by inducing the dimerisation of receptors, which could further lead to their internalization into clathrin-coated endocytic vesicles. Indeed, various studies have shown that lectins, initially bound in a unique form at the cell surface, are redistributed into clusters and undergo endocytosis, inducing redistribution and internalization of surface glycoproteins (Schwarz et al., 1982). It is also possible that XCL, comparably to Shiga and Cholera toxins (Sandvig et al., 1989; Shogomori and Futerman, 2001), binds to raft-associated glycolipids, and is further internalized by clathrin-coated vesicles.

In the second part of this study, we wanted to see if XCL could stimulate the uptake of poorly internalized molecules. We showed that the endocytosis of GFP and BSA was enhanced when these proteins were co-incubated with the lectin. The stimulatory effect exerted by XCL on endocytosis was abolished in the presence of chlorpromazine, an inhibitor of the clathrin-mediated pathway. This result leads us to hypothesize that XCL facilitates endocytosis via clathrin-coated pits. By its interaction with numerous glycoproteins of the cell surface, the lectin might stimulate the formation of clathrin-coated pits at the plasma membrane and thus enhance the uptake of molecules present in the medium. Alternatively, XCL (internalized via clathrin-mediated endocytosis) might be required to stimulate clathrin-independent uptake mechanisms. The finding that molecules are rapidly co-internalized with XCL could be of value for several applications in biotechnology. The specific binding of lectins has already been explored as a means of targeting and enhancing the delivery of therapeutic agents (Mody et al., 1995) or for gene transfer (Yanagita and Cheng, 1999; Yin and Cheng, 1994; Batra et al., 1994).

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References


