Biochimica et Biophysica Acta 1860 (2016) 392–401

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Uptake of *Marasmius oreades* agglutinin disrupts integrin-dependent cell adhesion

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ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 31 October 2015
Accepted 3 November 2015
Available online 10 November 2015

Keywords:
Lectin
Cysteine protease
Cell adhesion
Focal adhesion kinase
Integrin

ABSTRACT

Background: Fruiting body lectins have been proposed to act as effector proteins in the defense of fungi against parasites and predators. The *Marasmius oreades* agglutinin (MOA) is a lectin from the fairy ring mushroom with specificity for Galα1-3Gal containing carbohydrates. This lectin is composed of an N-terminal carbohydrate-binding domain and a C-terminal dimerization domain. The dimerization domain of MOA shows in addition calcium-dependent cysteine protease activity, similar to the calpain family.
Methods: Cell detachment assay, cell viability assay, immunofluorescence, live cell imaging and Western blot using MDCKII cell line.
Results: In this study, we demonstrate in MDCKII cells that after internalization, MOA protease activity induces profound physiological cellular responses, like cytoskeleton rearrangement, cell detachment and cell death. These changes are preceded by a decrease in FAK phosphorylation and an internalization and degradation of β1-integrin, consistent with a disruption of integrin-dependent cell adhesion signaling. Once internalized, MOA accumulates in late endosomal compartments.
Conclusion: Our results suggest a possible toxic mechanism of MOA, which consists of disturbing the cell adhesion and the cell viability.
General significance: After being ingested by a predator, MOA might exert a protective role by diminishing host cell integrity.

1. Introduction

Mushrooms are known to contain a wide variety of lectins with different structures and specificities [1]. Cytoplasmic fungal lectins, also referred to as fruiting body lectins, are part of the fungal defense system against parasites and predators [2]. When feeding on the content of the fungal cell, predators such as fungivorous nematodes ingest the cytoplasmic lectin, which then induces toxicity by an unknown mechanism after binding to specific glycans in the intestine of the worm [2–5].

The *Marasmius oreades* agglutinin (MOA) is a lectin from the fairy ring mushroom that was first reported to agglutinate blood group B erythrocytes [6]. The lectin has a dimeric structure composed of 293-residue protomers and specifically recognizes Galα1,3-containing structures, exhibiting the highest affinity for the branched blood group B trisaccharide Galα1,3(Fuc-α1,2)Gal [7,8]. Crystal structures of MOA in complex with two carbohydrate ligands revealed an N-terminal ricin B-chain like domain with three carbohydrate-binding sites and a C-terminal domain that is involved in dimerization [9,10]. The latter domain shows structural homology to enzymatically active proteins, and has been reported to be a calcium-dependent cysteine protease domain similar to the calpain and papain families [11,12].

MOA has been shown to exhibit a cytotoxic activity towards nematodes, which is dependent on the binding to plasma membrane glycosphingolipids and a cysteine protease activity [11]. Recently,

Abbreviations: MOA, *Marasmius oreades* agglutinin; MDCKII, Madin–Darby canine kidney strain II; FAK, Focal adhesion kinase; BAX, BCL-2-associated X protein; HUS, Hemolytic uremic syndrome; CME, Clathrin-mediated endocytosis; GSLs, Glycosphingolipids; DMEM, Dulbecco's Modified Eagle Medium; FCS, Fetal calf serum; PMP, D-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; StxB, Shiga toxin B-subunit; wt, Wild-type; FACS, Fluorescence-activated cell sorting; PBS, Phosphate buffered saline; PNPG, 4-Nitrophenyl α-D-galactopyranoside; PFA, Paraformaldehyde; BSA, Bovine serum albumin; PEI, Polyethyleneimine; HBSS, Hawks buffer saline solution; RIPA, Radio-immunoprecipitation assay; ECM, Extracellular matrix; Tf, Transferrin; ER, Endoplasmic reticulum.

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http://dx.doi.org/10.1016/j.bbagen.2015.11.002

Cordara *et al.* have demonstrated that MOA induces cytotoxicity in NIH/3 T3 cells, which correlates, at least in part, with its proteolytic activity [13]. Moreover, human BAX (BCL-2-associated X protein) has been identified as a potential target of MOA. In a separate study, MOA parenterally administered to mice caused symptoms comparable to the human Hemolytic Uremic Syndrome (HUS), going along with *in vitro* endothelial cell detachment [14]. However, no clear link has been established between cell detachment and the cysteine protease activity of MOA.

Cell adhesion is essential for tissue integrity. Processes that modify adhesion are tightly regulated by a wide range of proteins [15,16]. Abnormal disruption of adhesion attenuates nutrient and growth factor access and may induce cell death [15,17].

In this study, we investigated the possible role of the cysteine protease activity of MOA in the fungal defense system by studying its effect on cell adhesion in MDCKII cells. First, we demonstrate a cell detachment effect induced by MOA that is dependent on carbohydrate binding and cysteine protease activity, ultimately leading to cell death. Second, we show that cell detachment in response to MOA is preceded by disruption of the cytoskeleton, reduction of focal adhesion kinase (FAK) phosphorylation, internalization and degradation of β1-integrin. Finally, we show that MOA is mainly internalized by clathrin-mediated endocytosis (CME) and that it accumulates in late endosomal compartments. Our results suggest that MOA impairs cell survival by disturbing integrin-dependent cell adhesion signaling of the host in order to protect fungi against parasites and predators.

2. Materials and methods

2.1. Cell culture, generation of stable cell lines and depletion of glycosphingolipids (GSLs)

Madin–Darby canine kidney strain II cells (MDCKII, [18]) were grown at 5% CO_2 in DMEM (Dulbecco's Modified Eagle Medium, Life Technologies) containing 4.5 g/l glucose supplemented with 10% fetal calf serum (FCS) and 4 mM L-Glutamine. For the generation of a Gb3-expressing stable cell line, MDCKII cells were transfected with a plasmid encoding Gb3-synthase and a Geneticin resistance. After selection with 1000 μg/ml Geneticin, positive clones were maintained in DMEM containing 4.5 g/l glucose supplemented with 500 μg/ml Geneticin, 10% FCS and 4 mM L-Glutamine. For GSL depletion, cells were passaged for three days in the presence of 5 μM of the glucosylceramide synthase inhibitor D-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP, Santa Cruz Biotech) [19,20]. To control the depletion of GSLs, we used Cy5-labeled B-subunit of Shiga toxin (StxB-Cy5), which is known to interact specifically with the glycosphingolipid Gb3. StxB was purified as previously described [21].

2.2. Protein expression, purification and labeling

Wild-type (wt) MOA and the variant MOA(C215A) were expressed recombinantly in *Escherichia coli* and purified as previously described [7,8]. For fluorescence microscopy analysis and fluorescence activated cell sorting (FACS) measurements, both molecules were conjugated to activated Cy2, Cy3 or Cy5 dyes with kits (Amersham Life Sciences) according to the manual.

2.3. Cellular assays

For the cell detachment assay, cells were seeded (40,000 cells/well in a 24-well plate) and allowed to adhere overnight. The cells were washed with phosphate buffered saline (PBS) and fresh medium was added together with MOA. For the cysteine protease inhibition with E-64 (Sigma Aldrich) and for the blocking of MOA binding sites with soluble carbohydrates (4-nitrophenyl α-D-galactopyranoside, PNPG, Sigma Aldrich), MOA and E-64, or MOA and PNPG, were pre-incubated together for 10 min at room temperature before being added to the cells. As positive control, cells were washed with PBS, and 0.05% of trypsin-EDTA (Life Technologies) was added for 15 min at 37 °C to the cells. The number of detached cells per well was quantified by flow cytometry (Gallios, Beckman Coulter). For the cell viability assay, live cells were quantified by trypan blue exclusion (Sigma Aldrich).

2.4. Caspase assay

The Caspase-Glo® 3/7 assay (Promega) was used to quantify MOA protease activity. The reagent was added to MOA-containing DMEM and luminescence was measured using a plate-reader (BioTek).

2.5. Cell binding and internalization assay

After 1 h of stimulation with Cy2-labeled MOA, cells were washed with PBS, detached from culture dishes and distributed equally to two tubes. For measuring extra- and intracellular MOA fluorescence, cells were measured immediately by flow cytometry using a FACS-Gallios (Beckman Coulter). For measuring only intracellular MOA fluorescence, cells were suspended in 0.4% Trypan Blue for 1 min to quench any residual cell surface fluorescence [22,23], then intracellular MOA-Cy2 fluorescence was measured immediately by flow cytometry.

2.6. Immunofluorescence

Immunofluorescence studies were carried out as previously described [24]. Briefly, cells grown overnight on 24-well cover glasses (Menzel, 12 mm) were treated with Cy2/Cy3-labeled MOA in FCS-containing medium and fixed in 4% paraformaldehyde (PFA) at 4 °C for 10 min. Cells were washed and incubated with NH_4Cl at room temperature for 15 min before being permeabilized with PBS containing 0.02% saponin and 0.2% bovine serum albumin (BSA). Cells were stained with either anti-EEA1 (1:50, BD Transduction Laboratories), anti-Calnexin (1:100, Enzo Life Science), anti-CTR 433 (1:100, kind gift of Michel Bornens, Curie Institut), anti-Giantin (1:100, Abcam), anti-TGN46 (1:100, Sigma Aldrich), anti-Transferrin receptor (TfR, 1:100, Life Technologies), anti-Rab11 (1:100, Cell Signaling), anti-Lamp1 (1:200, BD PharMingen), anti-Rab7 (1:100, Santa Cruz Biotech) or anti-β1 integrin (1:100, R&D Systems) antibodies diluted in permeabilization buffer, followed by either donkey anti-mouse Cy3-labeled secondary antibody (1:100, Jackson ImmunoResearch), donkey anti-rabbit Cy3-labeled secondary antibody (1:100, Jackson ImmunoResearch) or donkey anti-goat Cy3-labeled secondary antibody (1:100, Jackson ImmunoResearch) diluted in permeabilization buffer. Nuclei were stained using DAPI (300 nM, Life Technologies).

2.7. Transfection and confocal microscopy

Cells were imaged on a confocal microscope (Nikon Eclipse Ti-E with A1R confocal laser scanner, 60× oil objective, NA = 1.49). For live-cell-imaging, cells were transfected using polyethyleneimine (PEI, linear, MW: 25 kDa, Polyscience) as described elsewhere [25]. The medium was exchanged 5 h post-transfection and cells were imaged 30 h post-transfection. After being washed once with PBS with Ca^{2+}/Mg^{2+} at 37 °C, cells on glass coverslips were mounted on a coverslip holder with warm (37 °C) HBSS solution (Hawks Buffer saline solution, PAN Technology) supplemented with 10 mM HEPES, 4.5 g/l glucose, 1% FCS and 4 mM L-glutamine. Before starting measurements, the recording medium was exchanged with fresh recording medium containing MOA-Cy5 (10 μg/ml). Measurements were carried out at 37 °C for 25 min. Image acquisition and co-localization analysis were performed with NIS-Elements (Nikon).

2.8. Clathrin and dynamin inhibition

Clathrin and dynamin were inhibited using Pitstop 2 ™ (Ascent Scientific) and Dynasore (Merck), respectively. Cells were pretreated with either 30 μM of Pitstop 2 ™ or with 150 μM of Dynasore for 30 min at 37 °C, followed by 10 min of incubation with MOA-Cy3 and Transferrin-Alexa 488 (Life Technologies). Cells were then fixed in 4% PFA at 4 °C for 10 min and imaged on a confocal microscope.

2.9. Western blot analysis

Western blot analyses were performed by standard procedures as described previously [26]. Briefly, for analysis of β1-integrin, FAK, phospho-FAK (pTyr397) and actin, cells were harvested and lysed with modified radio-immunoprecipitation assay (RIPA) buffer [20 mM Tris Base (pH 8), 0.5% Na-deoxycholate, 13.7 mM NaCl, 10% Glycerol, 0.1% SDS, 2 mM EDTA, 200 μM pefablock, 5 μg/ml leupeptin, 5 μg/ml approtinin, 1 mM Na-orthovanadate] for 30 min at 4 °C. The protein concentration of each extract was determined by using the Pierce® BCA Protein Assay Kit (Thermo Scientific). Equal amounts of proteins were separated by 8% Tris–glycine SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 3% BSA or 5% milk prepared with TBST [50 mM Tris (pH 7.6), 154 mM NaCl, 0.5% Tween 20]. Membranes were incubated with primary antibodies overnight at 4 °C. After washing, the membranes were incubated with secondary antibodies (horseradish peroxidase-labeled rabbit or mouse

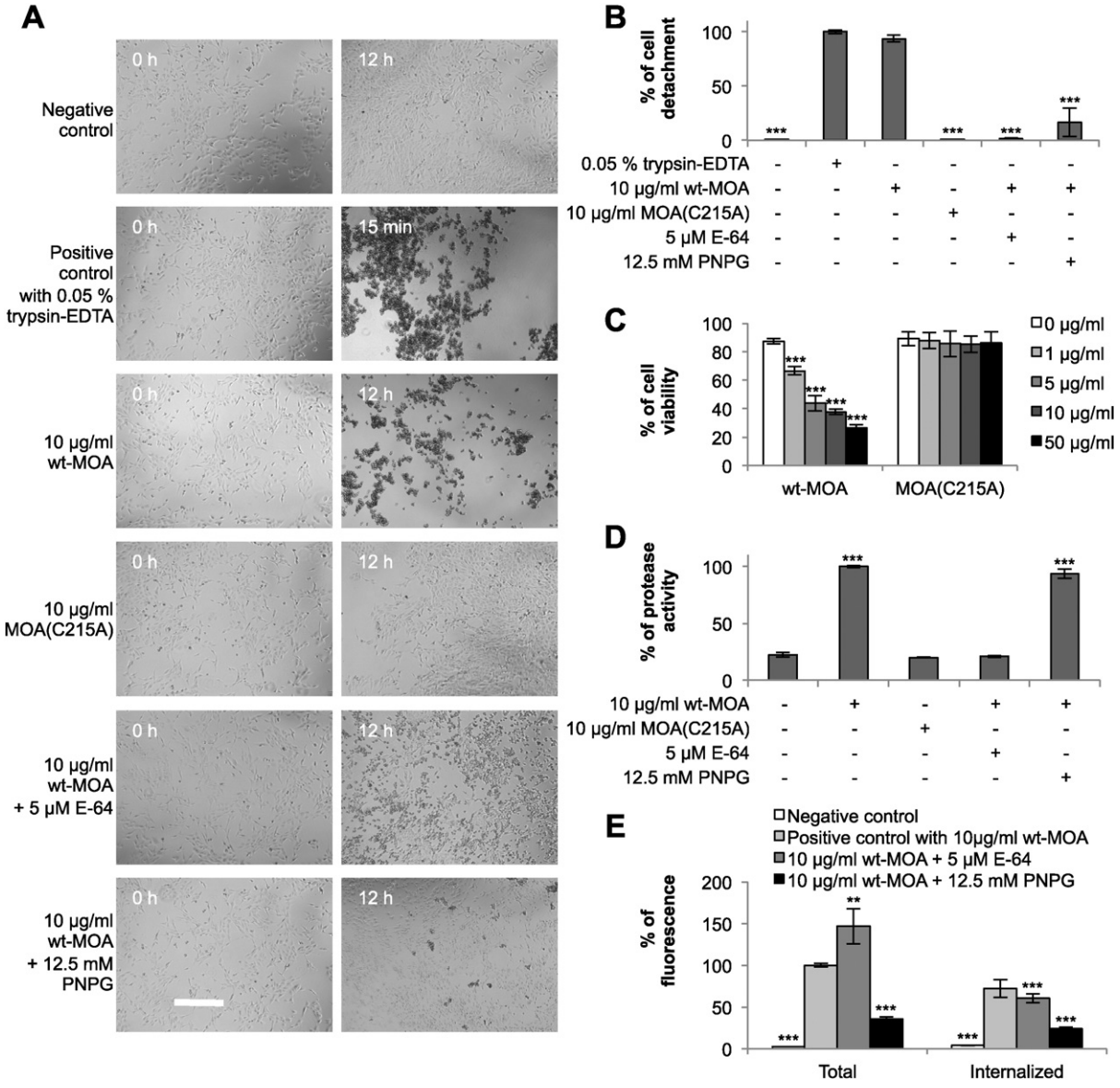

Fig. 1. MOA binding to MDCKII cells induces cell detachment via its protease activity. A) Adherent MDCKII cells were treated with MOA (10 μg/ml) for 12 h and observed by light microscopy. Wt-MOA caused rounding and detachment of cells whereas cells did not change morphology or detach in response to the variant MOA(C215A). Pre-incubation of wt-MOA with E-64, or with PNPG, prevented most changes in morphology and adherence. The scale bar represents 500 μm. B) Cell detachment caused by MOA treatment (12 h) was quantified by analyzing the supernatant with flow cytometry (means ± SEMs, $n = 3$). Significant difference compared to the positive control cells that had been detached with 0.05% trypsin-EDTA, $^{***}p < 0.001$. C) Wt-MOA treatment (1–50 μg/ml, 24 h) leads to cell death, whereas the variant MOA(C215A) (1–50 μg/ml, 24 h) did not affect cell viability as shown by the quantification using trypan blue exclusion (means ± SEMs, $n = 3$). Significant difference compared to the untreated control cells, $^{***}p < 0.001$. D) Quantification of MOA protease activity in FCS-containing medium using the Caspase-Glo® 3/7 assay. MOA enzymatic activity is inhibited for the variant MOA(C215A), or by pre-incubation with E-64 but not by pre-incubation with PNPG (means ± SEMs, $n = 3$). Significant difference compared to the negative control condition without MOA, $^{***}p < 0.001$. E) Binding and internalization capacity of MOA-Cy2 (10 μg/ml, 1 h) was decreased by pre-incubating MOA with PNPG. Trypan blue was used to quench external fluorescence and cells were analyzed by FACS (means ± SEMs, $n = 3$). Significant difference compared to the positive control, where cells were treated with 10 μg/ml of wt-MOA, $^{**}p < 0.01$, $^{***}p < 0.001$.

antibodies) for 1 h at room temperature. Actin was used as a loading control. Data shown are from at least three independent experiments. The densitometric analysis was performed using ImageJ software.

2.10. Statistical analysis

Statistical testing was performed with Microsoft Excel with data of $n \geq 3$ independent experiments. Differences to the control were analyzed for significance by using one-way ANOVA, with a Dunnett post-test compared to the control condition.

3. Results

3.1. MOA protease activity affects cell adhesion and leads to cell death

Warner *et al.* reported that MOA induces endothelial cell detachment after 18 h of MOA treatment [14]. Preliminary data with similar experimental results were acquired using NIH/3 T3 mouse fibroblast and Madin–Darby canine kidney (MDCK) cells. Since MDCK cells adhered much better to the support than NIH/3 T3 cells, we have chosen MDCK cells for further investigations. To determine the role of MOA

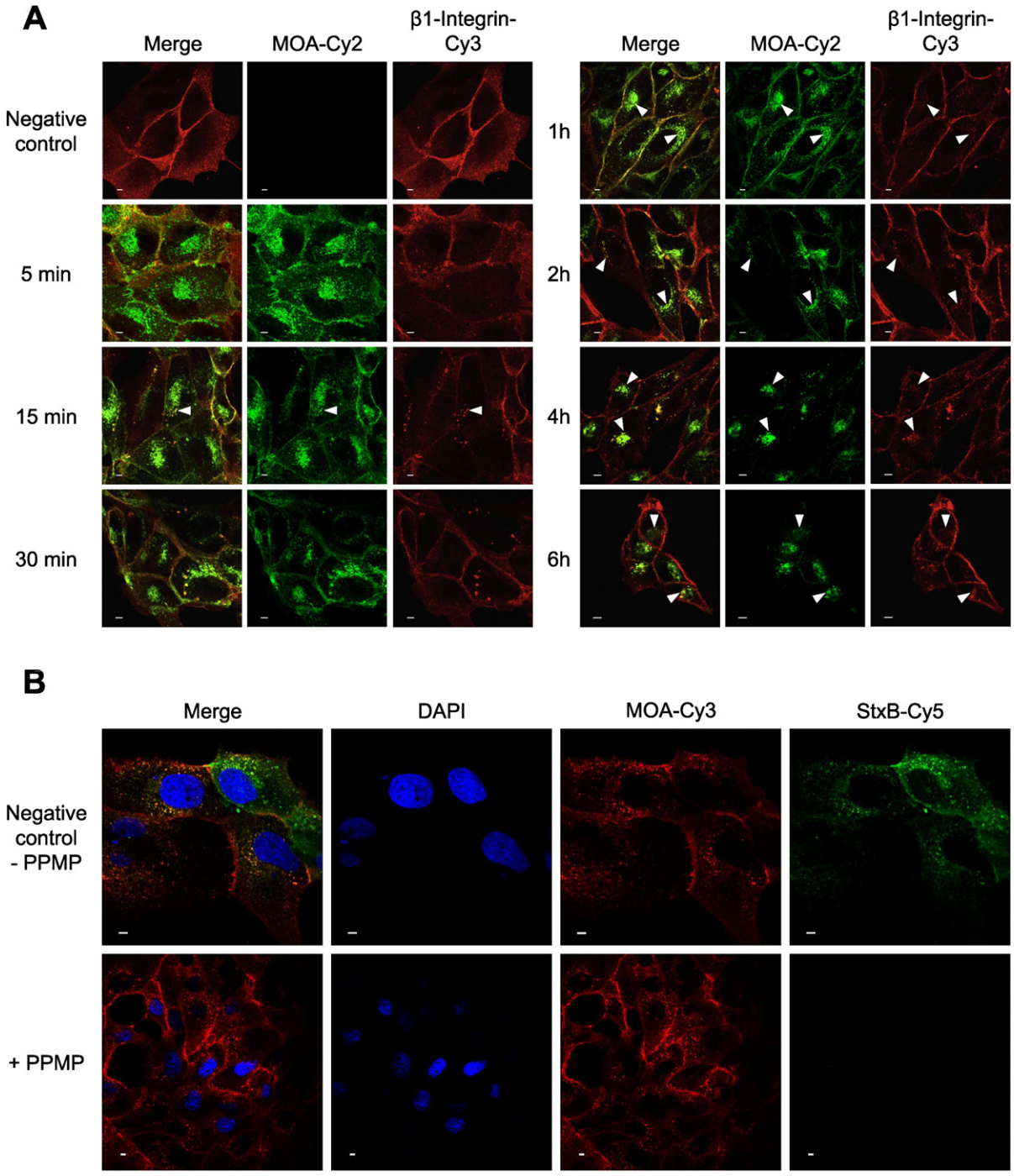

Fig. 2. MOA induces β1-integrin internalization. A) MDCKII cells were treated with MOA-Cy2 (10 μg/ml) and stained for β1-integrin. Treatment of MOA induces internalization of β1-integrin after 15 min, which then co-localizes with MOA in the perinuclear area (arrowhead). B) Gb3-synthase-expressing MDCKII cells were treated with MOA-Cy3 (10 μg/ml) and StxB-Cy5 (5 μg/ml) for 10 min, with or without PPMP treatment. After deletion of GSLs with PPMP treatment, no major changes were observed for MOA-Cy3, whereas StxB-Cy5 fluorescence was abrogated. For A) and B) the scale bars represent 5 μm. The shown pictures are representative for at least 3 independent experiments.

protease activity in cell detachment, MDCKII cells were incubated for 12 h with wild-type MOA or with the variant MOA(C215A), in which the cysteine residue of the putative catalytic triad was replaced by alanine [11]. Wild-type MOA induced cell detachment at a concentration of 10 μg/ml (Fig. 1A,B), whereas the same concentration of MOA(C215A) did not induce detachment of MDCKII cells (Fig. 1A,B). MOA-induced cell detachment was as effective as 0.05% of trypsin-EDTA, a solution commonly used to detach cells (Fig. 1A,B). Moreover, cell detachment did not occur when wild-type MOA was pre-incubated with 5 μM of E-64, an irreversible, highly selective cysteine protease inhibitor (Fig. 1A,B). In addition, cell detachment was largely reduced by adding 12.5 mM of PNPG as soluble, competing carbohydrate (Fig. 1A,B), suggesting that the binding of MOA to plasma membrane receptors is a prerequisite for cell detachment.

As the detachment of cells might lead to cell death [15,17], we analyzed the cell viability of adherent and detached MDCKII cells after

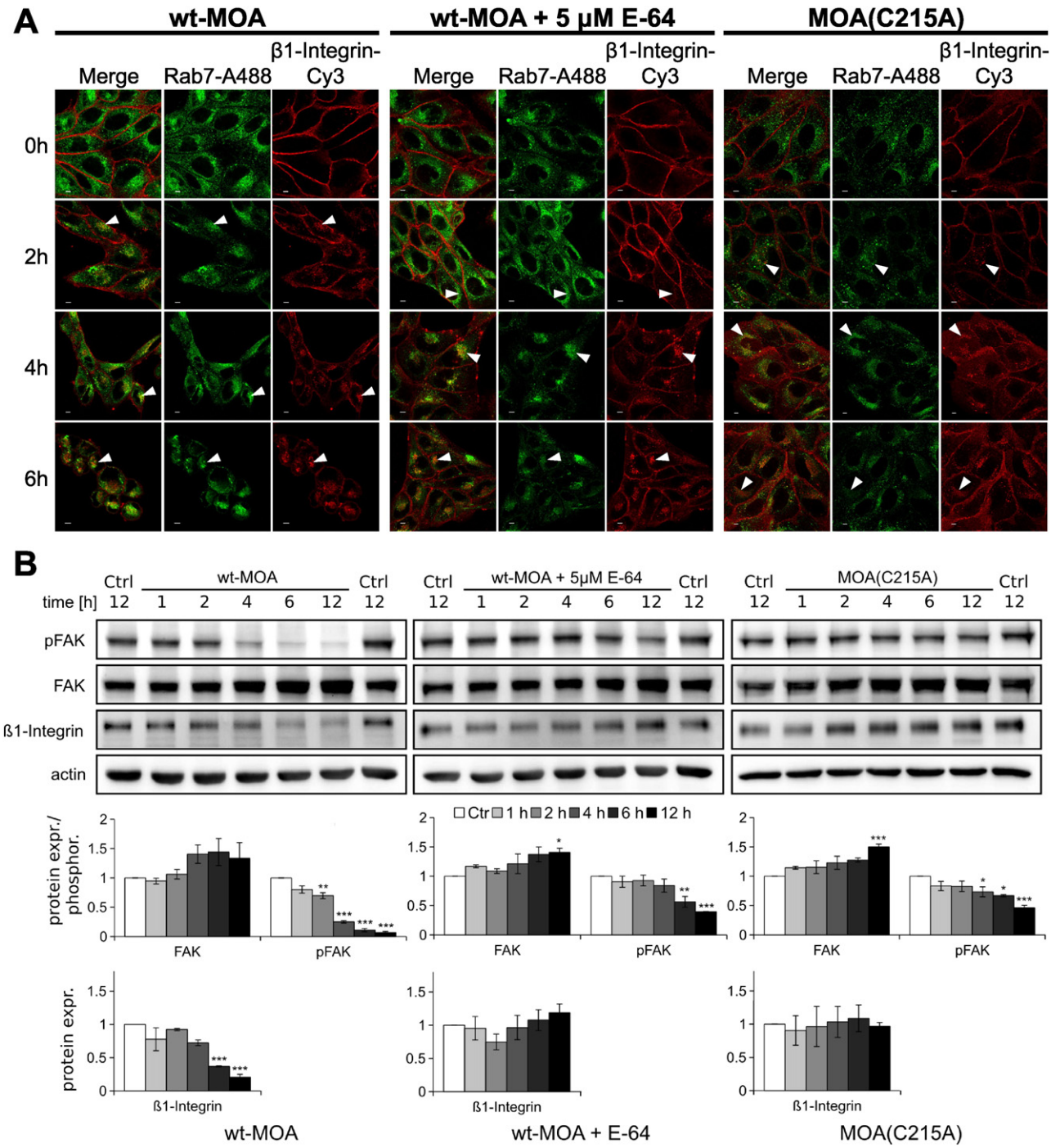

Fig. 3. MOA induces disruption of integrin-dependent cell adhesion signaling. A) MDCKII cells were either treated with wt-MOA (10 μg/ml), with wt-MOA pre-incubated with E-64 (10 μg/ml wt-MOA and 5 μM E-64) or with the variant MOA(C215A) (10 μg/ml) and stained for β1-integrin (red) and Rab7 (green). Inhibition of the protease activity prevented changes of cell morphology but did not prevent internalization of β1-integrin (arrowhead). Scale bars represent 5 μm. B) MOA protease activity induces β1-integrin degradation and reduces FAK phosphorylation, without degrading FAK, as shown by Western blots using β1-integrin, FAK and phospho-FAK (pTyr397) antibodies. Actin was used as a loading control (means ± SEMs, $n = 3$). Significant difference compared to the untreated control cells, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

24 h of MOA treatment using trypan blue exclusion. Wild-type MOA, which detaches the cells, induced cell death in a dose-dependent manner (from 1 μg/ml to 50 μg/ml), whereas the variant did not detach MDCKII cells neither did it affect cell viability (Fig. 1C).

The protease activity of MOA was tested with a Caspase-Glo® 3/7 kit. This assay is based on a luminogenic caspase-3/7 substrate containing the tetrapeptide sequence DEVD, which is highly similar to the cleaving site specific of MOA [11]. MOA showed protease activity in FCS-containing medium, whereas the activity was abrogated for the variant MOA(C215A) or by pre-incubating wild-type MOA with 5 μM of E-64 (Fig. 1D). The addition of 12.5 mM of PNPG, to which MOA binds, did not affect the protease activity of MOA (Fig. 1D).

To confirm that binding of MOA is required to affect cell adhesion by its enzymatic function, we analyzed the binding and the internalization capacity of MOA by FACS. Cells incubated with Cy2-labeled MOA with or without pre-incubation with E-64 (5 μM) showed similar fluorescence (Fig. 1E), revealing that the protease activity of MOA is not affecting the binding to the host cell plasma membrane. In contrast, addition of 25 mM of PNPG significantly reduced the cell-associated Cy2-MOA signal (Fig. 1E). These findings are supported by previous studies, where the toxicity of MOA was shown to be dependent on the carbohydrate-binding activity of the protein [11,14]. These data support the hypothesis that MOA binds selectively to specific carbohydrate-bearing receptors at MDCKII cells and induces cell detachment by its cysteine protease activity, ultimately leading to cell death.

3.2. MOA induces β1-integrin internalization

Given the importance of integrins for cell adhesion (reviewed in [27,28,29]), we investigated the possible effect of MOA on β1-integrin. Internalization and/or recycling of β1-integrin is critical for cell migration and adhesion and malfunctions in integrin trafficking are often associated to the loss of cell adhesion. For this reason, we analyzed integrin localization in MDCKII cells by immunofluorescence. Cells were incubated with Cy2-labeled MOA (10 μg/ml; from 5 min to 6 h), fixed, permeabilized, stained for β1-integrin and imaged by confocal microscopy. In untreated cells, β1-integrin was distributed over the cell membrane uniformly. Already after 15 min of MOA treatment, large amounts of the lectin were internalized. Internalization of β1-integrin was also observed after 1 h of incubation and co-localized with MOA in a perinuclear area (Fig. 2A), which was more pronounced after several hours of incubation (2 h–4 h–6 h).

To determine the type of the cellular receptors of MOA, we analyzed by fluorescence the binding of the lectin to a Gb3-expressing MDCKII cell line. Cells were treated with or without PPMP, an inhibitor of glucosylceramide synthase catalyzing the first step in the biosynthesis of glucosylceramide-based glycolipids (glucocerebrosides) [19,20]. Shiga toxin B subunit (StxB), which is known to bind specifically to the glycosphingolipid Gb3, bound to cells only when they were not treated with PPMP (Fig. 2B). MOA binding was apparently not affected by PPMP treatment, suggesting that the lectin does not bind to glucocerebrosides, but may bind either to galactosylceramide-based glycolipids (galactocerebrosides), or to glycoproteins. These results show that MOA does not interact with glucosylceramide-based glycolipids, but does induce β1-integrin internalization in MDCKII cells.

3.3. MOA induces the disruption of integrin-dependent cell adhesion signaling

The effect of MOA on β1-integrin localization was further examined by confocal fluorescence microscopy (Fig. 3A). As previously shown, untreated, adherent MDCKII cells showed mainly plasma membrane β1-integrin staining. After several hours of MOA exposure (10 μg/ml, 6 h), remaining adherent cells were less spread and showed prominent internalized β1-integrin staining located close to Rab7-positive late endosomal compartments. Incubation of MDCKII cells with wild-type MOA that had been pre-incubated with the protease inhibitor E-64 (5 μM), as well as incubation with the variant MOA(C215A) (10 μg/ml), did not change cell shape. The internalization of β1-integrin was not inhibited, suggesting that the protease activity of MOA occurs independently of β1-integrin endocytosis.

However, wild-type MOA induced significant β1-integrin degradation after 6 h, which was dependent on its protease activity (Fig. 3B). Since the loss of β1-integrin binding to extracellular matrix (ECM) proteins has previously been shown to perturb the FAK signaling pathway [31], the effect of MOA on FAK activation was examined by Western blots using antibodies specific to FAK and phosphorylated FAK (pTyr397). MDCKII cells treated with MOA (10 μg/ml) showed a

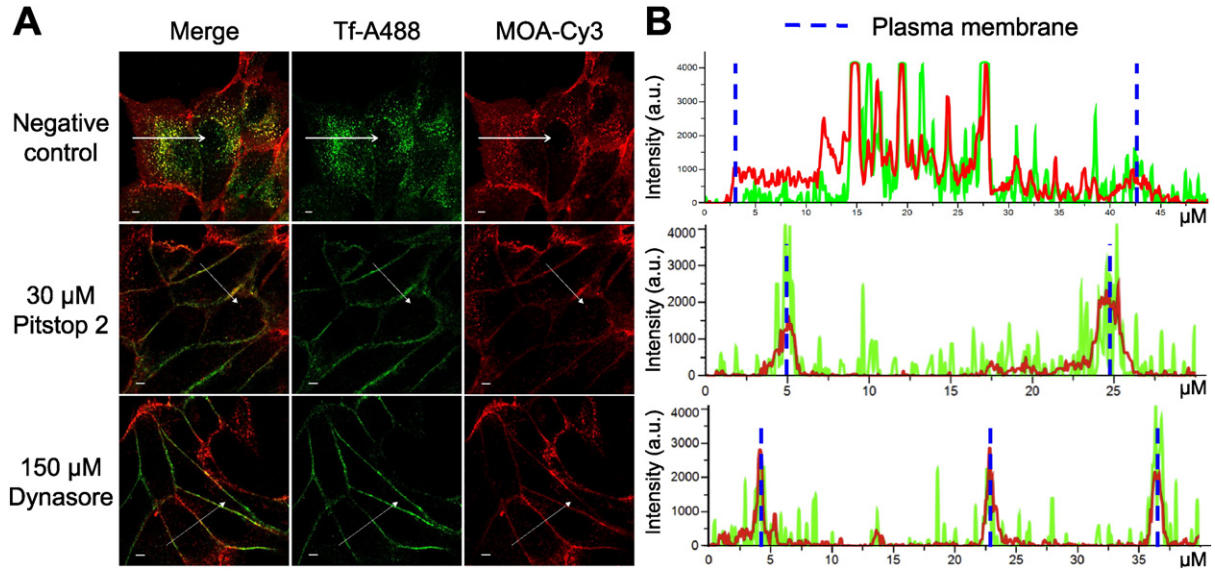

Fig. 4. MOA endocytosis requires clathrin and dynamin. A) MDCKII cells were pretreated with 30 μM of Pitstop 2 or 150 μM of Dynasore for 30 min at 37 °C, then treated with MOA-Cy3 (10 μg/ml) and Transferrin-Alexa 488 (5 μg/ml) for 10 min at 37 °C, fixed and examined by confocal microscopy. Inhibition of clathrin by Pitstop 2 as well as inhibition of dynamin by Dynasore, decreased internalization of MOA. Scale bars represent 5 μm. Representative pictures are observations of at least 3 independent experiments. B) Intensity profiles of single cells, along the arrows shown in A.

reduction in FAK activation down to 20% after 4 h that was sustained up to 12 h (Fig. 3B). Wild-type MOA co-incubated with the protease inhibitor E-64 (5 μM) as well as the variant MOA(C215A) (10 μg/ml) had a weaker effect on FAK activation than wild-type MOA. The fact that enzymatically inactive MOA induced a small effect on FAK activation suggests that other properties of the lectin also contribute to its toxicity. Such minor protease-independent toxicity was also reported in previous studies [11,13]. These results suggest that cell detachment caused by MOA protease activity is preceded by disturbed FAK signaling and involves the loss of integrin function.

3.4. MOA endocytosis is dependent on clathrin and dynamin

To further understand MOA uptake into MDCKII cells, we investigated the role of clathrin-mediated endocytosis (CME) in MOA internalization. As transferrin (Tf) is internalized exclusively through CME, Tf was used to assess the effects of inhibitors of CME [32]. The uptake of MOA-Cy3 and Tf-Alexa 488 into cells was measured while clathrin or dynamin activity was inhibited by treatment of cells with 30 μM Pitstop 2 ™ or 150 μM Dynasore, respectively. As expected, Tf endocytosis was blocked in the presence of one of the above-mentioned inhibitors (Fig. 4A,B). MOA was much less internalized under these conditions compared to untreated cells, suggesting that the lectin is mainly internalized through clathrin- and dynamin-dependent endocytosis.

3.5. MOA accumulates in late endosomes

Like Shiga toxin, many pathogen-produced proteins are known to be endocytosed and transported retrogradely to the Golgi apparatus and

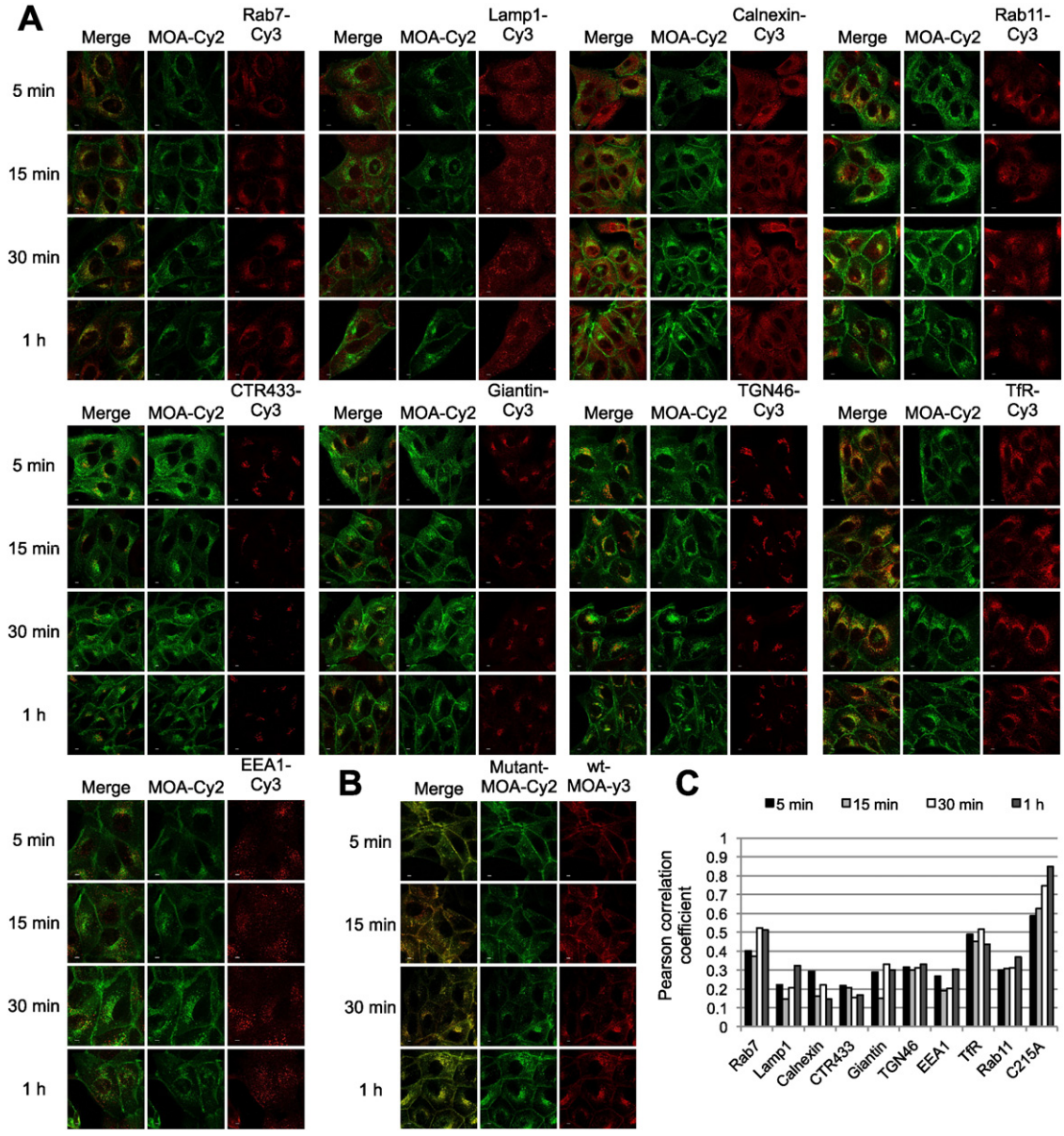

Fig. 5. MOA accumulates in late and recycling endosomes. MDCKII cells were A) treated with MOA-Cy2 (10 μg/ml) and stained with antibodies specific for Rab7 (late endosomes), or Lamp1 (lysosomes), or Calnexin (endoplasmic reticulum), or Rab11 (recycling endosomes), or CTR 433 (medial-Golgi), or Giantin (Golgi-complex), or TGN46 (trans-Golgi), or TfR (transferrin receptor, recycling pathway), or EEA1 (early endosomes). B) The variant MOA(C215A)-Cy2 was co-incubated with wt-MOA-Cy3 before addition to the cells. For A–B) the scale bars represent 5 μm. Representative pictures are observations of at least 3 independent experiments. C) Colocalization analysis using Pearson's correlation between green and red channels of the corresponding pictures.

the endoplasmic reticulum (ER) (reviewed in [33]). In previous studies, Wohlschlager *et al.* [11] reported that MOA protease activity can be regulated via pH and Ca^{2+}. They therefore hypothesized that MOA is endocytosed and transported from the early endosomal compartment via the Golgi to the ER, where the Ca^{2+} concentration is sufficiently high to activate its catalytic domain, thus leading to toxicity by degrading proteins that are in the folding process. We investigated the trafficking of MOA by immuno-fluorescence experiments using Cy2-labeled MOA (10 μg/ml) (Fig. 5A). After 5 min, 15 min, 30 min or 1 h of internalization of MOA, cells were fixed, quenched, permeabilized, and incubated with antibodies against the compartment markers Rab7, Lamp1, Calnexin, CTR 433, Giantin, TGN46, EEA1, TfR and Rab11. The intracellular distribution of MOA was similar for the wild-type and for the C215A variant (Fig. 5B,C). Thus, only the immunofluorescence data regarding the trafficking of wild-type MOA are presented in the following.

After 5 min of internalization of MOA-Cy2, an increase of the Pearson's correlation coefficient between the late endosomal marker Rab7 (red) and MOA (green) was observed in MDCKII cells (Fig. 5A; $r > 0.4$), demonstrating rapid MOA internalization. After 30 min of internalization, more overlap was observed, suggesting an accumulation of MOA in late endosomes. On the other hand, low/little co-localization was detected between the lysosomal compartment marker Lamp1 (red) and MOA (green) (Fig. 5A,C). Markers of the ER (Calnexin), Golgi (Giantin, CTR 433) and early endosomes (EEA1) showed no significant co-localization with internalized MOA. MOA showed slight co-localization with the trans-Golgi network marker TGN46 and the recycling endosome marker Rab11. These latter compartments seem to be juxtaposed to MOA-positive endosomes. Finally, MOA co-localized with TfR, which is known to be transported from early endosomes to recycling endosomes. As there is no significant co-localization with Rab11, these results suggest that MOA traffics along the first steps of the TfR endocytic pathway, but does not enter the recycling pathway.

In order to better characterize the intracellular trafficking of MOA, live-cell measurements were performed using MDCKII cells expressing either Rab5(Q79L)-mCherry or Rab7b-mCherry incubated with wt-MOA-Cy5 (10 μg/ml, Fig. 6). Expression of Rab5(Q79L), a constitutively active mutant of Rab5, induces enlarged early endosomes [34]. It is generally accepted that these structures are the consequence of enhanced membrane fusion that results from activated Rab5 and remains independent of the reported functional consequence of that expression [34–38]. As Rab5 is known to regulate endocytic trafficking at the level of early endosomes, we used the Rab5(Q79L) mutant to better visualize the first steps of endocytosis of the lectin in MDCKII cells. After 2 min of internalization of MOA-Cy5, the first vesicles containing MOA (green) were observed inside cells and co-localized with Rab5(Q79L)-positive compartments (red, Fig. 6A,B and Fig. A). Accumulation of MOA in these enlarged early endosomes increased over the time. In Rab7b-mCherry transfected MDCKII cells, first internalized MOA was also observed after 2 min of incubation, and a significant co-localization with Rab7b-positive compartments (red) was observed after 5 min of incubation (Fig. 6C,D and Fig. B). After 10 min of incubation, co-localization between MOA-Cy5 and Rab7b-mCherry reached a plateau, revealing an accumulation in the late endosomal compartment. These results confirm our previous data obtained with fixed samples, indicating a rapid internalization and accumulation of MOA at late endosomes.

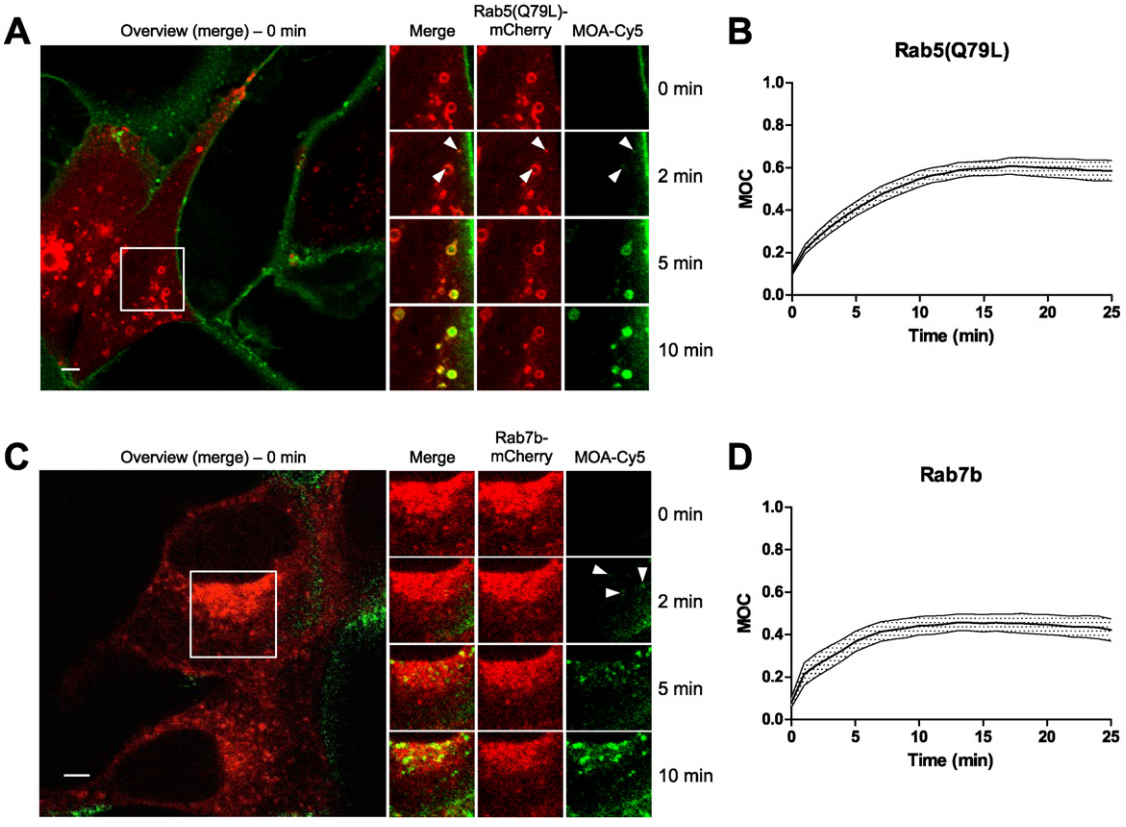

Fig. 6. Kinetics of MOA internalization and trafficking in MDCKII cells. A) Confocal fluorescence microscopy images of selected time-points of MDCKII cells transfected with a plasmid encoding for Rab5(Q79L)-mCherry (red) and treated with MOA-Cy5 (10 μg/ml, green) at 37 °C. First MOA-positive endosomes were observed at 2 min of incubation (arrowheads). B) Mander's overlap coefficient (MOC) between MOA and Rab5(Q79L) over the time. The analysis was performed from each single cell (means ± SEMs, $n = 11$). C) Confocal fluorescence microscopy images of selected time-points of MDCKII cells transfected with a plasmid encoding for Rab7b-mCherry (red) and treated with MOA-Cy5 (10 μg/ml, green) at 37 °C. First MOA-positive endosomes were observed at 2 min of incubation (arrowheads). For A) and C) the scale bars represent 5 μm. Representative pictures are observations of at least 5 independent experiments. D) Mander's overlap coefficient (MOC) between MOA and Rab7b over the time. The analysis was performed from each single cell (means ± SEMs, $n = 11$).

4. Discussion

Several studies support the role of fruiting body lectins in the fungal defense system against parasites and predators [2,11]. In agreement with the hypothesis, we have demonstrated that the fruiting body lectin MOA may exhibit toxicity through triggering of rapid disruption of integrin-dependent cell adhesion signaling and cell detachment of MDCKII cells leading to cell death. As the inhibition of the binding capacity as well as the protease activity of MOA significantly reduced cell detachment, we conclude that MOA activity is essential for its function in the fungal defense system. Our findings are supported by a previous study, in which MOA has been shown to be toxic towards nematodes in a carbohydrate binding- and an enzymatic activity-dependent manner [11]. However, the targeted receptors of MOA in *C. elegans* are GSLs, whereas the receptor in MDCKII cells is unclear. Thus, conclusions from our study with regard to the toxicity mechanism of MOA might not apply to the situation in nematodes. Moreover, Cordara *et al.* have demonstrated in a separate study that the proteolytic activity of MOA correlates partially with cytotoxicity in NIH/3 T3 cells [13].

Although MOA has already been reported to induce endothelial cell detachment in mice [14], no clear link has been established so far with the enzymatic activity of MOA. Cell detachment from ECM has adverse effects on cell survival. As cells in suspension are at a disadvantage as targets for growth factors as well as essential nutritional components, loss of adhesion promotes anoikis, a form of apoptosis specific to the absence of anchorage-dependent signaling (reviewed in [15,39]). This mechanism is controlled by mitochondria through activation of the Bcl-2 protein family and death is executed by effector caspases (reviewed in [17]). This kind of apoptosis plays important roles during development and tissue homeostasis.

The first goal of our study was to confirm the potential link between the catalytic activity of MOA and the cell detachment induced by MOA. We demonstrated that binding to the cell surface and internalization of MOA as well as its cysteine protease activity are required to disturb cell adhesion. The mechanism is, however, different from that induced by known serine proteases found in the digestive system, such as trypsin, where no host cell binding is required for cleavage of peptide chains.

In their nematoxicity study, Wohlschlager *et al.* proposed a possible toxicity mechanism, where binding of MOA to GSLs in the nematode intestinal epithelium leads to targeting of the protein to an intracellular compartment of high Ca^{2+}-concentration and pH where the protein would digest surrounding proteins and ultimately lead to disintegration of the intestinal epithelium [11]. Thus, the second goal of our study was to characterize the molecular mechanism of MOA-mediated toxicity in cells. The anchoring of cells to components of the ECM is mainly mediated by integrins. These proteins can sense mechanical forces arising from the matrix, thereby converting mechanical stimuli to downstream signals modulating cell viability, such as the FAK, MAPK, ERK-1/-2 signaling pathways (reviewed in [27]). MDCKII cells have been reported to form their own ECM by secreting proteins, e.g. laminin [40–42]. Moreover, β1-integrin has been established as a reporter for investigating cell adhesion in MDCKII cells [30,43,44].

MOA cysteine protease activity induces β1-integrin degradation. Interestingly, inhibition of MOA catalytic activity did not prevent β1-integrin internalization. Degradation of β1-integrin was preceded by dephosphorylation of FAK at Tyr397. As FAK was not degraded, we conclude that MOA protease activity might destabilize indirectly the focal adhesion complex. Hence, by disturbing FAK signaling and integrin function, MOA compromises the host cell integrity.

5. Conclusion

In plants, papain-like cysteine proteases are key players at molecular battlefields between plants and their invaders [45]. In accordance with this concept, our findings confirm the previously shown correlation between the cytotoxicity of MOA and its enzymatic activity [11,13,14], and suggest a possible toxic mechanism, which consists of disturbing cell adhesion and cell viability. Although further investigations are needed to gain a more detailed understanding of the mechanism of MOA cytotoxicity, our study strongly supports the hypothesis of a protective role of MOA in the fungal defense system.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2015.11.002.

Transparency document

The Transparency document associated with this article can be found in online version.

Acknowledgements

We thank Stefanie Schmieder and Therese Wohlschlager for supplying recombinant MOA and MOA(C215A). W.R. acknowledges the support by the Excellence Initiative of the German Research Foundation (EXC 294), by a grant from the Ministry of Science, Research and the Arts of Baden-Württemberg (Az: 33-7532.20) and by a starting grant of the European Research Council (Programme "Ideas"—call identifier: ERC-2011-StG 282105). This work was supported in part by the Excellence Initiative of the German Research Foundation (GSC-4, Spemann Graduate School). M.K. and N.S.J.v.d.V. are supported by a grant of ETH Zürich (ETH-34 11-2).

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