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Internalisation and multiple phosphorylation of γ-Conglutin, the lupin seed glycaemia-lowering protein, in HepG2 cells

Jessica Capraro [a], Chiara Magni [a,*], Franco Faoro [b], Dario Maffi [b], Alessio Scarafoni [a], Gabriella Tedeschi [c], Elisa Maffioli [c], Anna Parolari [d], Cristina Manzoni [d], Maria Rosa Lovati [d], Marcello Duranti [a]

[a] Department of Food, Environmental and Nutritional Sciences (DeFENS), Section of Chemistry and Biomolecular Sciences, Università degli Studi di Milano (UNIMI), Italy
[b] Department of Agricultural and Environmental Sciences, UNIMI, Italy
[c] Department of Veterinary Science and Public Health, UNIMI, Italy
[d] Department of Pharmacological and Biomolecular Sciences, UNIMI, Italy

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ABSTRACT

Lupin seed γ-Conglutin is a protein capable of reducing glycaemia in mammalians and increasing glucose uptake by model cells. This work investigated whether γ-Conglutin is internalised into the target cells and undergoes any covalent change during the process, as a first step to understanding its mechanism of action.

To this purpose, γ-Conglutin-treated and untreated HepG2 cells were submitted to confocal and transmission electron microscopy. Immune-revelation of γ-Conglutin at various intervals revealed its accumulation inside the cytosol.

In parallel, 2D-electrophoresis of the cell lysates and antibody reaction of the blotted maps showed the presence of the protein intact subunits inside the treated cells, whilest no trace of the protein was found in the control cells. However, γ-Conglutin-related spots with an unexpectedly low *pI* were also observed in the maps. These spots were excised, trypsin-treated and submitted to MS/MS spectrometric analysis. The presence of phosphorylated amino acids was detected.

These findings, by showing that γ-Conglutin is internalised by HepG2 cells in an intact form and is modified by multiple phosphorylation, open the way to the understanding of the lupin γ-Conglutin insulin-mimetic activity.

1. Introduction

In previous works, γ-Conglutin, a lupin seed glycoprotein [1], was shown to positively influence glucose uptake by various model cells [2,3] and to display glycaemia-lowering properties in animal [4] and healthy human [5] glucose overload trials.

γ-Conglutin is an unusual basic 7S protein present in lupin and other seeds. For a detailed description of its molecular properties see Ref. [1]. γ-Conglutin is a monomer of two different subunits: a large one, of about 30 kDa, which is mono-glycosylated, and a small one of about 17 kDa. The deduced amino acid sequence of the main expressed gene is available at UniProtKB/TrEMBL with the accession number of Q9FSH9_LUPAL [6]. Though deposited in the lupin seed protein bodies during seed development as any other seed storage protein, γ-Conglutin, does not behave as a storage protein, since it persists long after the onset of germination. Its physiological role in the seed is not known yet, despite sequence homology with fungal endo-xylanase inhibitors [7]. The protein is resistant to proteolytic enzymes at pH values close to neutrality, where it does exist as a tetramer [8,9].

A few years ago, the ability of this protein to lower plasma glucose concentrations upon glucose overload in mice was first shown [4]. Since then, experimental evidences on the peculiar biological effects of γ-Conglutin have accumulated. In particular, the effect of γ-Conglutin on the activation of differentiating myocyte signalling pathway closely resembled that of insulin [3]. As a matter of facts, γ-Conglutin cell stimulation resulted in the persistent activation of protein synthetic pathway kinases, and increased glucose transport, GLUT4 translocation, as well as muscle-specific gene transcription regulation. More recently, a relevant increase of glucose uptake by HepG2 cells, as well as a glucose lowering effect in chronically treated mice, were described [2]. Further studies on the oral administration of γ-Conglutin to animal models and healthy humans confirmed its remarkable capacity of decreasing glycaemia [5]. In a study aimed at identifying the metabolic fate of the protein, *in vitro* and *ex vivo* approaches showed that the protein can be transcytosed through a CaCo2 cell monolayer and cross the intestinal barrier in an intact form [10].

* Corresponding author. Address: DeFENS, Via Celoria 2, 20133 Milano, Italy. Fax: +39 02 503 16801.
E-mail address: chiara.magni@unimi.it (C. Magni).

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This set of findings is in line with the traditional medicine claims which include lupin seeds and flours amongst the natural anti-diabetic food products [11]. Now that the active principle responsible of this activity has unequivocally been identified, further studies are needed to assessing the modalities of interaction of this lupin protein with the target cells, so as to contribute unveiling its mechanism of action. With this aim, direct and indirect detection approaches were used in this work to show that γ-Conglutin is internalised in an intact form by HepG2 cells, and this process is accompanied by multiple phosphorylation of the protein.

2. Materials and methods

2.1. Cell culture

All culture reagents were obtained from Sigma–Aldrich, Italy. The human hepatoma cells (HepG2) were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown as described by Lovati et al. [2].

2.2. Confocal microscopy

HepG2 cells were grown on glass coverslips and incubated in DMEM (Dulbecco's Modified Eagle Medium) containing 11.1 mM glucose with/without 10 mM γ-Conglutin for 30 min, 3, 6 and 24 h. Afterwards, they were fixed with 4% paraformaldehyde, permeabilised with Tryton X-100 0.1% and washed with PBS. labelling was carried out overnight at 4 °C with anti-γ-Conglutin serum 1:100 v/v in PBS, followed by 2 h incubation at 20 °C in antirabbit conjugated with Alexa-Fluor 568 (1:200 v/v in PBS) and staining with DAPI (1:20,000 in PBS). Finally, coverslips were sealed with Mowiol on glass slides and examined with a video-confocal microscope (Vico-Nikon, Italy).

2.3. Isoelectric focusing, SDS–PAGE and western blotting

For IEF/SDS–PAGE analyses, cells were grown as above with/without 10 mM γ-Conglutin for 6 h. At the end of incubation, cell medium was removed and the monolayers were washed with cold PBS and then incubated in PBS added with 10 g/L heparin for 1 h in order to remove the excess of γ-Conglutin.

HepG2 cells were lysed by a solution of 8 mol/L urea, 20 mg/mL CHAPS and 65 mmol/L 1,4-dithiothreitol (DTT) (Amersham Biosciences, Milan, Italy). The protein extracts were centrifuged at 10,000 g for 30 min and immediately analysed or kept frozen at −80 °C until use.

Isoelectric focusing was performed on 7 cm pH 3−10 linear IPG strips (Amersham Biosciences, UK) following the procedure described by Capraro et al. [12]. The separation was performed on 9 cm × 7 cm 12% polyacrylamide SDS–PAGE gels using a mini-PROTEAN III cell (Bio-Rad, Milan, Italy). The 2-D separations were repeated three times for each analytical set.

The proteins in gels were transferred to 0.45 μ-pore nitrocellulose membranes (Protran, Whatman, Dassel, Germany) by using the TE 77 PWR Semidry Transfer Unit (Amersham Biosciences, UK), according to Towbin et al. [13]. γ-Conglutin was immune-detected as already described [14].

2.4. Mass spectrometry

For mass spectrometry analysis, each 2D-gel spot was excised and destained in 0.1% trifluoroacetic acid: acetonitrile 1:1 (v/v) and dryed in a Speed Vac. Gel pieces were rehydrated with trypsin (sequence grade, Sigma−Aldrich) solution (0.2 μg trypsin/spot in 50 μL 50 mmol/L ammonium bicarbonate), and incubated overnight at 37 °C. Peptides were extracted from the gel using 0.1% trifluoroacetic acid: acetonitrile 1:1 (v/v). The material was dried, resuspended in 10 μL 0.1% v/v formic acid and desalted using Zip-Tip C18 (Millipore) before mass spectrometric (MS) analysis.

Samples were separated by liquid chromatography using an UltiMate 3000 HPLC (Dionex, now Thermo Fisher Scientific). Buffer A was 0.1% v/v formic acid, 2% acetonitrile; buffer B was 0.1% formic acid in acetonitrile. Chromatography was performed using a PepMap C18 column (15 cm, 180 μm ID, 3 μm resin, Dionex). The gradient was as follows: 5% buffer B (10 min), 5−40% B (60 min), 40−50% B (10 min), 95% B (5 min) at a flow rate of 0.3 μL/min.

Mass spectrometry was performed using a LTQ-Orbitrap Velos (Thermo Fisher Scientific) equipped with a nanospray source (Proxeon Biosystems, now Thermo Fisher Scientific). Eluted peptides were directly electro-sprayed into the mass spectrometer through a standard non-coated silica tip (New Objective, Woburn, MA, USA) using a spray voltage of 2.8 kV. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350–2000) in the Orbitrap and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan. Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific).

Data Base searching was performed using the Sequest search engine contained in the Proteome Discoverer 1.1 software (Thermo Fisher Scientific). The following parameters were used: 10 ppm for MS and 0.5 Da for MS/MS tolerance, carbamidomethylation of Cys as fixed modification, Met oxidation and Ser/Thr/Tyr phosphorylation as variable modifications, trypsin (2 misses) as protease.

2.5. Transmission electron microscopy (TEM) and immune-gold labelling

HepG2 cells, plated and grown on Transwell®, were incubated as above with/without 10 mM γ-Conglutin for 30 min, 3 and 24 h. Transwell® membranes were washed with cold PBS and incubated for 1 h in PBS with 10 g/L heparin. Then, Transwell® membranes were fixed with 1.2% glutaraldehyde and 3.3% paraformaldehyde in 0.1 M PBS pH 7.4 at 4 °C for 3 h, dehydrated in an ethanol series and embedded in London Resin [15]. Immune-labelling was carried out on ultrathin sections at 4 °C overnight with an anti-γ-Conglutin polyclonal serum (1:100) and goat anti-rabbit antibody (1:20) conjugated with 20 nm gold particles. Ultrathin sections were stained with 2% uranyl acetate and lead citrate and examined with a JEOL 100SX TEM (Jeol Ltd., Tokyo, Japan).

2.6. Homology 3D modelling

γ-Conglutin 3D model was prepared by homology modelling using the programme ESyPred3D (available on-line at http://www.unamur.be/sciences/biologie/urbm/bioinfo/esypred/) with the amino acid sequence sequence Q9FSH9_LUPAL and the soybean homologous protein, Bg7S, 3D structure as the template (PDB accession number: 3AUP).

3. Results

3.1. Time course of γ-Conglutin uptake by HepG2 cells as assessed by confocal microscopy with anti-γ-conglutin antibodies

The uptake of γ-Conglutin by HepG2 cells, as monitored by fluorescently-labelled anti-γ-Conglutin antibodies in confocal microscopy, is shown in Fig. 1. 30 min after the treatment, it was