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

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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].

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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

already possible to observe some fluorescent spots around the cells (Fig. 1A), suggesting the presence of protein aggregates leaning against cell membranes. Whether these aggregates were only stacked outside the membrane or, at least in part, already inside the cells it was not clear at this time (Fig. 1A). Conversely, the intracellular uptake of some of these aggregates was more evident 3 h later (Fig. 1B). A very different distribution pattern of fluorescence was observed at 6 h after the treatment, when the whole cytoplasm showed a diffuse and intense fluorescence, suggesting that γ-Conglutin had spread in it, without a specific localisation or aggregation in dense bodies. Some fluorescence spots were still visible at the cell border, possibly outside membranes. This scenario completely changed at 24 h, when the diffused cytoplasm fluorescence was much less intense and large brilliant spots were again visible at the cell periphery. At any considered time, nuclei were not labelled.

3.2. Two-D electrophoresis and mass spectrometry evidence of γ-Conglutin internalisation and phosphorylation by HepG2 cells

In order to confirm the intracellular uptake of lupin γ-Conglutin by treated HepG2 cells and monitor the status of the internalised protein, 2D IEF/SDS–PAGE of the cell lysates at 6 h incubation, when the cells showed a 64% increase of glucose uptake (not shown), was performed. The resulting 2D electrophoretic maps of the untreated control cells and cells after 6 h treatment with γ-Conglutin are shown in Fig. 2, panels A and A', respectively. The two maps showed a very similar pattern. However, some spots, which were not present in the control sample, appeared in the γ-Conglutin-treated sample. These two positions in the electrophoretic map corresponded to that of γ-Conglutin subunits [16], which consists of a main small subunit around 17 kDa and *pI* 6.2 and large ones of 30 kDa and *pI 8.7*.

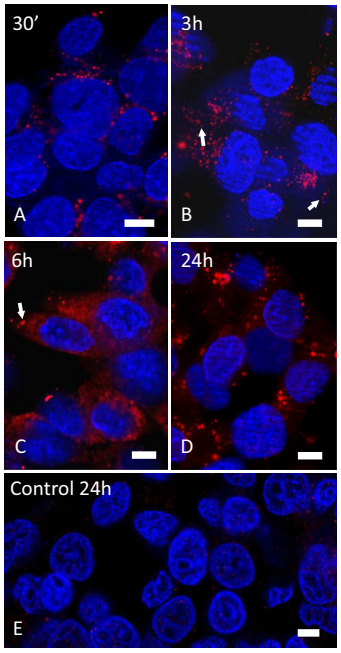

Fig. 1. Immune-detection by confocal microscopy of HepG2 cells at different incubation times with γ-Conglutin (red); nuclei are counterstained in blue by DAPI. Nuclei were not labelled at any considered time, as well as control cells without γ-Conglutin treatment (panel E). All bars = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For further evidence, the 2D maps of untreated and treated cells were blotted and revealed with anti-γ-Conglutin antibodies (Fig. 2B and B'). With the untreated cells, no labelling was visible throughout the map, suggesting that no protein component of the cells cross-reacted with γ-Conglutin antibodies. Conversely, in panel B', two main spots around 30 kDa and other less distinct ones at 17 kDa were detected by the antibodies, thus confirming the presence of intact γ-Conglutin subunits.

Other unforeseen spots reacting with γ-Conglutin antibodies were found in the blotted map (Fig. 2, panel B') at the large subunit position, but with unusually low *pI*s. The acidic spots in the blotted map, indicated as spot 1 (S1) and spot 2 (S2), as well as a main spot in the canonical position of γ-Conglutin large subunit (S3), were excised, trypsin treated and submitted to MS/MS spectrometry, as described under Methods. The results of mass analyses are detailed in Table 1. The analysis allowed to unequivocally identify the 3 spots as γ-Conglutin, in agreement with the Western blot data. Moreover, the presence of phosphorylated amino acids was detected in various tryptic peptides of spots 1 and 2, whilest none was seen in the unmodified γ-Conglutin. The position of the phosphorylated amino acids in γ-Conglutin sequence is shown in Table 1. The analysis of the predicted 3D structure of γ-Conglutin confirmed the expected location of the modified amino acids at the surface of the molecule (not shown). However, due to the incompleteness of the sequence coverage, the presence of other phosphorylated amino acids could not be excluded.

3.3. Uptake and fate of γ-Conglutin in HepG2 cells by TEM immune-labelling

To shed light on the intracellular uptake pathway and the presence of γ-Conglutin aggregates, TEM immune-localisation of the protein at 30 min, 3 h and 24 h was performed. γ-Conglutin aggregation occurred around the numerous microvilli present on HepG2 cell membranes (Figs. 3A and B), consistently with the above mentioned fluorescent spots leaning against cell membranes. These microvilli seemed to trap the protein, sometimes in large aggregates (Fig. 3B). At 3 h treatment, some small aggregates were visible both stacked to the cell membrane (Fig. 3C) or inside the cytoplasm (Fig. 3D), with no clear signs of endocytosis, possibly because the lack of osmication during the fixation process prevented membrane visualisation. Moreover, intense labelling was localised both in the protein aggregates stacked to microvilli and in some dense bodies in the cytoplasm (Fig. 3E). These bodies, being only labelled in some parts, were possibly formed by other substances, besides γ-Conglutin, and corresponded to the brilliant fluorescent spots observed in the cytoplasm by confocal microscope at the same time course. Intriguingly, dense bodies were not present at 24 h, but the labelling was scattered in the cytoplasm, with rare aggregation (Fig. 3F). Serial sectioning showed that some of these protein aggregates were invaginated into the cell membrane, however still outside the cell (Fig. 3G).

4. Discussion

Evidence of systemic biological effects of a number of proteins present in foods is accumulating in the last years [17]. Several plant peptides and proteins, including soybean storage proteins, lectins, enzyme inhibitors and others (for a review, see Scarafoni et al. [18]) appeared to be involved. However, the mechanism of action of these bioactive proteins/peptides in the modulation of cell activities has rarely been elucidated. Anyway, the prerequisite for any biological activity is the interaction with or entry into the target cells. This, in turn, implies that the protein can reach the district to perform its activity in an intact or, at least, still active form.

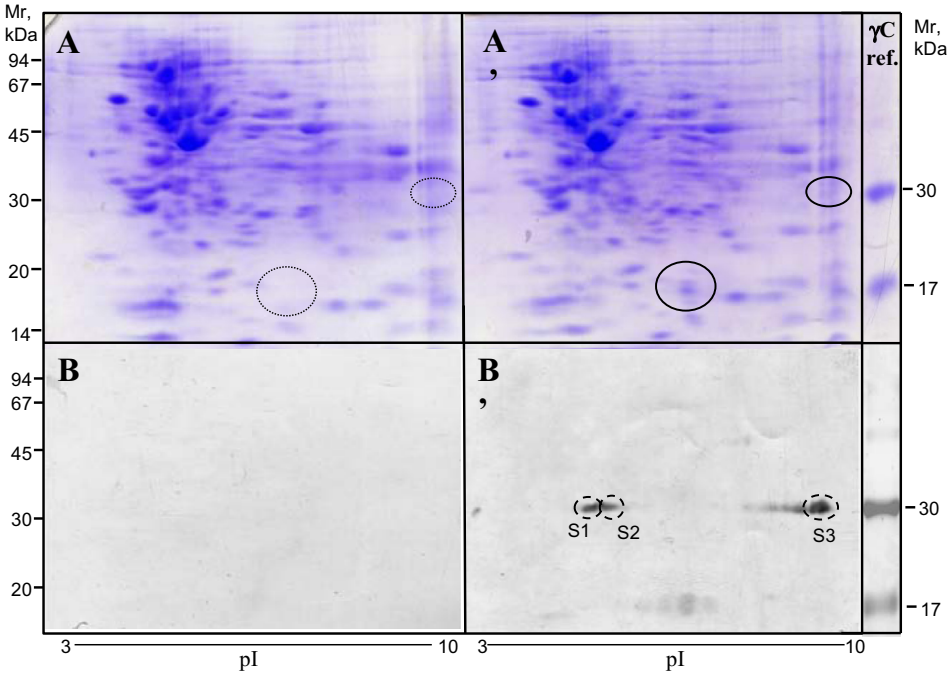

Fig. 2. Two-D electrophoretic maps of proteins from HEPG2 cell lysates incubated for 6 h in DMEM without (A) and in presence (A') of the lupin seed protein γ-Conglutin (10 mmol/L). Figure B and B', show antibody revelation of γ-Conglutin in the blotted maps of cells incubated with (B') or without (B) γ-Conglutin. Reference γ-Conglutin is also showed. In panel A', the spots corresponding to the 30 kDa and 17 kDa γ-Conglutin subunits were circled after identification by comparison to the reference map (see the white lupin 2D reference map on http://www.lupinproteinteam.unimi.it). These spots are not present in the untreated sample (panel A, dotted circles to locate the empty areas). Antibody revelation evidences the large (marked as Spot 3) and small subunits of γ-Conglutin in the treated sample (panel B'). Two acidic spots, revealed by the same antibodies, are marked Spot 1 and 2, respectively.

Table 1
List of phosphorylated peptides in 2D-gel spots S1, S2 and S3.

Sequence	Identified phosphorylated residue	Position[a]	XCorr	Charge	*m/z* (Da)	MH^+(Da)
Spot S1						
HSIFEVFTQVFANNVPK	T	36	1.58	3	686.33496	2056.99033
RTPLMQVPVLLDLNGK	T	298	1.74	3	625.34088	1874.00809
KISGGVPSVDLIMDK	S	327	1.87	4	410.46106	1638.82241
AVGPFGLCYDTKK	Y	321	1.43	2	768.34937	1535.69145
SCSNLFDLNNP	S	409	1.60	2	680.76617	1360.52507
Spot S2						
RTPLMQVPVLLDLNGK	T	36	2.07	3	625.33844	1874.00077
KISGGVPSVDLIMDKSDVVWR	S	327	1.42	3	799.72986	2397.17502
IPQFLFSCAPTFLTQK	T	149	1.11	3	659.99481	1977.96988
Spot S3						
Various peptides belonging to γ-Conglutin sequence	No phosphorylated residues	–	–	–	–	–

[a] The residue position is referred to sequence Q9FSH9.

Notably, a number of plant and seed proteins have been found to be fully or partially resistant to proteolytic enzymes into the gastro-intestinal tract in force of their peculiar amino acid sequences and or structures [19–21]. In this respect, γ-Conglutin was shown to undergo an 'all or none' mechanism of proteolytic degradation *in vitro*: when γ-Conglutin native conformation was lost, the protein became susceptible to proteolytic enzymes; conversely, if the native structure was preserved, as it occurs at any pH value greater than 3.0, no degradation was observed [8].

In this work we used a dual approach to get complementary information on the internalisation and possible covalent modifications of γ-Conglutin in HepG2 cells, as the basis for further studies aimed at unveiling the mechanism of action of this protein.

Remarkably, microscopy showed that the protein did get in contact with the cell in the form of molecular aggregates. The spontaneous formation of these aggregates at neutral pH values [22] may facilitate the interaction with the cell structures devoted to captation of foreign materials, such in the cases of albumin in astrocytes [23] and human 1-acid glycoprotein (AGP) [24].

Intracellular accumulation of γ-Conglutin inside the cells is particularly evident in confocal microscopy at 6 h incubation, when also glucose uptake is significantly increased. At the same time of treatment, the lupin protein was detected in the HepG2 cell lysates by 2D electrophoresis, and the position of its two subunits in the map suggested that the covalent integrity of the protein was preserved. However, a shift in the map of γ-Conglutin related spots to more acidic pH values denoted a significant *pI* change of some polypeptides. Mass spectrometry unequivocally showed that this *pI* drift was due to multiple phosphorylation of the protein. No phosphorylation events have been previously described for γ-Conglutin.

A prerequisite for any biological activity is the interaction with or entry into the target cells. Our findings, by showing that γ-Conglutin can be taken up by HepG2 cells in an intact form and is

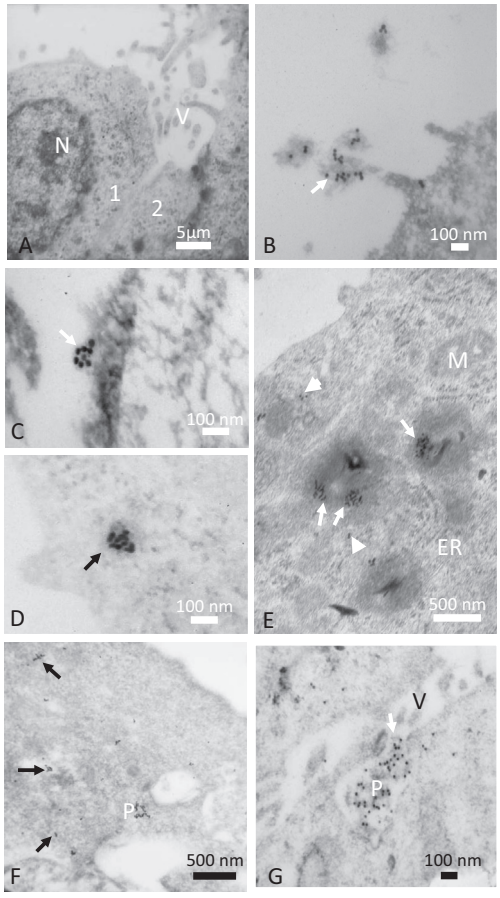

Fig. 3. TEM ultrathin sections of HepG2 cells at 0 min (A), 30 min (B), 3 h (C-E) and 24 h (F,G) incubation with γ-Conglutin. γ-Conglutin is sometime localised in electron-dense bodies (panel E, arrows) or scattered throughout the cytoplasm (panel E, arrowheads) and rare protein aggregates (P) are present. (G) Serial sectioning of an aggregate showing that its invagination into the cell membrane (arrow) (V, microvilli). ER, endoplasmic reticulum; M, mitochondrion.

modified by multiple phosphorylation, open the way to more focused studies aimed to understand the mechanisms of action of this bioactive lupin seed protein able to lower glycaemia in animals and humans.

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