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FELLOWSHIP FINAL REPORT

The role of glycosylation in the functional activity and pathological consequences of serpin proteins

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ABSTRACT

This project focus on the molecular basis of a peculiar class of conformational diseases, called Serpinopathies, with a special emphasis to glycosylation, an important post-translational modification which rules the functional and pathological behaviour of the proteins responsible for the diseases. The authors exploited their expertise on protein biophysics and glyco-biochemistry to set up a long-term program for the studies on the role of glycosylation in the functional activity and pathological consequences of serpin proteins. An experimental work was accomplished to start the expression and production of two serpins, neuroserpin and C1-inhibitor, in a novel eukaryotic expression model. Further, the program was given a wider scope by consolidating a European network of researchers working on closely related issues.

1- Introduction

Serpinopathies are a class of genetic diseases related to the deficiency of a serpin (SERin Protease Inhibitor) and/or its accumulation as polymer chain in the cell of synthesis [1]. For instance, the best-known α1-antitrypsin deficiency is caused by mutations in alpha1-antitrypsin determining polymer accumulation in the hepatocytes and lack of inhibition of lung proteases [2]; the Hereditary Angio-Edema is caused by a poor activity of mutated C1-inhibitor [3]; the Familial Encephalopathy with Neuroserpin Inclusion Bodies (FENIB) is related to the accumulation of neuroserpin polymers within neuron endoplasmic reticulum [4]. FENIB and in general all serpinopathies are incurable disorders also due to the incomplete understanding of polymer structure and formation, which makes it difficult to develop a successful therapeutic approach based on polymerisation inhibitors [5]. To date, all the biochemical and biophysical research performed on neuroserpin has been carried out using recombinant neuroserpin expressed in bacteria, thus non-glycosylated [6]. However, our recent data indicate that neuroserpin polymerisation is hindered by the presence of glycosidic chains at two sites that are glycosylated when expressed in mammalian cells [7]. Also, preliminary experiments show that N-linked glycosylation relates to the stability and polymerization of alpha1-antitrypsin, as well as to the molecular conformation of the unstructured domain of C1-inhibitor. The main aim of the present project is to highlight the role of glycosylation in the conformational stability, functional activity and polymerisation propensity of neuroserpin and C1-inhibitor. Also, we aim at establishing a

European-wide network for research in this field.

2- Experimental details

Expression of human neuroserpin. *Leishmania tarentolae* P10 cells (Jena Bioscience, Jena, Germany) were transfected with a pLEXSY-sat2-hNS plasmid (kindly provided by S. Ricagno). The latter construct was built by inserting the human neuroserpin gene (hNS), fused with a polyhistidine tag and a secretion signal peptide, into the pLEXSY-sat2 plasmid (Jena Bioscience), used for constitutive expression [8]. Transfection was performed by electroporation as in Belaz et al. 2015 [9].

Purification of human neuroserpin. Cells were grown following the manufacturer protocol in BHI medium enriched with hemin, generic pen-strep antibiotic and selective antibiotic nurseotricin. After 48hr cell growth, cells were harvested and the medium was filtered and loaded into a 5ml HiTrap Chelating HP Amersham column (GE Healthcare Europe GmbH, Freiburg, Germany) charged with 0.1M $NiSO_4$, and equilibrated with 20 mM Tris-HCl buffer (pH 8). The protein was eluted by a high content Imidazole buffer by using an AKTA liquid chromatography system, then dialised and concentrated for further measurements.

Cloning of human C1-inhibitor gene. We also started the cloning of a construct to start the expression in Leishmania of another serpin, the C1-inhibitor. In this case, the C1-inhibitor gene was amplified by PCR enabling the upstream addition of a XbaI restriction site and a sequence coding for a 6xhis-tag, and the downstream addition of a NotI restriction site. The two restriction sites are then used for the insertion of the amplicon in the pLEXSY-hyg2 plasmid (Jena Bioscience), which is equivalent to the other plasmid used for neuroserpin but with a different selection antibiotic (hygromycin). As for neuroserpin, the his-tag is added between the N terminus of the protein and the secretion signal peptide to avoid any interference in the mobility of the C-terminus, which is know to critically influence serpin dynamics and function [7].

3- Results and discussion

The experimental part of the project was centered on the production of glycosylated serpins in a eukaryotic expression system, as an alternative to the more typical bacterial *E. coli* expression. The choice felt on *Leishmania tarentolae* (*L. tarentolae*) and in particular on *L. tarentolae* P10 cells, produced by Jena Bioscience and engineered to safely express secretion proteins [8]. This expression model is able to provide a glycosylation pattern, which closely resemble that of mammalian cells. Its advantage respect to other eukaryotic or mammalian cell lines rely on its extreme simplicity in cultivation and its expected high yield.

We performed the cloning of human C1-inhibitor gene into a plasmid suitable for constitutive expression in Leishmania. Although C1-Inhibitor is currently purified from plasma, the achievement of a handy expression system would allow to easily express mutated variants either relevant for the pathology or suitable to study the effect of glycan chains on structure and conformational stability. As a side project, we measured the structures of two commercial variants of C1-inhibitor with different glycosylation patterns. The measurements were performed by Small Angle X-ray Scattering at Synchroton Soleil, Paris, France (proposal 20170083) and show a clear difference in the overall structure of the two variants [*to be published*].

In the case of human neuroserpin, the lack of purified proteins makes the achievement of an efficient expression system particularly important. In close collaboration with the group of S. Ricagno, we challenged the expression and purification neuroserpin in Leishmania. We performed the first control tests by size exclusion chromatography, non-denaturant and SDS gel electrophoresis, circular dichroism, absorption spectroscopy, along with polymerisation and activity assays (as described in Noto et al. 2015 [5f]). Also, we verified the presence of glycosylation comparing bacterial recombinant neuroserpin with leishmania neuroserpin digested with PNGase, and by mass spectroscopy. The work is still on going, also to optimize both the culture and the purification protocols.

4- Conclusion

Glycosylation is an ubiquitous post-translational modification, which is important for the folding and stability of many glycoproteins [10], as well as for their cellular fate and the activation of cell quality control [11]. Serpins are glycoproteins mainly working as inhibitors of serine proteases. Pathological mutations in serpin gene may cause a conformational failure, which brings to a deficiency of serpins in the place of action (lack of function) or their polymerisation and toxic deposition in cells (gain of function) [1]. Although its *bona fide* importance [11,12], little is known about the role of glycosylation in folding and polymerisation of neuroserpin and, more in general, in the molecular details of serpin conformational stability [7].

This project was the onset of an ambitious working platform to study the role of glycosylation on the molecular aspects of serpins. On one hand, an experimental work was initiated by using the parasite Leishmania as a novel eukaryotic model for protein expression. The aim is to gain the capability to produce mature glycosylated serpins with the same easiness and high yield typically obtained with *E.coli*. This may allow one to perform comprehensive biophysical studies on serpin function and structure, including the rare pathological variants.

A further outcome of the project was the strengthening of a European network of researchers working on this subject. Indeed, this was a main activity in the overall project, not secondary to the plain experimental work. The results were achieved by involving many collaborators directly into the experimental work, and also by organizing a LE STUDIUM conference on "The role of Glycosylation on Serpin Biology and Conformational diseases". The aim of the conference was to drive the attention and the interest of serpin scientists on the biophysical aspects of serpinopathies related to glycosylation. The conference has seen participants with different background, from medical doctors to molecular biophysicists. Notwithstanding the recognized importance of the field, the conference was likely the first one explicitly dedicated to this subject; its success encouraged participants to plan other meeting modelled on this one.

5- Perspectives of future collaborations with the host laboratory

The deliberate plan is to implement a research line which will exploit the joint effort initiated by the authors and their groups during this project. The host laboratory lead by R. Daniellou is the the unit of Enzymology and Glycobiochemistry, a branch in the field of biochemistry of the ICOA, Institute of Organic and Analytical Chemistry of CNRS. The group is focused on understanding the mechanisms and structures of enzymes involved in synthesis (Glycosyltransferases), or breaking (Glycosidases) of carbohydrates, and on a wider perspective on the discovery of novel bioactive molecules having potential applications as drugs or as components of cosmetic formulation. The other laboratory lead by the LE STUDIUM fellow M. Manno is a branch of the Institute of Biophysics of the National Research Council of Italy (CNR). This group works on the stability of biological molecules, including extracellular vesicles and proteins involved in conformational diseases, with a particular focus on serpins and serpinopathies. The two groups are currently committed to develop an experimental research program to study the role of glycosylation in the functional and dysfunctional properties of serpin molecules.

6- Scientific communication in the framework of the fellowship

(A) Invited Lectures, seminars and workshops

M. Manno. The molecular bases of serpinopathies, a class of conformational diseases. Vrije Universiteit Brussel (organised by K. Pawels and P. Tompa). Brussel, Belgium, May 9, 2017.

M. Manno. Physics, biophysics and glycobiochemistry: A multidisciplinary route to Serpinopathies, a class of conformational

diseases. LE STUDIUM LECTURE. Orléans, France, April 6, 2017.

M. Manno. Serpin conformational diseases: From molecular studies to therapeutic intervention. 15h ADOC Conference-Colloque on "Fundamental research, support for applied research" (organised by ADOC, Association des Doctorants du CBM Orléans). Orléans, France, May 04, 2017.

M. Manno. Non-classical polymers of alpha1-antitrypsin: a multidisciplinary approach. 1° Italian Research Meeting on alpha1-antitrypsin deficiency. Brescia, Italy, February 25, 2017.

(B) Conferences

M. Manno. The well-tempered polymerisation of human neuroserpin. A biophysics perspective. Workshop on "The role of glycosylation in serpin biology and conformational diseases", Orléans (France), September 27-29, 2017.

J. Ati. Glycosylate me, LEXSY! Workshop on "The role of glycosylation in serpin biology and conformational diseases", Orléans (France), September 27-29, 2017.

M. Manno, L. Randazzo, S. Raccosta, R. Noto, M.R. Mangione, J. Ati, P. Lafite, R. Daniellou, E. Miranda, R. Russo, A. Barbiroli, M. Bolognesi, S. Ricagno, V. Martorana. The well-tempered polymerization of human neuroserpin: The mechanism and its prevention in related pathologies. Conference on "Biophysical Approaches to Protein Folding and Disease". Edinburgh, UK, July 20-21, 2017.

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