We are grateful to the CNRS (Centre National de la Recherche Scientifique) through the TGE high-resolution FT-ICR for supporting the mass spectrometry experiments.

Dicer-2 / Argonaute-2 / R2D2 interaction network during antiviral response in *D. melanogaster* cells.

Yann Verdier[1] ; Iman Haddad[1], Karim Majzoub[2] ; Jules A. Hoffmann[2], Jean-Luc Imler[2] ; Joëlle Vinh[1]

[1] Spectrométrie de Masse Biologique et Protéomique, CNRS USR 3149, ESPCI ParisTech, 10 rue Vauquelin, 75231 Paris, France.
[2] Institut de Biologie Moléculaire et Cellulaire, CNRS UPR9022, 15 rue René Descartes, 67084 Strasbourg, France

Introduction

Fighting viral infections is hampered by the scarcity of viral targets and their variability resulting in development of resistance. In Drosophila, viral infections induce various and specific immune responses, including RNA degradation by iRNA mecanism. Viral RNA are recognized and processed into 21-nucleotide-long siRNA complexes by Dicer-2. siRNAs are then loaded onto AGO-2, a central component of the RNA Induced Silencing Complex (RISC), and one strand is discarded, while the other is used to guide RISC towards complementary RNA molecules. These mechanisms are allowed by R2D2, which helps directing Dicer-2 to viral RNA, and loading siRNA onto RISC [1,2]

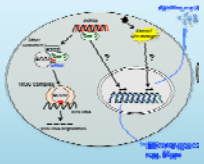

Fig1. RNAi response in Drosophila [2]

Aim of the work

This work aims to caracterize the Dicer-2 / Argonaute-2 /R2D2 complex during cellular response against Drosophila C Virus (DCV) infection.

Material and Method

Material:

Argonaute-2 (AGO), Dicer-2 and R2D2 were inserted into the insect expression vector pIZ/V5-His (Invitrogen) containing the biotin-tag sequence at the 5' or 3' end. Drosophila S2 cells (Invitrogen) expressing the biotin ligase BirA, were used for protein expression and labelling. Cells were either mock infected or infected with DCV at multiplicity of infection (MOI) 1. Protein purification and identification was performed as described [3] at t=0, t=8h and t=16h.

Mass spectrometry analysis

The on-beads tryptic digestion method was used on streptavidin-purified samples [4]. Beads were discarded by filtering through C18 Tips (Proxeon). 5% of the total amount of peptides were purified on a capillary reversed phase column (nano C18 Acclaim PepMap 100 Å; 75 µm internal diameter, 15 cm length; Dionex), at a constant flow rate of 220 nL/min.
The MS analysis was performed on a FT ICR mass spectrometer (LTQ-FT Ultra, ThermoFisher Scientific, San Jose, CA) with the "top 7" acquisition method: MS resolution 60,000; mass range 500-2000 Th; followed by 7 MS/MS (LTQ) on the 7 most intense peaks, with a dynamic exclusion for 90 s.
Each sample was first analyzed in triplicate then an exclusion list was added for 3 other runs. The database search was done using Xtandem search engine on the 17D melanogaster database (16535 sequences). The following parameters were used: up to 2 miss cleavages; MS tolerance 10 ppm, MSMS tolerance 0.5 Da, full tryptic peptides, partial modifications: carbamidomethylation (C), oxydation (M, H, W), phosphorylation (Y). Protein networks were visualized using Cytoscape (v2.8.3).

Label-free protein quantification

Peaks were aligned and areas calculated using MassChroQ software [5]. To allow intersample comparison, areas were normalized using the values of 10 ubiquitary, constant peptides. Then, areas from all the peptides of each protein were summed for each sample, allowing the comparison of this protein quantity between all the samples.

Biological assay

dsRNA against selected genes were designed using the online E-RNAi algorithm (http://www.dkfz.de/signaling/e-rnai3). 30,000 S2 cells were bathed with dsRNA 4 days at 24°C . Silenced cells were infected with DCV (MOI 1) for 16 h. Viral RNA load was measured by RT-Q-PCR using the "Cell to Ct" SYBR green kit (Ambion) and by immunofluorescence. Results were validated only if there were no effects on cell viability and proliferation, and if the viral load was at least 50% different from the control.

Results

Time course of protein identification in R2D2/AGO2/Dicer2 samples after Drosophila C Virus (DCV) infection.

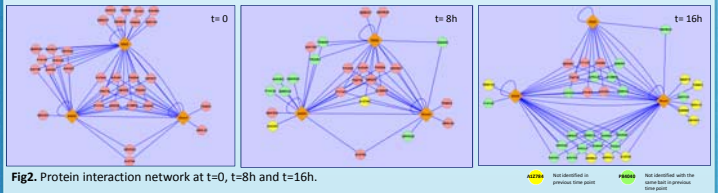

Fig2. Protein interaction network at t=0, t=8h and t=16h.

- 39 proteins were identified.
- The analysis of the complex at different time points shows the dynamism of the molecular events.

Quantitative assessment of protein concentration in the complex: example of protein P11146

Fig3. Peak area of peptides from protein P11146 in the whole complex (left) and in samples purified with r2d2, Dicer-2 and AGO-2 baits (right).

- This protein was identified with the 3 baits, at the 3 time-points. Its quantity in the whole complex was constant during the experiment (left).
- However, quantification analysis suggests that this protein is less associated with Dicer2 at t=8h (right).

Biological significance of the results.

Out of the 39 proteins, 28 have been tested for biological significance.
The inhibition of the expression of 13 of them (46%) influences the antiviral response.
P11146 protein is among the functionally significant proteins.

Conclusions & Perspectives

Our work allowed the caracterisation of the Dicer-2 / Argonaute-2 /R2D2 complex during antiviral response in Drosophila. 39 proteins were identified. Functional analysis showed that a large ratio of them are functionaly significant for antiviral response.
From a methodological point of view, the use of FT ICR mass spectrometer allowed a very good measure stability during all the experiment. The accuracy of this instrument was also useful for acquisitions with exclusion list and for peak alignment necessary for label-free quantification.
Further work is needed for a better caracterization of this complex, in particular when stimulated with other viruses. The biological function of some identified proteins will also be studied.

References

[1] Deddouche et al. Nature Immunology 2008; 9: 1425–32.
[2] Ding. Nature Reviews 2010; 10: 632–44.
[3] Fukuyama, et al. J Proteomics 2012; 75(15):4610-9.
[4] Fukuyama, et al. Proc Natl Acad Sci U S A. 2013; 110(26):10717-22.
[5] Valot, et al. Proteomics, 2011; 11:3572-7.

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Contact: yann.verdier@espci.fr