Acta Biochim Biophys Sin, 2018, 50(7), 727–729
doi: 10.1093/abbs/gmy054
Advance Access Publication Date: 1 June 2018
Lab Note

Lab Note

A novel dense granule protein NcGRA23 in *Neospora caninum*

Weirong Wang†, Pengtao Gong†, Pu Wang, Jingquan Dong, Xiaocen Wang, Zhengtao Yang, Jianhua Li*, and Xichen Zhang*

College of Veterinary Medicine, Jilin University, Changchun 130062, China

†These authors contributed equally to this study.
*Correspondence address. Tel: +86-431-87836155; Fax: +86-431-87981351; E-mail: jianhuali7207@163.com (J.L.)/xczhang@jlu.edu.cn (X.Z.)

Neospora caninum is an intracellular parasite first discovered by Bjerkas in 1984 in young canine suffering from myositis and meningitis [1]. *Neospora caninum* can infect canines, bovines, and other animals, leading to abortion or stillborn foetuses in bovines and motor nervous system diseases in nascent calves, severely damaging the animal agricultural industry. Tranas *et al.* [2] showed that *N. caninum* could also be detected in human serum, but no human *N. caninum* infections have been reported. *Neospora caninum* has brought great losses to the animal husbandry industry; thus, it has received much attention from veterinarians and is a subject of many studies.

Dense granule proteins (GRAs) are secreted by *Toxoplasma gondii* and *N. caninum* [3–6], and play important roles in forming parasitophorous vacuoles (PV) [7,8]. GRAs are thought to modify the microenvironment within the PV, and influence intracellular survival and replication [9]. In 2013, Liu *et al.* [10] expressed NcGRA14 and showed that it was associated with PV membrane (PVM) formation. However, the functions of most other granule proteins remain unknown.

In this study, the *N. caninum* GRA23 (NcGRA23) gene (NCLIV_006780) was analyzed by BLAST based on the homologous sequence of the *T. gondii* GT1 GRA23 gene (TGGT1_297880) in GenBank. BLAST analysis of nucleotide and amino acid sequences of *N. caninum* and *T. gondii* GRA23 gene showed that nucleotide homology was 63.96%, and amino acid homology was 57.92% (**Supplementary Fig. S1**).

To obtain the NcGRA23 protein and its polyclonal antibodies, cDNA of NcGRA23 was amplified from RNA (**Supplementary data**) by PCR and then cloned into *Escherichia coli*. The primers used to amplify the full-length NcGRA23 gene were PF (5′-ATAGAATTCC TCGCGTCCGCCGACGAAGCTTT-3′) and PR (5′-ATACTCGAG TTAGTTCTTTCGCGCGAGCACTGA-3′). The purified protein (0.6 mg/ml) was verified by SDS-PAGE (**Supplementary Fig. S2A**) and western blot analysis using sera immunized with *N. caninum* NC-1 tachyzoites (**Supplementary Fig. S2B**).

Rabbits were immunized subcutaneously three times with the GRA23 protein, at 10-day intervals. Freund's complete adjuvant was used (1:1) in the first immunization (500 μg/per rabbit), and Freund's incomplete adjuvant was used (1:1) in the second and third immunizations (300 μg/per rabbit). Blood was collected from the heart 10 days after the last immunization. After blood was centrifuged at 3000 *g* for 15 min, the serum was separated, and the antibody titer was detected by indirect ELISA and stored at −20°C for future use. The NC-1 tachyzoites were lysed and analyzed by western blot analysis using anti-recombinant NcGRA23 antibodies. The result showed that anti-recombinant NcGRA23 antibodies are specific (**Supplemented Fig. S2C**).

NcGRA6 is located in the PV network [11]. To determine the location of NcGRA23, we co-localized GRA6 with GRA23. MDBK cells (Cell Bank of the Shanghai Institutes for Biological Sciences, Shanghai, China) were inoculated with NC-1 tachyzoites (MOI = 3) for 48 h and washed three times (15 min each) with pre-cooled PBS. Then, cells were treated in 0.5% Triton-X-100 at room temperature for 15 min and washed with PBS. Subsequently, cells were blocked with 5% BSA at room temperature for 30 min and then incubated with diluted primary rabbit anti-GRA23 and rat anti-GRA6 polyclonal antibodies overnight at 4°C. After wash with PBS, cells were incubated with FITC-labeled goat anti-rabbit IgG and Cy3-labeled goat anti-rat IgG (Earthox, Millbrae, USA) for 1 h. Finally, intracellular parasites were observed and mapped on a laser scanning confocal microscope (Fv1000; Olympus, Tokyo, Japan). The results showed that *N. caninum* GRA23 was distributed in the front of the parasite, while GRA6 was located in the back of the parasite (**Fig. 1A**). The GRA6 was co-localized with the newly discovered compact granule protein GRA23 by immunofluorescence (**Fig. 1A**). GRA6 was mainly distributed in the PV. After *N. caninum* invasion, GRA6 was nearly absent from the PVM (**Fig. 1B**). GRA23 was distributed on the edge of the PV and existed in the PVM (**Fig. 1B**). The co-localization with GRA6 revealed that GRA23 is secreted in the PV and partially secreted at the PV margins.

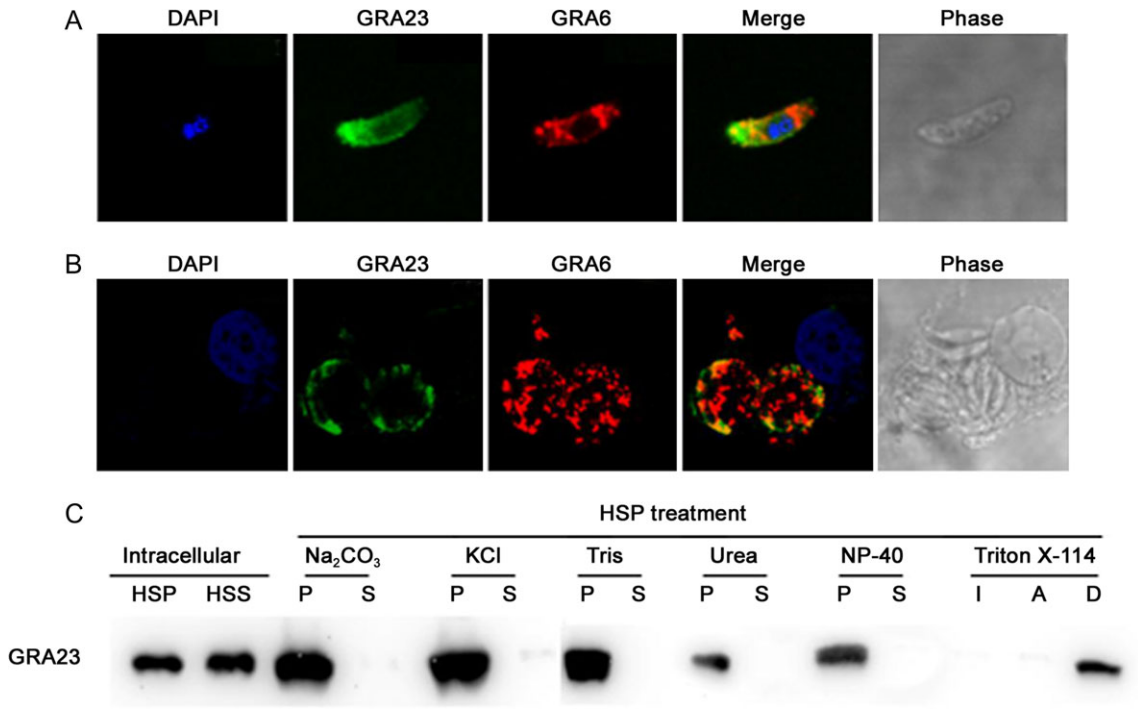

Figure 1. Extracellular and intracellular staining of *N. caninum* tachyzoites and western blot analysis (A,B) Indirect immunofluorescence assay was performed on NC-1 extracellular parasites (A) and on MDBK cells infected with parasites for 48 h (B). (C) Western blot analysis of cell fractions. HSP: high speed pellet; HSS: high speed supernatant.

To analyze the major forms of the GRA23 protein in the PV, the infected MDBK cells were fractionated. Cells were suspended in pre-cooled PBS (without calcium). After several passes through a 27-gauge needle, the parasite and cell debris were removed by centrifugation at 2500 *g* for 10 min. The supernatant was then fractionated into soluble and membrane-related fractions by ultracentrifugation at 100,000 *g* for 2 h. Then the supernatant and the pellet were collected separately and subject to 5%–12% SDS-PAGE. Western blot analysis was performed using a primary antibody against GRA23 protein at 1:1000 and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (TransGen Biotech, Beijing, China) at 1:3000. Results showed that GRA23 exhibited soluble and membrane-related forms in the PV (**Fig. 1C**).

To reveal the nature of the interaction between GRA23 and the PVM, the pellet fractions were treated with several denaturants. The results showed that the pellet is insoluble in 50 mM Tris and partially soluble in urea and NP-40. After treatment with Triton X-114, NcGRA23 was predominantly present in the aqueous phase (**Fig. 1C**). These data suggest that GRA23 is not an integrated membrane protein, but rather a membrane protein.

The signal peptide and transmembrane domain of the GRA23 were predicted using the SignalP 4.1 Server and the TMHMM v2.0 Server. The bioinformatics analysis results showed that GRA23 has a signal peptide, but no transmembrane domain, however immunofluorescence and fractionation showed that GRA23 is a transmembrane protein distributed in the PV network and the PVM, which is inconsistent with the bioinformatics analysis results. Therefore, we re-analyzed the ORF of GRA23. Genomic sequence analysis showed that the GRA23 start codon should be from 85 to 87 rather than from 160 to 162 as published in GenBank. Bioinformatics analysis using our GRA23 ORF showed that GRA23 has a signal peptide sequence and transmembrane domain (**Supplementary Fig. S3**).

In conclusion, this research showed that NcGRA23 can participate in the formation of parasitic PV and it is expressed in the PV. Immunofluorescence showed that NcGRA23 is present in the PV. In addition, NcGRA23 is expressed both in soluble form and in membrane-associated form in the PVM. Our results indicate that NcGRA23 is present in both soluble and membrane-associated forms in the PV of the IVN. Similar to TgGRA2, NcGRA23 is a membrane protein. Further studies are required to reveal its function in PV formation.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Funding

This work was supported by a grant from the National Basic Science Research Program China (No. 2015CB150300).

References

1. Bejrkas I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Z Parasitenkund* 1984, 70: 271–274.
2. Tranas J, Heinzen RA, Weiss LM, McAllister MM. Serological evidence of human infection with the protozoan *Neospora caninum*. *Clin Diagn Lab Immunol* 1999, 6: 765.
3. Ahn HJ, Kim S, Nam HW. Nucleolar translocalization of GRA10 of *Toxoplasma gondii* transfectionally expressed in HeLa cells. *Korean J Parasitol* 2007, 45: 165–174.

4. Zhang W, Deng C, Liu Q, Liu J, Wang M, Tian KG, Yu XL, *et al*. First identification of *Neospora caninum* infection in aborted bovine foetuses in China. *Vet Parasitol* 2007, 149: 72.
5. Nam HW. GRA proteins of *Toxoplasma gondii*: maintenance of host-parasite interactions across the parasitophorous vacuolar membrane. *Korean J Parasitol* 2009, 47: S29–S37.
6. Cesbron-Delauw MF. Dense-granule organelles of *Toxoplasma gondii*: their role in the host-parasite relationship. *Parasitol Today* 1994, 10: 293.
7. Arab-Mazar Z, Fallahi S, Koochaki A, Mirahmadi H, Tabaei SJ. Cloning, expression and immunoreactivity of recombinant *Toxoplasma gondii* GRA5 protein. *Iran J Microbiol* 2016, 8: 331–337.
8. Liu Q, Li FC, Elsheikha HM, Sun MM, Zhu XQ. Identification of host proteins interacting with *Toxoplasma gondii*, GRA15 (TgGRA15) by yeast two-hybrid system. *Parasit Vectors* 2017, 10: 1.
9. Adjogble KD, Mercier C, Dubremetz JF, Hucke C, Mackenzie CR, Cesbron-Delauw MF, Däubener W. GRA9, a new *Toxoplasma gondii* dense granule protein associated with the intravacuolar network of tubular membranes. *Int J Parasitol* 2004, 34: 1255.
10. Liu G, Cui X, Hao P, Yang D, Liu J, Liu Q. GRA 14, a novel dense granule protein from *Neospora caninum*. *Acta Biochim Biophys Sin* 2013, 45: 607–609.
11. Dong J, Li J, Wang J, Li F, Yang J, Gong P, Li H, *et al*. Identification and characterization of GRA6/GRA7 of *Neospora caninum* in MDBK cells. *Acta Biochim Biophys Sin* 2017, 49: 361–366.