New Phenomenon

GRA 14, a novel dense granule protein from Neospora caninum

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Dense granule protein (GRA) is a secreted protein in Neospora caninum and Toxoplasma gondii, which plays an important role in forming parasitophorous vacuole (PV) [1–4]. GRAs are thought to remodel and maintain the environment of the PV for intracellular survival and replication in T. gondii [5]. The precise function of GRAs is still unknown [6–9]. TgGRA14 protein is a new GRA protein that is highly expressed and targeted to PV membrane and intravacuolar [5]. However, the GRA14 of N. caninum (NcGRA14) has not been identified.

The NcGRA14 gene was predicted in ToxoDB (http://toxodb.org/toxo/). NcGRA14 has 60.5% nucleotide similarity with the TgGRA14. DNA sequencing analysis indicated that NcGRA14 gene contained a long open reading frame (1215 bp) without introns. NcGRA14 appeared to contain one methionine translation start codon in the N-terminal portion of the encoded protein (Fig. 1). The whole amino acid (aa) sequences is 404 aa. The predicted potential site of signal peptidase is in aa 1–36, while the transmembrane domain is in aa 285–304. The predicted size of the mature peptide after removal of the signal sequence is about 44.6 kDa. The bioinformatics prediction of NcGRA14 protein is a transport protein involved in telomerase ribonucleoprotein complex–RNA binding.

To determine whether the putative NcGRA14 gene encoded a functional protein, the cDNA of NcGRA14 was amplified by PCR. Primers used for amplification of the full length of GRA14 gene were P1F (5'-ATGCGAGG CGCAACGCGGG-3') and the P1R (5'-TTAGTAGACCG AGTTACCTGAGG-3'). In addition, the primers P2F (5'-cgGGATCCATGGGCTCGAGGCGAGATTCG-3') and P2R (5'-cggCTCGAGGCGAGACTTGGCAGCTCCGGAT-3') were designed to amplify the expression sequence of NcGRA14 (747 bp). Then the product was cloned into the prokaryotic expression vector pET-28a (Novagen, Gibbstown, USA) and transformed into Escherichia coli. The recombinant NcGRA14 (rNcGRA14) fused to a His6-tag was expressed and purified using HisTrap FF purification columns (Novagen). The purified protein was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis using sera from mice immunized with Nc1 tachyzoites as previously described for Toxoplasma tachyzoites [10]. A polyclonal antibody against the rNcGRA14 recognized an approximate 45-kDa GRA14 protein (Fig. 2).

To confirm GRA localization, intracellular and extracellular immunofluorescence assay (IFA) was carried out as described previously [5]. For intracellular IFA, N. caninum tachyzoites were used to infect the human foreskin fibroblasts on coverslips for 16–24 h. The coverslips were fixed in 3.5% formaldehyde/phosphate buffered saline (PBS) for 15 min. The coverslips were then washed in PBS and blocked in PBS/3% bovine serum albumin (BSA). For complete permeabilization of formaldehyde-fixed samples, cells were permeabilized in a solution containing PBS/3% BSA/0.25% Triton X-100 for 30 min. Samples were then incubated with primary antibody (1:100) in PBS/3% BSA or PBS/3% BSA/0.25% Triton X-100 for 1 h. The samples were then washed five times in PBS and incubated with the fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Protein Tech Group, Chicago, USA) diluted 1:100 in PBS/3% BSA. For extracellular parasites IFA, N. caninum tachyzoites were harvested and purified. Then tachyzoites were resuspended in 200 μl of cold PBS, spotted on glass slides, air-dried, fixed, permeabilized and then processed for immunofluorescence as intracellular IFAs described above. All observations were performed on a confocal microscope (Leica TCS SP5 II; Leica, Solms, Germany). Immunofluorescence analysis showed that NcGRA14 protein existed in tachyzoites and secreted to the PV in 24 h (Fig. 3).

In conclusion, we present a novel secreted GRA protein in N. caninum that has not been reported, further work needs to be done in the future including (i) whether NcGRA14 protein is a diagnosis antigen or cross-reacting antigen in T. gondii? (ii) whether the NcGRA14 protein is a vaccine candidate? (iii) can overexpression or knockdown of GRA14 strains affect the invasion in vitro or in vivo?
Figure 1 Polypeptide sequence of NcGRA14 and alignment produced by ClustalW with the homologous protein in *T. gondii* (TgGRA14). The amino acid sequence of NcGRA14 protein contains a polypeptide of 404 amino acids. The putative signal peptide at the N-terminus was in red italics, whereas the blue italics indicated the potential transmembrane domain.

Figure 2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. (A) SDS-PAGE and Coomassie blue staining of the recombinant NcGRA14 protein from induced *Escherichia coli* lysates. Lane 1, whole *E. coli* lysates; lane 2, soluble proteins; and lane 3, inclusion bodies. Arrow indicates the position of the recombinant NcGRA14 protein. The molecular weight markers are shown on the right side. (B) The recombinant NcGRA14 protein was detected by western blot analysis.

Figure 3 Extracellular and intracellular staining of *Neospora caninum* tachyzoites. Indirect immunofluorescence assay performed on Nc1 extracellular parasites (A) or on human foreskin fibroblast (HFF) cells infected for 2 h (B), 24 h (C) with Nc1 strain parasites and incubated with the mouse serum anti-NcGRA14. Dynamic observation the GRA14 protein show that it traffics from anterior and posterior of the parasite to PV membrane shortly after 24 h invasion.
and (iv) what is the precise role of GRA protein in intracellular? These unresolved questions need to be answered in further study.

**Funding**

This work was supported by the grants from the National Special Research Programs for Non-Profit Trades (Agriculture) (200903036-08) and Research Fund for the Doctoral Program of Higher Education of China (20110008110006), and supported by the earmarked fund for Modern Agro-industry Technology Research System.

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