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NUCLEOCAPSID (NP) AND PHOSPHO-(P) PROTEINS OF NEWCASTLE DISEASE VIRUS: IDENTIFICATION OF REGIONS ON NP THAT FORM PARTICLES AND INTERACT WITH P

By

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May 2003

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The nucleocapsid protein (NP) of Newcastle disease virus (NDV) plays an important role in the replication of the viral genomic RNA. The NP is closely associated with the viral phosphoprotein (P) and this association is crucial in ensuring the specific binding of NP to the viral RNA. In order to have a better understanding of the structure and functions of the NP, deletion mutagenesis was carried out to characterise and localise regions involved in NP-NP and NP-P interactions.

The NP and a fusion derivative (NP_{cfus}) containing a hexa histidine tag at its C-terminus were produced abundantly in *Escherichia coli*. These proteins were fractionated on sucrose gradient centrifugation and microscopic analysis showed that both the NP and NP_{cfus} proteins self-assembled predominantly into ring-like particles with the diameter of 24 ± 2 nm around a central hole of 7 ± 1 nm. Some of these ring-like particles stacked together to form herringbone-like particles which are heterogenous in length with a diameter of 20 ± 2 nm

and a central hollow of 5 ± 1 nm. Fusion of the C-terminal end to 29 amino acids inclusive of the *myc* epitope and His-tag did not impair ring assembly but inhibit the formation of the long herringbone particles. Immunogold labelling of the ring-like particles with the anti-*myc* antibody showed that the C-terminus of the NP_{cfus} protein is exposed on the surface of the particles.

The essential subunit of NDV nucleocapsid is the NP, a polypeptide with 489 amino acids. In order to identify the contiguous sequence on NP that self-assembles into ring- or herringbone-like particles, a total of 11 N- or C-terminally deleted NP mutants were constructed and self-assembly studied in *E. coli* showed that a large part of the N-terminus of the NP encompassing amino acids 1 to 375, was required for the formation of herringbone-like particle. In contrast, the C-terminal end covering amino acids 376 to 489 was dispensable for the formation of this particle. Nevertheless, a region located between amino acids 376 to 439 may play a role in regulating the length of the herringbone-like particle.

As NP and some of its mutants assemble into particles, this feature was exploited to carry or display foreign peptides. Hepatitis B virus core antigen (HBcAg) and the C-terminal fragment of the N protein of Nipah virus were separately fused to a truncated NP protein, $NP_{\Delta C391}$, and expressed in *E. coli*. Antigenicity analysis of the chimeric proteins by using ELISA showed that the foreign peptide was at least partially exposed on the surface of the chimeric protein particles.

An *in vitro* binding assay was established to identify the regions on NP that interact with the P. A highly interactive region was located at the first 26 amino acids of the N-terminus of NP. The interaction between these two proteins remained strong even with the removal of 114 amino acids from the C-terminal end of NP. It is most likely that the last 49 amino acids of the NP might form another contact region for P, but is not as important as the N-terminal end.

As a whole, this study has provided a valuable insight into the structure of the NP as well as the delineation of its key functional regions. This knowledge will be useful for detailed exploration of the NP protein.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PROTEIN NUKLEOKAPSID (NP) DAN FOSFO (P) PENYAKIT SAMPAR AYAM: PENENTUAN BAHAGIAN NP YANG MEMBENTUK PARTIKEL DAN BERINTERAKSI DENGAN P

Oleh

KHO CHIEW LING

Mei 2003

Pengerusi: **Profesor Datin Khatijah Yusoff, Ph.D.**

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Protein nukleokapsid (NP) virus penyakit Sampar Ayam (NDV) memainkan peranan penting dalam replikasi RNA genomik virus. Protein NP berkait rapat dengan fosfoprotein (P) virus dan penyatuan ini adalah amat penting untuk memastikan protein NP hanya berikat secara spesifik kepada RNA virus. Untuk memahami struktur dan fungsi NP dengan lebih terperinci, mutagenesis potongan telah dijalankan dan mengenal pasti bahagian yang terlibat dalam interaksi NP-NP dan NP-P.

NP dan satu terbitan gabungan (NP_{cfus}) yang mengandungi tag enam histidin pada terminal C telah dihasilkan dengan banyak dalam *Escherichia coli*. Protein tersebut telah diasingkan dengan pengemparan kecerunan sukrosa. Analisis mikroskopik menunjukkan kedua-dua protein NP dan NP_{cfus} gabung sendiri membentuk partikel seperti cincin dengan diameter luaran berukuran 24 ± 2 nm mengelilingi satu lubang tengah yang berdiameter 7 ± 1 nm. Sesetengah partikel berbentuk cincin ini bertimbun dan membentuk

struktur menyerupai "herringbone" yang mempunyai saiz yang berlainan dengan diameter luaran berukuran 20 ± 2 nm dan lubang tengah yang berdiameter 5 ± 1 nm. Pencantuman 29 asid amino termasuk epitop *myc* dan tag-histidin pada terminal C protein NP tidak mengganggu pembentukkan struktur cincin tetapi menghalang pembentukkan struktur panjang "herringbone". Pelabelan imuno-emas partikel cincin dengan antibodi anti-*myc* menunjukkan terminal C protein NP_{cfus} adalah terdedah di permukaan partikel cincin ini.

Subunit utama nukleokapsid NDV adalah NP, polipeptida yang mengandungi 489 asid amino. Untuk menentukan turutan asid amino protein NP yang diperlukan bagi pembentukan struktur cincin dan "herringbone", sejumlah 11 mutan NP yang samada terminal N atau C-nya disingkirkan telah dibina dan kajian penyatuan dalam *E. coli* menunjukkan sebahagian besar terminal N protein NP yang merangkumi asid amino dari 1 hingga 375 adalah diperlukan bagi pembentukan struktur "herringbone". Sebaliknya, bahagian terminal C protein NP meliputi asid amino dari 376 ke 489 tidak diperlukan bagi pembentukan struktur ini. Namun begitu, kawasan yang terletak di antara asid amino 376 dan 439 mungkin memainkan peranan dalam mengawal saiz struktur "herringbone".

Oleh sebab NP dan sesetengah mutannya membentuk partikel, ciri ini telah dieksploit untuk membawa atau mempamerkan peptida asing. Antigen teras virus hepatitis B (HBcAg) dan fragmen terminal C daripada N protein

virus Nipah telah digabungkan kepada terminal C protein NP terpotong, $NP_{\Delta C391}$ dan diekspres dalam *E. coli*. Analisis keantigenan protein kacukan dengan menggunakan teknik ELISA menunjukkan sebahagian peptida asing terdedah pada permukaan partikel kacukan.

Satu asai pengikatan protein *in vitro* telah digunakan untuk mengenalpasti bahagian protein NP yang berinteraksi dengan protein P. Satu bahagian NP protein yang berinteraksi kuat dengan protein P telah dikenalpasti terletak pada permulaan 26 asid amino dari terminal N protein NP. Interaksi di antara kedua-dua protein ini tetap tinggi walaupun sejumlah 114 asid amino dari terminal C protein NP telah disingkirkan. Berkemungkinan besar 49 asid amino pada terminal C protein NP membentuk satu kawasan yang berinteraksi dengan P, tetapi ia tidak sepenting terminal N protein NP.

Secara keseluruhan, kajian ini telah membekalkan maklumat terperinci mengenai struktur NP dan juga bahagian berfungsi pada protein tersebut. Pengetahuan ini adalah amat berguna untuk mengkaji protein NP dengan lebih mendalam pada masa yang akan datang.

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I certify that an Examination Committee met on 13th of May 2003 to conduct the final examination of Kho Chiew Ling on her Doctor of Philosophy thesis entitled "Nucleocapsid (NP) and Phospho-(P) Proteins of Newcastle Disease Virus: Identification of Regions on NP that form Particles and Interact with P " in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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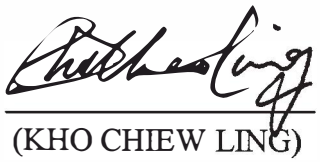
DECLARATION

I hereby declare that the thesis is based on my original work except for equations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



(KHO CHIEW LING)

Date: 30/5/2003

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ATP	adenosine triphosphate
BCP	1-bromo-3-chloro-propane
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Ci	curie
CITE	cap-independent translation enhancer
cpm	counts per minute
C-terminus	carboxy terminus
DEPC	diethylpyrocarbonate
DNA	deoxy-ribonucleic acid
DNase	deoxyribonuclease
DTT	1, 4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
h	hour
HBcAg	hepatitis B core antigen
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L	large protein

LB	Luria Bertani
µg	microgram
µl	microlitre
µM	micromolar
M	molar
mA	milliampere
min	minute
M_r	relative molecular mass
mRNA	messenger RNA
NBT	nitro blue tetrazolium
NDV	Newcastle disease virus
NEP-gel	sodium-phosphate-EDTA-gelatin buffer
ng	nanogram
nm	nanometre
NP	nucleocapsid protein
NP-40	nonidet P40
nt	nucleotide
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline

pH	*Puissance hydrogene*
PCR	polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
RNase	ribonuclease
rNTP	ribonucleoside triphosphate
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SDS	sodium dodecyl sulphate
SV5	simian virus 5
TAE	Tris-acetate-EDTA buffer
TBS	Tris-buffered saline
TEMED	tetramethyl ethylenediamine
U	unit
UV	ultraviolet
VLP	virus-like particle
vol	volume
VSV	vesicular stomatitis virus
w/v	weight/volume

CHAPTER 1

INTRODUCTION

Protein-protein interactions are involved in essentially all cellular processes. Many major research topics in biology such as DNA replication, transcription, translation, protein trafficking, cell cycle control, signal transduction, and intermediary metabolism are cellular events in which protein complexes have been implicated as essential components (Phizicky & Fields, 1995). Therefore, a comprehensive understanding of protein-protein interactions as well as the elucidation of their functional domains has become one of the major goals of modern biology. With the advent of recombinant DNA technology, the study of protein-protein interactions and subsequently the localisation of protein interaction domains have become more feasible than ever. Now, it is possible to clone virtually any genes of interest, and translate them into proteins by inserting the genes into appropriate expression systems. Using the approach of site-directed mutagenesis, the nucleotide sequence of a gene can then be modified to make any conceivable variant of the original protein. These techniques have launched the new technology of protein engineering, which has had a major impact on the study of protein structure, function and stability (Creighton, 1993).

Like many other negative-sensed RNA viruses, the RNA genome of Newcastle disease virus (NDV) encodes a core of three polypeptides common