Six-helix bundle assembly and characterization of heptad repeat regions from the F protein of Newcastle disease virus

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Paramyxoviruses may adopt a similar fusion mechanism to other enveloped viruses, in which an antiparallel six-helix bundle structure is formed post-fusion in the heptad repeat (HR) regions of the envelope fusion protein. In order to understand the fusion mechanism and identify fusion inhibitors of Newcastle disease virus (NDV), a member of the Paramyxoviridae family, we have developed an E. coli system that separately expresses the F protein HR1 and HR2 regions as GST fusion proteins. The purified cleaved HR1 and HR2 have subsequently been assembled into a stable six-helix bundle heterotrimer complex. Furthermore, both the GST fusion protein and the cleaved HR2 show virus–cell fusion inhibition activity (IC50 of 1.07–2.93 µM). The solubility of the GST–HR2 fusion protein is much higher than that of the corresponding peptide. Hence this provides a plausible method for large-scale production of HR peptides as virus fusion inhibitors.

Newcastle disease virus (NDV), one of the major animal pathogens for the poultry industry, is a member of the family Paramyxoviridae, which is composed of enveloped negative-stranded RNA viruses. The attachment and subsequent fusion lead the viral core into the cell. Fusion mediated by parvoviruses requires two glycoproteins, fusion (F) protein and haemagglutinin–neuraminidase (HN, or homologue H and G) protein (Lamb, 1993), though some viruses in the family, such as simian parainfluenza virus 5 (SV5) and respiratory syncytial virus (RSV), do not absolutely require HN (or its homologue) to participate in the fusion process (Horvath et al., 1992; Bagai & Lamb, 1995; Karron et al., 1997). The mechanism underlying this difference is not fully understood.

The F protein is synthesized initially as a precursor, F0, which is cleaved into a disulfide-linked heterodimer of F1 and F2 by a furin-like enzyme of the host cell (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). During the attachment and fusion process, the F protein undergoes conformational changes that expose the fusion peptide in F1 and results in the fusion peptide embedding in the host membrane, with or without the help of the HN protein. The F protein contains at least three heptad repeat regions (HR1, HR2 and HR3) (Young et al., 1997, 1999; Ghosh et al., 1998; Dutch et al., 1999; Matthews et al., 2000; Sergel et al., 2000). HR1 is located at the carboxyl terminus of the fusion peptide, while HR2 is located adjacent to the amino terminus of the transmembrane domain. Both HR1 and HR2 are important for fusion function, but the importance of HR3 is yet to be unravelled. Mutations of amino acids in each region of HR1 and HR2 decrease the fusion activity (Buckland et al., 1992; Sergel-Germano et al., 1994; Reitter et al., 1995; Sergel et al., 2001). Studies on enveloped viruses show that there are similar domains to HR1 (also called HR-A, N-peptide) and HR2 (also called HR-B, C-peptide) in many other enveloped viruses (see review by Bentz, 2000; Eckert & Kim, 2001).

Fusion of NDV does require both F and HN proteins. Previous studies on HR1 and HR2 of the NDV F protein indicated that HR2 could inhibit the F protein-mediated fusion (Young et al., 1997, 1999), whereas the inhibitive effect of HR1

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could only be seen if it was added to cells prior to the cleavage of the F protein in an in vitro transfection system, which represents an artificial state. Nonetheless it is not clear whether the HR1 and HR2 of NDV could form a heterotrimer (trimer of the HR1/HR2 heterodimer) and whether it adopts a similar fusion mechanism to other enveloped viruses because of its absolute requirement for HN protein in the fusion process. However, a recent crystal structure of F in the metastable state indicates the possible existence of different conformation states of the NDV F protein (Chen et al., 2001).

In this article, by using an E. coli expression system, we have expressed, purified and characterized HR1 and HR2 of the F proteins derived from a virulent and an avirulent NDV strain. The F gene clones of a Chinese virulent isolate, F48E9 (GenBank no. AF079172), and a Chinese avirulent isolate, Changchun (GenBank no. AF400614), were used in this study. The helical wheels of the HR1 (amino acids 137–198) and HR2 (amino acids 449–503) are depicted in Fig. 1(A). The HR regions for virulent and avirulent strains were named v-HR1, v-HR2 and a-HR1, a-HR2 respectively. Using the known HR1/HR2 structure from SV5 (sequence identities compared to NDV in the regions are 32.3% and 34.6% respectively, but the a and d sites of the helical wheel are highly conserved) as a template (Baker et al., 1999), the modelling program (Modeller, http://guitar.rockefeller.edu/modeller/modeller.html) yields a model of the NDV HR1/HR2 heterotrimer structure (Fig. 1B). This structure shows clearly an anti-parallel coiled coil and the same model has been obtained for a-HR1/HR2 and v-HR1/HR2, though there are three amino acid differences between v-HR1 and a-HR1 (S139A, N145K, N192K) and six amino acid differences between v-HR2 and a-HR2 (L451Q, V456I, W461D, D479N, S486R, G497S) in our
sequences used for the study. These different amino acids locate either in the outside part of the coiled coil, which would not have any effect on the overall structure (at b, c, g sites of the helical wheel for HR1; or c, e, f sites for HR2; but not at the important sites a and d), or would not be included in the model.

HR1 and HR2 DNA fragments of F gene from virulent and avirulent NDV which cover the amino acid regions of 136–198 for HR1 and 449–503 for HR2 were cloned into pGEX-6p-1 (Pharmacia) and expressed as GST (glutathione S-transferase) fusion proteins according to the manufacturer’s protocol. The expressed fusion proteins (Fig. 2A) were soluble in PBS and were easily purified in a glutathione–Sepharose affinity column (Pharmacia). The HR1 and HR2 proteins were subsequently cleaved from the fusion protein by PreScission protease (Pharmacia) and purified by a Sephasil C4 reverse phase column. Mass spectroscopy analysis (data not shown) indicated that the molecular masses of purified a-HR1, a-HR2, v-HR1 and v-HR2 were 7107, 6309, 7103 and 6261 Da respectively, close to the predicted molecular masses of 7128, 6333, 7107 and 6269 Da. For heterotrimer (trimer of the HR1/HR2 heterodimer) assembly of the purified HR1 and HR2, 50 μM of a-HR1 and a-HR2 or v-HR1 and v-HR2 or a-/v-HR1 and a-/v-HR2 combinations were mixed and incubated at room temperature for 1 h. The mixture was then loaded on a Superdex G75 (Pharmacia) column for gel filtration. One major peak of about 40 kDa was observed on the profile (Fig. 2B). The peak after SDS–PAGE in Tricine gels gave a protein band with a molecular mass of 40 kDa (Fig. 2C), which matches the molecular mass of a trimer of the HR1/HR2 heterodimer. The band disappeared in 2% SDS at 100 °C (into HR1 and HR2 monomer, indicating the existence of both HR1 and HR2 in the peak) but not in 2% SDS at room temperature (Fig. 2C). HR1 and HR2 derived from a combination of virulent and avirulent strains also yielded heterotrimer formation, i.e. a-HR1/v-HR2 or v-HR1/a-HR2 heterotrimers were observed.

In the circular dichroism (CD) analysis (Jasco J-715 spectrophotometer), 10 μg/ml of a-HR1 or v-HR1 had a CD spectrum exhibiting double minima at 208 and 222 nm, showing a characteristic α-helix (Fig. 3A). Their α-helix content was 33±1% and 36±1% respectively. The a-HR2 or v-HR2 appeared in a non-structured unfolded form (Fig. 3B). The heterotrimer of a-HR1/HR2 or v-HR1/HR2 showed obvious double minima and its α-helix content was more than 80% (Fig. 3C). A thermal stability test indicated that the heterotrimers of a-HR1/HR2 or v-HR1/HR2 were extremely stable and did not melt up to 98 °C (Fig. 3D). The α-helix of a-HR1 was more stable than v-HR1, as their melting points were different, a-HR1 at 50 °C and v-HR1 at 40 °C (Fig. 3E). Whether or not this difference has something to do with virulence needs to be addressed in a future study.

For the cell fusion assay, monolayers of Hela T4 cells were infected with virus (strain F48E9, kindly provided by the Chinese Institute of Animal Medicine Supervision) at 0 p.f.u. per cell for 1 h at 37 °C. The inoculum was removed and DMEM with 2% FCS was added to the cells with or without a series of dilutions of HR samples. The cells were scored for fusion at 24 h after incubation at 37 °C in a 5% CO₂ incubator. After staining with Giemsa, cell fusion was measured by polyclonal formation and recorded as the percentage of nuclei numbers in polyclonies to numbers of total nuclei. At least five random different fields under a light microscope were counted and the IC₅₀ values were calculated according to the Reed–Muench method (Welkos & O’Brien, 1994). Syncytium formation was inhibited by treatment with the GST fusion or free form of v-HR2 or a-HR2, while the syncytia were

Fig. 2. Protein expression SDS–PAGE gel (A), gel filtration profile of the HR1/HR2 heterotrimer complex (B) and the HR1/HR2 complex SDS–PAGE gel (C). (A) Proteins were run on a 12% SDS–PAGE. Lanes 1, protein marker (indicated in kDa); 2, control of BL21 (DE3) cell lysates; 3, GST alone; 4, a-HR1–GST; 5, a-HR2–GST; 6, v-HR1–GST; 7, v-HR2–GST. (B) A peak around 40 kDa was detected in the HR1/HR2 mixture in the gel filtration. The relative positions of the standard protein markers are indicated (kDa). (C) Profile of the 10% SDS–PAGE Tricine gel. Lanes 1, protein marker (indicated in kDa); 2, sample from 40 kDa peak but heated (100 °C) with 2% SDS, indicating the existence of both HR1 and HR2 in the peak.
normally formed in the untreated monolayers after infection with virus at 0.1 p.f.u. per cell (Fig. 4a). The IC$_{50}$ values (Fig. 4b, c) of free/GST-fused v-HR2 and a-HR2 were 1.07/1.16 µM and 1.14/1.25 µM respectively. The results indicated that HR2 has strong inhibition activity on virus-induced cell fusion.

For the plaque reduction assay, HR samples of a series of twofold dilutions together with appropriately diluted F48E9 virus were added to monolayers of chicken embryo fibroblast cells. After 1 h of incubation at 37 °C, the HR and inoculum were removed and overlaid with 2% white agar mixed at 1:1 with 2 × DMEM containing 4% FCS. Four days after overlaying, the cells covered with agar were stained with neutral red and the number of plaques was counted (Habel & Salzman, 1969). The IC$_{50}$ values were calculated using the Reed–Muench method (Welkos & O’Brien, 1994). The plaque reduction test showed that virus–cell fusion was inhibited by v-HR2 or a-HR2, while plaques were normally formed in untreated monolayers infected with 10$^{-6}$ p.f.u. of allantoic fluid virus (Fig. 4a). The IC$_{50}$ values of free/GST-fused v-HR2 and a-HR2 were 2.93/2.80 µM and 2.50/2.92 µM respectively (Fig. 4b, c). This indicated that HR2 is a strong inhibitor of NDV in virus plaque formation.

Under the same conditions, neither the GST fusion nor the free form of HR1 (a-HR1 or v-HR1) or the heterotrimer showed any inhibition activity in virus–cell fusion or virus plaque formation (Fig. 4).

At the molecular level, the details of virus–cell fusion mechanisms of paramyxovirus are largely unknown. Conformational changes of fusion proteins are probably involved in the fusion process (Lamb, 1993). A common post-fusion thermo-stable homologous HR1/HR2 heterotrimer structure has been observed in the fusion proteins of a number of enveloped viruses, e.g. retrovirus [including human and simian immunodeficiency viruses (HIV and SIV)], Ebola virus, influenza virus (see review by Bentz, 2000; Eckert & Kim, 2001). Moreover, the heterotrimer structures of the HR1/HR2 of the F proteins of paramyxoviruses SV5 and RSV have been unravelled lately (Baker et al., 1999; Zhao et al., 2000). A similar post-fusion structure has been proposed for the NDV F protein due to common characteristics of the HR1/HR2 region. Nevertheless, NDV and SV5 or RSV are fundamentally different in terms of fusion as HN protein is absolutely required for NDV, but not for SV5 or RSV. A recent crystal structure of NDV F$_{0}$ protein (Chen et al., 2001) in the metastable (prefusion) state has shown that its specific characters, in addition to the common features, could be compared to well-studied influenza virus (HA for influenza A, HEF for influenza C), e.g.

**Fig. 3.** CD spectra at 37 °C and measurement of thermal stability of HR1, HR2 and the HR1/HR2 complex. Both a-HR1 and v-HR1 showed a typical α-helix (A), but the a-HR2 and v-HR2 were unfolded (B). The complex of HR1 and HR2 gave an α-helix structure (C). Thermodynamic stability was measured at 222 nm from 25 °C to 98 °C with a 1 °C increase every minute and the HR1/HR2 complex showed extreme stability (D), but the HR1 melted at 40 °C for v-HR1, 50 °C for a-HR1 (E).
Fig. 4. Inhibition of cell fusion or plaque reduction. (a) Infected Hela T4 cells were treated with 12.5 µM of v-HR2 (A) or 12.5 µM of v-HR2–GST (C). Syncytia were obvious in the untreated cells (B). Infected chicken embryo blast cells were treated with 50 µM of v-HR2 (D) or 50 µM of v-HR2–GST (F). The plaques were obvious in the untreated cells (E). (b) Inhibition curves of cell fusion and plaque reduction by HR samples as indicated. As clearly showed, only HR2 or HR2–GST had an inhibition effect in all cases (curves G, H, I, J). (c) IC50 values of inhibition of fusion and plaque reduction calculated according to the Reed–Muench method (Welkos & O'Brien, 1994).
the central triple-stranded coiled coil in the NDV F protein and influenza HA is oriented in the opposite directions in these two molecules. Therefore, a generalized extrapolation of structural conformational changes might not be appropriate and more experimental data need to be accumulated. In this study, our data showed the assembly of such a heterotrimer complex for NDV. Our data also indicate that creating GST fusion proteins is an effective way of preparing virus envelope HR peptide inhibitors. Other HR-homologue inhibitors should be pursued in the same way for quick and cost-effective preparation. This is especially relevant to HIV fusion inhibition peptides, e.g. T-20 in clinical trials (Kilby et al., 1998).

Consistent with the previous report (Young et al., 1997), our study showed that HR2 gave strong inhibition activity against virus-cell fusion, while HR1 did not show any. More importantly, in our system the GST fusion HR2 showed a similar inhibition effect to free HR2. Young et al. (1999) did not see any fusion inhibition by HR1 but found that an amino acid change (F115G) at the F0 cleavage site (F0 into F1 and F2) in a system in which the F0 cleavage was controlled by externally added trypsin, could lead to the inhibition by HR1 of fusion mediated by F and HN proteins only if the HR1 was added before the F0 was cleaved by trypsin. As F0 is cleaved into F1 and F2 by a furin-like enzyme in the Golgi membrane and the virus surface F protein is processed into F1 and F2, these results may represent an artefact. It is noteworthy that all the other viruses whose HR1 or HR1 homologues show fusion inhibition do not have HN or HN homologues, e.g. HIV/SIV, Ebola virus. The HR1 derived from paramyxovirus SV5 does show some fusion inhibition (Joshi et al., 1998), whereas HR1 derived from other paramyxoviruses, e.g. Sendai virus and measles virus, does not show any (Rapaport et al., 1995; Wild & Buckland, 1997). HR1 proteins derived from all those paramyxoviruses whose fusion absolutely requires HN protein do not appear to have any fusion inhibition, whereas the HR1 from SV5, whose fusion does not absolutely require HN protein, shows the fusion inhibition effect. Therefore it is possible that HN protein (or its homologues) plays an important role in the HR1/HR2 domain exposure in the pre-hairpin state of the F protein. This could be due to either the direct steric constraint of HN protein for the HR1/HR2 accessibility or HN-induced conformational change of the F protein during the fusion process if HN is absolutely required. This should be addressed in the future by testing HR1 inhibition of more paramyxoviruses, e.g. by including more HN (or homologue)-independent and -dependent viruses, and of NDV F protein mutants (Sergel et al., 2000) whose fusion does not require HN protein.

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References


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