Chimeric pneumovirus nucleocapsid (N) proteins allow identification of amino acids essential for the function of the respiratory syncytial virus N protein

H. L. Stokes,† A. J. Easton and A. C. Marriott

University of Warwick, Department of Biological Sciences, Coventry CV4 7AL, UK

The nucleocapsid (N) protein of the pneumovirus respiratory syncytial virus (RSV) is a major structural protein which encapsidates the RNA genome and is essential for replication and transcription of the RSV genome. The N protein of the related virus pneumonia virus of mice (PVM) is functionally unable to replace the RSV N protein in a minigenome replication assay. Using chimeric proteins, in which the immediate C-terminal part of the RSV N protein was replaced with the equivalent region of the PVM N protein, it was shown that six amino acid residues near the C terminus of the N protein (between residues 352–369) are essential for its function in replication and for the ability of the N protein to bind to the viral phosphoprotein, P.

The nucleocapsid (N) proteins of the pneumoviruses (family Paramyxoviridae, genus Pneumovirus) are the major structural proteins involved in encapsidation of the RNA genome and are essential for replication and transcription of the genome (Collins et al., 1996; Yu et al., 1995; Yunus et al., 1998). The N proteins of human respiratory syncytial virus (RSV) and bovine RSV (bRSV), expressed individually in insect cells, retain the ability to form filamentous nucleocapsid-like structures in the absence of other viral proteins or viral RNA (Meric et al., 1994; Samal et al., 1993). This suggests that the N protein is a major determinant of the structure of the functional virus nucleocapsid complex. The N–RNA complexes purified from insect cells expressing the RSV N protein are morphologically distinct from those of other members of the Paramyxoviridae (Bhella et al., 2002). Within RSV-infected cells, the N protein associates with the P (phosphoprotein) and M2-1 proteins to form cytoplasmic inclusions, believed to be the site of virus replication (Garcia et al., 1993b). The minimal protein complement required for replication is the N, P and L proteins; the M2-1 protein is required in addition for efficient transcription (Collins et al., 1996; Grosfeld et al., 1995; Yu et al., 1995). The interaction of the RSV N and P proteins has been well documented (Garcia-Barreno et al., 1996; Khatat et al., 2001; Samal et al., 1993; Slack & Easton, 1998) and the C terminus of the P protein has been demonstrated to be the most important site for binding to the N protein (Garcia-Barreno et al., 1996; Hengst & Kiefer, 2000; Slack & Easton, 1998). Human and bovine RSV N proteins are 93% identical at the amino acid level and are both 391 residues in length. Regions of the N protein involved in binding to the P protein have been variously mapped to residues 46–65, 241–260 and 305–335 or 334–352 for human RSV (Garcia-Barreno et al., 1996; Murphy et al., 2003; Murray et al., 2001), and to two regions, 244–290 and 338–364, for bRSV (Khatat et al., 2000). These studies made use of deleted N proteins or synthetic peptides. Unlike other paramyxovirus N proteins, the N proteins of pneumoviruses do not possess a hypervariable C-terminal tail required for interaction with the polymerase (Lamb & Kolakofsky, 2001). However, the invariant motif FX4YX4SYAMG seen in paramyxovirus N proteins can be aligned with an equally well conserved LX4((F/C)X4SVVLG motif (residues 312–326) in pneumovirus N proteins (Barr et al., 1991). This motif is thought to function in nucleocapsid assembly (Lamb & Kolakofsky, 2001). A recent study suggests that a protein containing only residues 1–91 of the N protein is able to bind to RNA and form nucleocapsid-like structures (Murphy et al., 2003).

The N protein can be assayed for its function in RNA replication, transcription and encapsidation using a minigenome replication assay, in which plasmids encoding the minigenome and the N, P, L and M2-1 proteins are transfected into an appropriate cell line (Marriott & Easton, 1999). An attempt to map functionally important regions on the bRSV N protein by means of deletions found that all internal deletions resulted in inactivity, and deletion of as little as two residues from the N terminus or one residue from the C terminus was sufficient to reduce activity by 95–98% (Khatat et al., 2000). This suggests that the structural integrity of the N protein is compromised by deletions, resulting in a loss of the active conformation. An alternative approach to deletion mapping of a protein is to substitute cognate regions from a homologous protein to...
generate a chimeric protein, which is much more likely to retain an active conformation than a deleted form of the protein. In this report we describe the use of chimeric proteins to identify a region of the RSV N protein essential for its template activity, as well as binding to the P protein.

Pneumonia virus of mice (PVM), a member of the genus Pneumovirus, causes a severe respiratory infection in laboratory mouse colonies (Horsfall & Hahn, 1939, 1940). PVM, or a serologically related virus, is also widespread in the human population (Pringle & Eglin, 1986). The N protein of PVM shows 60% identity to the RSV N protein, with a highly conserved region (residues 245–315) showing 96% identity (Barr et al., 1991) (Fig. 1). Analysis of deletion mutants of the PVM N protein suggested that a region between residues 309 and 381 was important in binding to the PVM P protein (Barr & Easton, 1995). A series of chimeric plasmids were constructed in which the C-terminal part of the RSV N gene ORF was replaced with the equivalent region of the PVM N gene ORF. Joins were in regions of exact identity between the protein sequences in order to maintain protein integrity (Fig. 1, underlined) and were achieved by ligating together PCR products with Hgal restriction sites at the position of the join (Hgal is an enzyme that cleaves to one side of its recognition site such that the recognition site is removed from the ligated product). Details of plasmid constructions are available from the authors upon request. Western blot analysis showed that each plasmid directed production of a protein of the expected size, 43 kDa, when transfected into cells expressing T7 RNA polymerase (data not shown).

Functionality of the N protein chimeras was analysed using the RSV plasmid-based minigenome system, as described previously (Marriott et al., 1999, 2001). The minigenome used contains the CAT reporter gene flanked by the minimal cis-acting RSV genomic termini (minigenome RS/RS in Marriott et al., 2001). ORFs for the N, P, L and M2-1 proteins were inserted into the vector pTM1 (Moss et al., 1990) or a modified derivative of pTM1. All constructs were transcribed from T7 promoters after transfection into HEP-2 cells, which had been infected with 1 p.f.u per cell of the T7 RNA polymerase-expressing vaccinia virus, vTF7-3 (Fuerst et al., 1986). Addition of plasmids encoding the

Fig. 1. Alignment of the RSV and PVM N proteins. The PVM strain 15 N protein sequence is aligned with that of RSV strain RSS-2. Shaded areas show regions of amino acid identity. Junctions in chimeric N proteins used in this study are underlined. Chimeric proteins are identified by the C-terminal residue corresponding to the RSV sequence.
N, P, L and M2-1 proteins of RSV (strain RSS-2) to cells along with the RS/RS minigenome resulted in replication and transcription of the minigenome, and hence production of the CAT protein (Fig. 2a, lane 1). Omission of the L plasmid abolished replication and transcription (Fig. 2a, lane 3). Replacing the RSV N plasmid with a PVM N plasmid resulted in an undetectable level of CAT expression (Fig. 2a, lane 2). Chimeric N plasmids RPN223, RPN293, RPN333 and RPN351 were equally as inactive as the PVM N plasmid when substituted for the RSV N plasmid. However, chimera RPN374 showed activity in terms of CAT protein production (Fig. 2a, lane 4) and also replication of minigenome RNA (Fig. 2b, lane 4). Replicated RNA was detected using a positive-sense riboprobe following digestion with micrococcal nuclease: replication products encapsidated with the N protein are resistant to digestion. CAT production averaged 32% of that obtained when the equivalent amount of the RSV N plasmid was used. It is not clear whether this reduction is due to an effect on replication or transcription, or both, since, in the assay used, replication and transcription are coupled, and the Northern blot assay used was not quantitative. In chimera RPN374, the C-terminal 17 residues of the RSV N protein are replaced by the C-terminal 19 residues of the PVM N protein (Fig. 1). The resulting chimeric protein is clearly able to function in replication, transcription and encapsidation of the RSV minigenome. Chimera RPN351 is inactive in replication, suggesting that a functionally critical region is located between residues 352 and 374 of the RSV N protein. Furthermore, 17 of 23 amino acids in this region are identical between the RSV and the PVM proteins, such that the non-functional chimera RPN351 has only six amino acid changes from the functional chimera RPN374 (residues 352, 356, 359, 361, 367 and 369).

The ability of the chimeric N proteins to interact with the RSV P protein was tested by co-immune precipitation from transfected cells using a P-specific monoclonal antibody (021/2P; Garcia et al., 1993a). Whereas anti-P antibody was able to co-precipitate RSV N protein and RPN374 protein, along with the P protein, the other chimeric N proteins were not precipitated (Fig. 3). This suggests that the six amino acid changes from chimera RPN374 to RPN351 are also responsible for disrupting the interaction between the N and the P proteins.

Previous studies on the RSV N protein using deletion mutations have shown that most deletions result in non-functional N proteins, at least in terms of ability to bind the P protein (Hengst & Kiefer, 2000; Slack & Easton, 1998). A deletion of 39 residues from the N protein C terminus was shown to retain its ability to co-precipitate the P protein, although this mutant lost its ability to form cytoplasmic inclusions when expressed in HEp-2 cells. Larger deletions resulted in loss of interaction with N-specific antibodies, suggesting a failure to fold properly (Garcia-Barreno et al., 1996). The only study to date in which the template functions of the N protein were assayed showed that deletions as small as two amino acids could ablate the function of the bRSV N protein (Khattar et al., 2000). Point mutations made to the C-terminal leucine residue (to Ala, Gly, Lys or Glu) also destroyed N protein function. The strategy of making chimeric proteins presented in this report shows that the C terminus of the PVM N protein can functionally replace the equivalent region of the RSV N protein, with this peptide segment contributing to maintaining the overall structural integrity of the N protein.

The bRSV N protein retains the ability to co-precipitate P...
protein and to encapsidate RNA, with the C-terminal 27 residues deleted, despite being inactive in replication (Khattar et al., 2000). Deletion of a further single amino acid abolished both P protein binding and RNA encapsidation. This suggests that residues 365–391 are dispensable for P protein binding. If this is applicable to human RSV N protein, this would further delineate the residues critical for binding to P protein, to $A^{352}, Q^{356}, E^{359}$ and $G^{361}$.

The ability of the N protein to encapsidate RNA into nuclease-resistant nucleocapsids was not addressed directly in this study. Encapsulation appears to be relatively non-specific in terms of RNA sequence (Meric et al., 2001) and cellular RNA is encapsidated in the absence of other viral proteins (Meric et al., 1994). By analogy with Sendai virus, it is hypothesized that the P protein enables the N protein to encapsidate the viral genome specifically (Curran et al., 1995). Chimeric RPN374 N protein is clearly able to encapsidate and protect the viral minigenome (Fig. 2b, lane 4), but encapsidation in the absence of replication was below the level of reliable detection in our blots. However, a longer exposure of the blot in Fig. 2(c) did suggest that the RPN223 protein is also able to encapsidate RNA (Fig. 2c). The smear of hybridizing material seen in Fig. 2(c, lane 8) may represent partial loss of nuclease-resistance by the RPN293 protein.

Loss of ability of the N protein to support replication in this study maps to the same six amino acid changes as loss of binding to the P protein, suggesting that the N–P interaction is more important to RSV replication than the N–RNA interaction. Although this hypothesis requires further investigation, this would imply that reagents able to interfere with N–P binding may be highly inhibitory to RSV replication and thus be potential therapeutic reagents for RSV respiratory disease.

We suggest that the strategy of producing chimeric proteins from homologous, structurally related proteins is a useful one for mapping functional residues or regions on a protein, which is less likely to be affected by incorrect folding than the traditional deletion approach. We, and others, have applied the chimeric protein approach recently to analyse the functional domains of the RSV M2-1 transcriptional enhancer protein (unpublished data; Zhou et al., 2003).

ACKNOWLEDGEMENTS

We would like to thank Dr J. Melero for the gift of monoclonal antibodies against the P protein. H. L. S. was supported by a research studentship from the Medical Research Council.

REFERENCES


Grosfeld, H., Hill, M. G. & Collins, P. L. (1995). RNA replication by respiratory syncytial virus (RSV) is directed by the N, P and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. J Virol 69, 5677–5686.


