Epitope mapping of human respiratory syncytial virus 22K transcription antitermination factor: role of N-terminal sequences in protein folding

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The reactivity of a panel of 12 monoclonal antibodies raised against the human respiratory syncytial virus 22 kDa (22K) protein was tested by Western blotting with a set of 22K deletion mutants. The results obtained identified sequences in the C-terminal half of the 22K polypeptide required for integrity of most antibody epitopes, except for epitope 112, which was lost in mutants with short N-terminal deletions. This antibody, in contrast to the others, failed to immunoprecipitate the native 22K protein, indicating that the N terminus of this protein is buried in the native molecule and exposed only under the denaturing conditions of Western blotting. In addition, N-terminal deletions that abolished reactivity with monoclonal antibody 112 also inhibited phosphorylation of the 22K protein previously identified at Ser-58 and Ser-61, suggesting that the N terminus is important in regulating the 22K protein phosphorylation status, most likely as a result of its requirement for protein folding.

The 22K protein is encoded by the first open reading frame (ORF1) of the M2 gene, which is unique to viruses of the subfamily Pneumovirinae. It was originally proposed as a second matrix protein based on solubility properties (Huang et al., 1985) and thus it was renamed M2-1. However, the 22K protein associates with characteristic cytoplasmic inclusions found in HRSV-infected cells that also contain at least N and P as other viral components (García et al., 1993). In agreement with this subcellular localization, it has been reported that the 22K protein acts as a transcription elongation factor, allowing the synthesis of full-length mRNAs (Collins et al., 1996). In addition, the 22K protein enhances read-through transcription at gene junctions to generate polycistrionic RNAs (Hardy & Wertz, 1998). It is possible that both effects reflect a transcription antitermination activity of the 22K protein (Fears & Collins, 1999) that functions by a presently unknown mechanism, although it has been reported that the 22K protein interacts with the P protein (Mason et al., 2003) and that it binds RNA (Cuesta et al., 2000) without sequence specificity (Cartee & Wertz, 2001).

The 22K protein exists in at least two different isoforms identified by one-dimensional SDS-PAGE. The slower-migrating 24 kDa form is phosphorylated at Ser-58 and
Ser-61 and is the most abundant isoform when expressed in the absence of other HRSV components (Cartee & Wertz, 2001). In contrast, the faster-migrating 22 kDa isofrom is not phosphorylated and is the most abundantly expressed species in HRSV-infected cells.

The 22K sequence has a Cys3-His1 motif (C-X7-C-X5-C-X3-H) spanning aa 7–25 that has been proposed to bind zinc (Hardy & Wertz, 2000), by analogy to a similar motif found in the transcription factor Nup475 (Worthington et al., 1996). Critical residues in this motif are essential for protein function in a minireplicon system (Hardy & Wertz, 2000; Zhou et al., 2003) and for virus infectivity (Tang et al., 2001).

To gain structural information about the 22K protein, two sets of specific monoclonal antibodies (mAbs) were prepared: (i) mAbs 22K1 to 22K7 (Fig. 1a) were obtained from BALB/c mice immunized with affinity-purified 22K protein expressed in HRSV-infected HEp-2 cells; and (ii) mAbs 65, 112, 129 and 312 (Fig. 1a) were obtained from BALB/c mice inoculated with a GST–22K chimera expressed in bacteria.

Antibody specificities were confirmed by immunoprecipitation of the 22K protein synthesized in a cell-free system (Fig. 1a). Most antibodies precipitated a 22K doublet containing a major 24 kDa band and a minor 22 kDa band, which were the main translation products; in addition, some antibodies (in particular 22K3, 37M2, 65 and 129) precipitated a 19 kDa band whose origin is not known but which is also found in HRSV-infected cells (not shown). The only exception was mAb 112, which did not immunoprecipitate either the 22 kDa doublet or the 19 kDa band, although its specificity for the 22K protein was confirmed by Western blotting (see below). To rule out incorrect folding of the in vitro-synthesized protein or some other effect of the cell-free system, mAb 112 was used to immunoprecipitate radiolabelled 22K protein from HRSV-infected cell extracts. As shown in Fig. 1(b), the 22K protein was precipitated efficiently by other mAbs (shown only for 37M2 and 22K4) but only traces of this protein were immunoprecipitated with mAb 112. It should be noted that the cell-free synthesized protein was mainly in the 24 kDa isoform (Fig. 1a), whereas the majority of the protein synthesized in infected cells was in the 22 kDa isoform (Fig. 1b). Thus, epitope 112 was not accessible for antibody binding in the native structure of either isoform.

To map the antibody epitopes in the 22K primary structure, a set of 22K deletion mutants was prepared. The ORF1 sequence of the M2 gene (Long strain) was amplified by PCR from the previously described plasmid L22K (Garcia et al., 1993) and inserted into the pGEM-4 vector (pGEM/M2-1) under the control of a T7 promoter. A series of deletion mutants was constructed (Fig. 2a) and used for transfection of HEp-2 cells previously infected with the vaccinia recombinant vTF-7.3 (Fuerst et al., 1986). Expression of the mutant proteins was monitored by Western blotting with a polyclonal rabbit antiserum raised against purified 22K protein (serum z22K; Fig. 2b). Specific bands that were absent in untransfected cells (Fig. 2b, Mock) were observed in extracts of cells transfected with each of the plasmids. The electrophoretic mobility of these bands reflected the extent of the deletions. Differences in band intensity may reflect variability of transfection efficiencies.
with different plasmids, or different expression levels or stability of each mutant protein.

The same cell extracts were tested for reactivity with individual mAbs. Fig. 2(b) shows representative results obtained with mAbs 37M2, 22K4, 312 and 112. mAb 37M2 reacted with all the different mutant proteins except Δ111–166 and ΔC6. mAb 22K4 did not react with mutants ΔC6, Δ111–166 and ΔC6. mAb 312 lacked reactivity with ΔC46 and ΔC6. These results indicated that epitopes 37M2, 22K4 and 312 were located in the C-terminal half of the 22K primary structure, although they did not include the C terminus of the protein since the three antibodies reacted with ΔC6. In contrast, mAb 112 reacted with all C-terminally truncated mutants but did not react with ΔN2–7 and ΔN2–67, indicating that N-terminal residues of the 22K polypeptide were essential for the integrity of the 112 epitope.

A summary of the Western blot results obtained with all the mAbs is presented in Fig. 2(a), except for mAbs 22K2 and 22K6, which reacted poorly in this assay and whose
pattern of reactivity could not be assigned unambiguously. Four reactivity patterns were discerned. The pattern observed with mAb 37M2 was reproduced with mAbs 22K3, 65 and 129. A second pattern was shared by mAbs 22K1, 22K4, 22K5 and 22K7. Finally, mAbs 312 and 112 each generated a unique reactivity pattern.

These results allowed us to locate tentatively the epitopes recognized by the different antibodies in the 22K protein primary structure (Fig. 2b). Since antibody reactivity was evaluated by Western blotting, we assumed that all epitopes were linear and that lack of reactivity of a given antibody with certain mutants indicated that some or all of the deleted amino acids were part of the epitope. With these considerations, epitope 37M2 (and 22K3, 65 and 129) was placed between residues 128 and 148 of the 22K polypeptide. Epitope 22K4 (and 22K1, 22K5 and 22K7) was located in the segment encompassing residues 148 to 166. Epitope 312 was located between residues 166 and 188. Finally, epitope 112 was placed between residues 2 and 6.

The results of Figs 1 and 2 led us to conclude that epitope 112 is buried in the 22K molecule but exposed under the denaturing conditions of Western blotting. Thus, we tried to refine the sequence requirements of epitope 112 and relate them to folding of the native 22K protein. Phosphorylation of the 22K protein is influenced not only by mutations at the sites of phosphorylation (Ser-58 and Ser-61) but also by changes in relevant amino acids distantly located in the protein primary structure (Hardy & Wertz, 2000). Thus, phosphorylation can be used as a test for correct folding of the 22K protein.

The 24 kDa isomer of the 22K protein was expressed and labelled with $^{32}$Porthophosphate in HEp-2 cells infected with vaccinia vTF7-3 and transfected with the plasmid pGEM/M2-1 (Fig. 3a, wt). Identity of this band was confirmed by immunoprecipitation using a pool of anti-22K mAbs (Fig. 3b). In contrast, an analogous $^{32}$P-labelled band could not be detected in extracts from cells transfected with the plasmid carrying the $\Delta2$–7 mutation either before (Fig. 3a) or after (Fig. 3b) immunoprecipitation. However, both proteins were expressed to comparable levels, as assessed by Western blotting with a pool of anti-22K mAbs (Fig. 3c). Identity of the $\Delta2$–7 mutant was confirmed by lack of reactivity with mAb 112 in a Western blot (Fig. 3d). Thus, the $\Delta2$–7 mutation prevented phosphorylation of the 22K protein, probably as a consequence of improper folding. In contrast to the $\Delta2$–7 mutation, elimination of an equivalent number of amino acids at the C terminus ($\Delta$C6) had no effect on expression or phosphorylation of the 22K protein (Fig. 3).

Next, another set of mutants was made that eliminated aa 2 ($\Delta2$), aa 2–4 ($\Delta2$–4) or aa 2–6 ($\Delta2$–6) of the 22K protein; in addition, a point mutation Ser2Ala (S2A) was introduced into the 22K protein to rule out the possibility of phosphorylation of the wild-type protein at this residue (although this was reported not to occur by Carter & Wertz, 2001), which could account for lack of phosphorylation of the $\Delta2$–7 mutant. All of these mutants were phosphorylated when expressed in transfected HEp-2 cells (Fig. 3a) and were immunoprecipitated with the antibody pool (Fig. 3b), except for mutant $\Delta2$–6, which could not be labelled with $^{32}$P, despite being expressed at levels similar to those of other mutants (Fig. 3c). Reactivity of the new mutants in Western blots with mAb 112 (Fig. 3d) also allowed us to refine the sequence requirements of this epitope. Elimination of aa 2–4 partially inhibited reactivity with mAb 112 and the $\Delta2$–6 mutation inhibited mAb 112 reactivity completely.

In conclusion, the results presented here indicate that epitope 112 is not exposed in the native 22K protein. Since most linear epitopes generally span six to ten contiguous amino acids, epitope 112 could include aa 5–6 to 12–16 of the 22K polypeptide and one or two of the first cysteines in the Cys$_2$–His$_3$ motif. While the structure of this motif is not known for the 22K protein, the partial structure of the analogous motif in the transcriptional factor Nup475 has
been determined by multidimensional nuclear magnetic resonance (Worthington et al., 1996). In this case, a zinc ion is coordinated by the three cysteines and the histidine. Interestingly, amino acids located N-terminal to the Cys3-His1 motif in Nup475 interact with the His residue, indicating a very compact local structure for this part of the protein. By analogy, the lack of reactivity of mAb 112 with the native 22K protein, and the influence of N-terminal amino acids (aa 4–6) in the folding of this molecule, could be explained by formation of a similar compact structure around the Cys3-His1 motif of the 22K protein.

Since all antibodies analysed in this study, with the exception of 112, recognized epitopes located in the C-terminal half of the 22K protein and they could immunoprecipitate the native molecule, it is likely that a substantial part of the C-terminal half of the 22K protein is exposed and thus accessible to antibodies. This is consistent with results reported by Tang et al. (2001) and Zhou et al. (2003) indicating greater flexibility of the C-terminal half of the protein for sequence changes than the N-terminal 30 aa. The antibodies described here may prove valuable tools to study structural aspects of the 22K protein, interactions of this molecule with viral and cellular partners, and the mechanism of action of this transcription antiterminator.

Acknowledgements

This study has been carried out with financial support from the Commission of the European Communities, specific RTD programme ‘Quality of Life and Management of Living Resources’, QLK2-CT2001-01225, ‘Towards the design of new potent antiviral drugs: structure function analysis of Paramyxoviridae RNA polymerase’. It does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.

References


