Abstract

Rotavirus is the most important agent of viral gastroenteritis in humans and animals and is responsible for about 600,000 deaths per annum. Most of these deaths occur in the developing countries and India is estimated to account for about a quarter of these deaths.

Rotavirus nonstructural protein, NSP5 is encoded by genome segment 11 and is 200 amino acids in length. Phosphorylation generates different isoforms of NSP5 with molecular masses ranging from 26-34 kDa. Segment 11 also codes for another nonstructural protein, NSP6 from an out-of-phase reading frame (compared to NSP5) which is 92 amino acids in length. Yeast two hybrid analysis has indicated that NSP5 interacts with NSP6 and the interaction domain was mapped to the C-terminal 35 amino acids in NSP5. Along with NSP5, another nonstructural protein, NSP2, is localized in the viroplasm of infected cells. It is a basic protein possessing RNA-binding activity. Co-expression of NSP2 and NSP5 in uninfected cells generates viroplasm-like structures and results in hyperphosphorylation of NSP5. Towards understanding the functional interaction of NSP5, NSP2 and NSP6, we have generated a series of deletion and amino acid substitution mutants of these proteins and identified the mutual interacting regions using yeast two-hybrid and pull-down assays. Our results indicate that the region from position 190 and 195 at the C-terminus is important for dimerization/oligomerization of NSP5. Further, while the amino acid substitution mutants NSP5ΔC5 Q195A, NSP5ΔC5 QQ191-195AA maintained the ability to interact with wild type full length NSP5, the self-interaction of NSP5ΔC5 Q195A is severely affected, suggesting that substitution of a single conserved residue between position 190 and 195 affects dimerization of the protein. NSP5 C-terminal deletion mutants □C10, □C25, □C35 and □C70 showed highly reduced or negligible interaction with full-length NSP5, but they exhibited a level of interaction comparable to that exhibited by full-length NSP5 in homotypic interaction assays.

Yeast two-hybrid analysis of NSP2 and NSP5 interaction suggests that 3 regions in NSP2 are involved in interaction with NSP5: N-terminal region of about 24 amino acids, C-terminal region of 34 amino acids and a major internal site spanning amino acid positions 85 to 114. Yeast two-hybrid analysis of NSP5-NSP6 interaction revealed that the region between amino acids 5 and 10 from the C-terminus of NSP5 is important for NSP6 interaction. Results also indicate that the N-terminal region between amino acid position 34 and 55 of NSP6 is critical for NSP5 interaction.

Since NSP5 is a major component of viroplasm structures, we investigated the oligomeric status of NSP5 by gel filtration, glycerol gradient fractionation, dynamic light scattering (DLS) and glutaraldehyde cross-linking. The full length NSP5 protein and mutants having deletions up to 70 amino acids from the C-terminus (□C70) existed as a high molecular weight complex in solution as

suggested by the migration of these proteins in 8% native PAGE, size exclusion chromatography and glycerol gradient centrifugation. DLS data suggests that these multimeric proteins are of homogenous species and not non-specific aggregates. Trypsin proteolytic analysis of NSP5, NSP5ΔC70 and NSP5ΔC95 showed that NSP5ΔC95 is extremely sensitive to trypsin digestion and was degraded completely within 5 min of incubation at 37°C suggesting it to be highly unstructured.

Since NSP5 occurs in multiple phosphorylated forms in infected cells, investigations were carried out to identify the putative kinase (or kinases) involved in phosphorylation of NSP5. We attempted to address this issue through *in vitro* phosphorylation of NSP5 in cell lysates treated with specific kinase inhibitors as well as phosphorylation of NSP5 in immune complexes of specific cellular kinases. Our results indicate that rotavirus NSP5 purified from *E. coli* can be phosphorylated by cellular kinase/s *in vitro* but is not a suitable substrate for hyperphosphorylation. Full length NSP5 containing a GST, MBP or His tag at its N-terminus was phosphorylated but only in the presence of mammalian cell extracts. However, we could not detect phosphorylation of untagged NSP5, suggesting that a tag at the N-terminus is necessary for phosphorylation *in vitro*. Among all the deletions tested □C5, □C10, □C25 and □C35 and the full-length proteins were phosphorylated to a comparable extent, but there was a drastic reduction in the level of phosphorylation of □C70, □C95 and □N150. NSP5 phosphorylation was enhanced by NSP2□N85 and NSP2□C203. Results also demonstrate that GST-NSP6□C34□can up regulate NSP5 phosphorylation. Investigations were carried out to identify the different cellular protein kinases that might participate in the phosphorylation of NSP5 by examining the effect of various kinase inhibitors on NSP5 phosphorylation. Analysis of the inhibition of NSP5 phosphorylation did not reveal involvement of a specific kinase but it appears that many kinases may be involved in phosphorylation of NSP5. Among all the inhibitors tested, U0126 was found to be the most potent inhibitor suggesting the importance of MEK1/MEK2 in phosphorylation of NSP5.

Results indicate that components of MAPK pathway kinases, as well as pathways involving PKA, CKII and PI3K-like kinases are involved in NSP5 phosphorylation. Several cellular kinases such as MEK1/2, ERK1/2, mTOR, MSK1, CKI, CKII, MNK1 and AKT were observed to phosphorylate NSP5 *in vitro*. In line with the prediction of several putative sites for many kinases, our results suggest that NSP5 is a target for several kinases in the infected cell.