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Identification of nucleocapsid protein residues required for Sendai virus nucleocapsid formation and genome replication

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Alanine substitution mutations in the Sendai virus nucleocapsid (NP) protein have defined highly conserved hydrophobic and charged residues from amino acids (aa) 362 to 371 that are essential for function of the protein in RNA replication. Mutant NP362, which had the change F362A, was incapable of supporting *in vitro* RNA replication. NP362 expressed alone formed extended oligomers which exhibited an abnormal morphology and density suggesting that these particles were not associated with any RNA. Mutant NP364, which had changes L362A and G365A, was also inactive in RNA replication; however, this was because the protein was unstable and did not form NP–NP complexes. Mutant NP370 mutant, which had changes K370A and D371A, was inactive in *in vitro* replication, although it could form the required NP_0–P and NP–NP protein complexes. The self-assembled nucleocapsid-like particles formed by NP370 alone had a morphology like that of wild-type NP and banded in CsCl as ribonucleoprotein particles, suggesting that they contained cellular RNA. These data suggest that the replication defect of NP370 may be in the ability to specifically encapsidate Sendai virus genome RNA. Mutant NP373, where nonconserved charged residues at aa 373 and 375 were substituted with alanine, gave a wild-type phenotype. Thus these amino acids are not required for either protein–protein interactions or *in vitro* Sendai virus RNA replication.

Introduction

Sendai virus is a member of the family *Paramyxoviridae* and has a single-stranded, negative-sense (−) RNA genome 15 384 nucleotides (nt) in length. Based on the rule of six (Calain & Roux, 1993), the genome of Sendai virus is encapsidated by 2564 molecules of the NP protein [524 amino acids (aa)] forming the nucleocapsid (NC). The viral RNA-dependent RNA polymerase, consisting of the large (L, 2228 aa) and phosphoprotein (P, 568 aa) subunits, is associated with the nucleocapsid in the virion. The RNA polymerase utilizes the nucleocapsid and not naked RNA as the template for both transcription and replication of the genome RNA (for a review see Kingsbury, 1991). Encapsidation by NP is coupled to viral RNA replication and all replicative products are resistant to nuclease. In the current model for encapsidation (Lamb & Kolakofsky, 1996), there is an initial sequence-specific binding of NP to viral leader RNA, followed by the cooperative assembly of NP on the growing chain, presumably through nonspecific NP–RNA interactions. This model predicts that there should be a specific encapsidation signal in the nascent genomic RNA and a specific RNA-binding site on NP, as well as perhaps less-specific RNA-binding domain(s) leading to the final NP–RNA assembled complex. Finally, there is also an NP–NP-binding domain. For the rhabdovirus vesicular stomatitis virus (VSV) it has been shown that the binding affinity of the nucleocapsid protein for (+) strand leader RNA is approximately 10 times greater than for nonspecific RNA (Blumberg *et al.*, 1983), and that the encapsidation signal is in the 3′-terminal 15–17 nt of the (+) strand leader RNA (Blumberg, 1983; Moyer *et al.*, 1991; Smallwood & Moyer, 1993). The RNA-binding domain(s) in the nucleocapsid protein has not been identified.

Both Sendai and measles virus (MV) nucleocapsid proteins, as well as the N protein of VSV, have been shown to self-assemble into nucleocapsid-like particles when they are expressed alone, thus providing support for an NP–NP interaction (Buchholz *et al.*, 1993, 1994; Spehner *et al.*, 1991; Fooks *et al.*, 1993, Sprague *et al.*, 1983). In addition to the

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NP–RNA and NP–NP complexes, an NP_0–P interaction occurs (Homann *et al.*, 1991), where P protein is thought to act as a chaperone for NP, preventing its self-assembly (Curran *et al.*, 1995). The soluble NP_0–P complex is required for genome replication *in vitro* (Horikami *et al.*, 1992) as it is the substrate for encapsidation, and it is believed that the P protein is released once NP binds to the nascent RNA. An NP_0–P complex has also been demonstrated for other paramyxoviruses, including MV (Huber *et al.*, 1991), respiratory syncytial virus (RSV) (Garcia *et al.*, 1993), parainfluenza virus (PIV) types 2 and 3 (Nishio *et al.*, 1996; Zhao & Banerjee, 1995), simian virus 5 (Precious *et al.*, 1995), as well as for the rhabdoviruses, VSV and rabies virus (Davis *et al.*, 1986; Peluso & Moyer, 1988; Fu *et al.*, 1994; Chenik *et al.*, 1994).

Individual domains in the Sendai virus NP protein for the various interactions have not been specifically mapped, but previous studies using deletion analysis have shown that the first 400 aa of NP were required for both self-assembly and activity in RNA replication *in vitro* (Buchholz *et al.*, 1993; Curran *et al.*, 1993). Protein blotting analysis suggested that the first 400 aa were also required for the formation of the soluble NP_0–P complex (Homann *et al.*, 1991; Buchholz *et al.*, 1994). To identify specific domains in NP, we have analysed mutants generated by alanine substitution for their ability to form various protein–protein complexes and for biological activity in viral RNA replication. This approach has enabled us to identify specific amino acids required for NP–RNA binding and for the self-assembly of nucleocapsid-like particles.

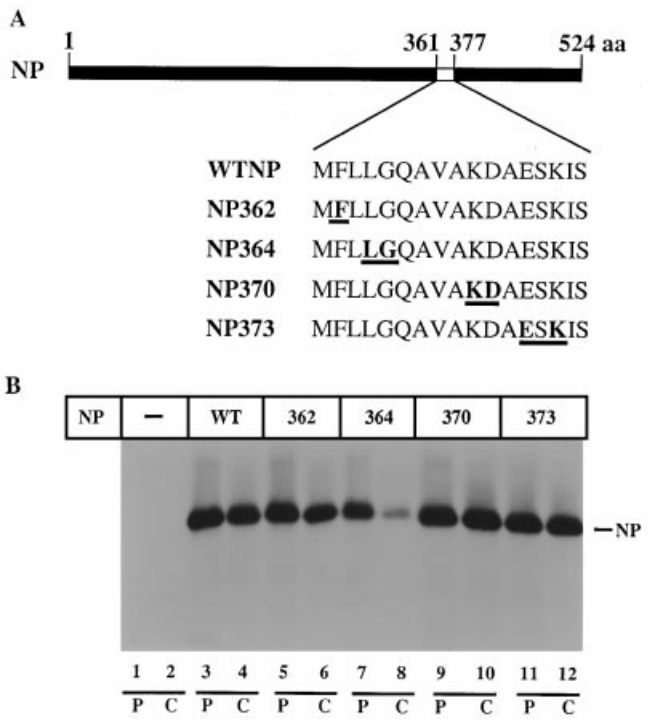

Fig. 1. Schematic of the NP mutants and pulse–chase analysis of the proteins. (A) The sequence from aa 361–377 of the NP protein is shown. Ala was substituted for either one or two residues of NP (bold and underlined amino acids) as shown. (B) VVT7-infected A549 cells were transfected in duplicate with no plasmids (–), the wt (WT) or mutant NP plasmids as indicated. At 5·5 h p.t. the cells were labelled for 30 min with Tran^{35}S-label and cytoplasmic cell extracts prepared immediately (pulse, P) or at 16 h p.t. following a chase (C) as described in Methods. NP was immunoprecipitated with α-SV antibody and analysed by SDS–PAGE. The position of the NP protein is indicated.

Methods

■ **Cells, viruses and plasmids.** Wild-type (wt) Sendai virus and the Sendai virus defective interfering particle, DI-H (Harris strain), were propagated in the allantoic fluid of 9-day-old embryonated chicken eggs and purified as described previously (Horikami *et al.*, 1992). Recombinant vaccinia virus containing the gene for phage T7 RNA polymerase (VVT7) (Fuerst *et al.*, 1986) was grown in Vero cells. Protein and RNA synthesis were performed in human A549 cells (ATCC). The plasmids pGEM-NP, pGEM-P/C and pGEM-L were described previously (Curran *et al.*, 1991). The plasmid pTM1-GST–P containing the glutathione *S*-transferase (GST) gene fused in-frame to the 5′ end of the Sendai virus P gene (GST–P) was described previously (Chandrika *et al.*, 1995*a*). All of the viral genes were cloned downstream of the phage T7 promoter.

■ **Construction of NP mutants.** Alanine substitution mutagenesis targeted aa 362–375 of NP, generating four mutants, NP362, NP364, NP370 and NP373, shown in Fig. 1. PCR was used to create the mutants using two overlapping complementary mutagenic oligodeoxynucleotide primers and two standard outside oligodeoxynucleotide primers (Table 1) as described previously (Higuchi *et al.*, 1988). The PCR products were digested with *Bst*XI and *Eco*RV and subcloned into these sites (nt 833–1339) in pGEM-NP. For screening of mutant clones, each mutagenic primer introduced a new silent restriction site as shown in Table 1. The mutation in each clone was confirmed by double-stranded dideoxy sequencing of the entire subcloned fragment.

■ ***In vitro* RNA synthesis.** Subconfluent A549 cells in 60 mm dishes were infected with VVT7 at an m.o.i. of 2·5 p.f.u. per cell and transfected in duplicate with wt or mutant pGEM-NP (2 μg), pGEM-P/C (5 μg) and pGEM-L (0·5 μg) plasmids at 37 °C with lipofectin (Gibco-BRL). At 18 h post-transfection (p.t.) cytoplasmic cell extracts were prepared by lysolecithin permeabilization at 4 °C, and DI-H RNA replication was assayed *in vitro* with the addition of [α-^{32}P]CTP and purified detergent-disrupted (dd) DI-H (2·2 μg) as described previously (Horikami *et al.*, 1992), except that the actinomycin D concentration was raised to 20 μg/ml. The nuclease-resistant nucleocapsid products were purified by banding on CsCl gradients, the RNA extracted, analysed by gel electrophoresis and the dried gels were exposed to Kodak X-Omat film. The replication products were quantified on a phosphorimager (Molecular Dynamics). Samples (10%) of the extracts were analysed by immunoblot using a rabbit anti-Sendai virus (α-SV) primary antibody (Carlsen *et al.*, 1985) and an alkaline phosphatase-conjugated secondary antibody and developed with NBT–BCIP.

■ **Protein analysis.** VVT7-infected A549 cells were transfected with the wt or mutant NP plasmids (2 μg) and labelled with Tran^{35}S-label (ICN; 66 μCi/ml) at 5·5 h p.t. for 30 min in methionine- and cysteine-free medium. Cell extracts were prepared immediately (pulse, P), or following a chase (C), in medium containing 10-fold excess methionine and cysteine, for various times as indicated in the figure legends. For overnight labelling, the cells were labelled in medium containing 0·1 × unlabelled methionine and cysteine. Cell extracts were prepared at 4 °C in 300 μl reaction mix salts (RM salts: 0·1 M HEPES, pH 8·5, 0·05 M NH_4Cl, 7 mM KCl and 4·5 mM magnesium acetate) containing 0·25% NP40 and 1 μg/ml aprotinin. The lysate was clarified at 13 000 r.p.m. for

Table 1. Oligodeoxynucleotide primers

The plus and minus symbols in parentheses following the primer number refer to the messenger (+) or genomic (−) sense of the oligodeoxynucleotide and the sequences are presented 5′ → 3′. The restriction enzyme sites used for screening are indicated (right) and are underlined in the sequence.

Mutant	Mutagenic primers	Enzyme
NP362	SM291(+)-GGAAATGGCCTTACTTGGCCAAGCC	*Bal*I
	SM294(−)-GGCTTGGCCAAGTAAGGCCATTTCC	
NP364	SM290(+)-GTTCTTAGCAGCACAAGCCGTG	*Bso*FI
	SM293(−)-CGGCTTGTGCTGCTAAGAAC	
NP370	SM209(+)-CGTGGCCGCGGCTGCTGAATCG	*Sac*II
	SM208(−)-CAGCAGCCGCGGCCACGGCTTG	
NP373	SM289(+)-GATGCTGCTAGCGCGATCACGAGTGCC	*Nhe*I
	SM292(−)-CGTGATCGCGCTAGCAGCATCC	
Mutant	**Standard outside primers**	
NP370	SM180(+)-GCCATGGCTTACAGTAG	
	SM089(−)-CCCCTAGCGTCCTGGTCC	
NP362, NP364 and NP373	SM090(+)-GGTTGAGACCCTTGTGAC	
	SM089(−)-CCCCTAGCGTCCTGGTCC	

30 min at 4 °C to remove the nuclei. For immunoprecipitation, samples of the ^{35}S-labelled supernatant were incubated with α-SV antibody (1 μl) and the antigen–antibody complex was collected with inactivated *Staphylococcus aureus*, Cowan strain, as described previously (Carlsen *et al.*, 1985). For analysis of NP_0–P complex formation, VVT7-infected cells were transfected with pGEM-NP (2 μg) alone or together with pTM1-GST–P (1 μg) or with pTM1-GST–P (1 μg) alone. The cells were labelled overnight, cytoplasmic cell extracts were prepared and samples (75 μl) immunoprecipitated as above. For bead-binding ^{35}S-labelled extracts (75 μl) were incubated with glutathione–Sepharose 4B beads (Pharmacia Biotech) according to the manufacturer's protocol and the immunoprecipitated and bound proteins analysed by 9% SDS–PAGE.

■ **Self-assembly of NP proteins.** To measure the self-assembly of the NP protein, cells transfected with the wt or mutant pGEM-NP (2 μg) were labelled with Tran^{35}S-label as described in the figure legends. Cell extracts were separately analysed by sedimentation on either 5 ml CsCl step-gradients [layered from the bottom: 1·4 ml 40% (w/w) CsCl, 1·4 ml 30% (w/w) CsCl, 1·4 ml 20% (w/w) CsCl, 0·6 ml 30% (v/v) glycerol in 10 mM HEPES, pH 8·5] in an SW55 rotor at 36000 r.p.m. for 16 h at 4 °C (Buchholz *et al.*, 1993) or on 12 ml linear 20–40% (w/w) CsCl gradients in TNE (25 mM Tris–HCl, pH 7·5, 50 mM NaCl and 2 mM EDTA) in an SW41 rotor at 36000 r.p.m. for 16 h at 4 °C. Fractions (0·7 ml for the step gradients and 1 ml for the linear gradients) were collected from the top with the pellet resuspended in the last fraction and the densities determined with a refractometer. Samples of each fraction were immunoprecipitated with α-SV antibody and the proteins analysed by 9% SDS–PAGE. The NP band in each fraction was quantified on a phosphorimager.

■ **Electron microscopy.** For electron microscopy of the self-assembled proteins, VVT7-infected A549 cells were transfected with the NP, NP362 and NP370 plasmids. After incubation for 20 h the unlabelled cell extracts were fractionated by centrifugation through 30% (v/v) glycerol (5 ml) in an SW55 rotor at 50000 r.p.m. for 90 min at 4 °C. The pellets containing the assembled particles were collected in 75 μl ET buffer (1 mM Tris–HCl, pH 7·5, and 1 mM EDTA) and visualized by negative staining with 2% uranyl acetate by the Electron Microscopy Core Laboratory in the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Results

Effect of mutagenesis on protein stability

Based on the amino acid alignments of paramyxovirus NP proteins (Miyahara *et al.*, 1992), we selected for site-directed mutagenesis the region between aa 362 and 375 of the Sendai virus NP protein (Fig. 1), which includes residues conserved in 10 or more of 13 different NP proteins. We targeted the conserved hydrophobic residues (aa 362, 364 and 365), two conserved charged residues (aa 370 and 371) and two unconserved, but adjacent charged residues (aa 373 and 375, Fig. 1A). To try to decrease the chance of disrupting the secondary structure of the protein (Cunningham & Wells, 1989; Bass *et al.*, 1991), the targeted amino acids were changed to alanine (Ala). The expression and stability of each of the NP mutants was determined using pulse–chase analysis. VVT7-infected cells were transfected with each of the NP plasmids, pulse-labelled for 30 min and extracts prepared immediately (pulse, P) or following a 16 h chase (C). Immunoprecipitation analysis showed that wild-type (wt) NP protein was synthesized in the pulse and was stable to the chase (Fig. 1, lanes 3 and 4, respectively), as were the mutants NP362, NP370, and NP373 (Fig. 1, lanes 5 and 6, 9 and 10, 11 and 12, respectively). In contrast, the NP364 protein was synthesized but was not stable to the overnight chase (Fig. 1, lanes 7 and

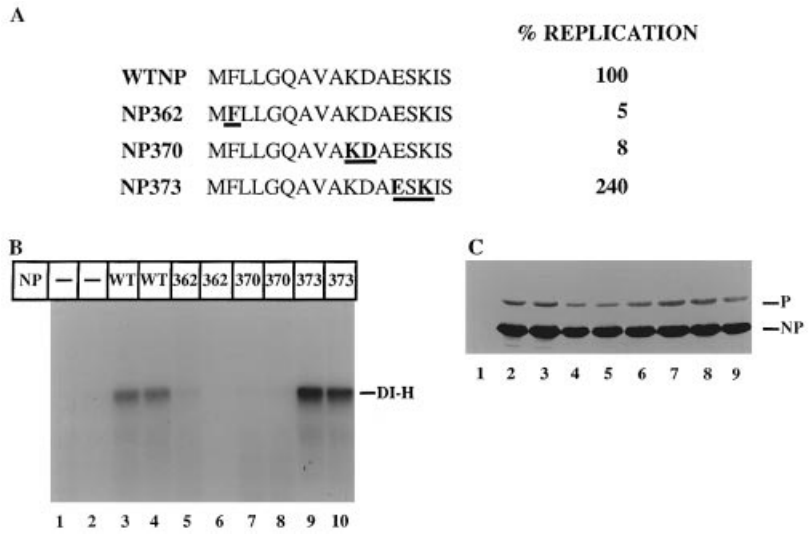

Fig. 2. *In vitro* DI-H RNA replication with the mutant NP proteins. (A) The sequence from aa 361–377 of the wt and mutant NP proteins is shown as described in Fig. 1. (B) VVT7-infected A549 cells were transfected with no plasmids (–) or cotransfected with the P and L plasmids together with the indicated wt or mutant NP plasmids in duplicate. Cytoplasmic cell extracts were incubated with dd DI-H in the presence of [α-^{32}P]CTP, nuclease-resistant nucleocapsid products purified and the RNA extracted and analysed by gel electrophoresis as described in Methods. The position of DI-H RNA is indicated. The amount of product in three experiments was quantified on a phosphorimager and calculated relative to wt NP designated as 100% (A), where the values varied by <5%. (C) Immunoblot analysis on samples of the cytoplasmic cell extracts with α-SV antibody, where the positions of the P and NP proteins are indicated. One mock sample was lost so the lanes are mock (1), wt NP (2 and 3), NP362 (4 and 5), NP370 (6 and 7) and NP373 (8 and 9).

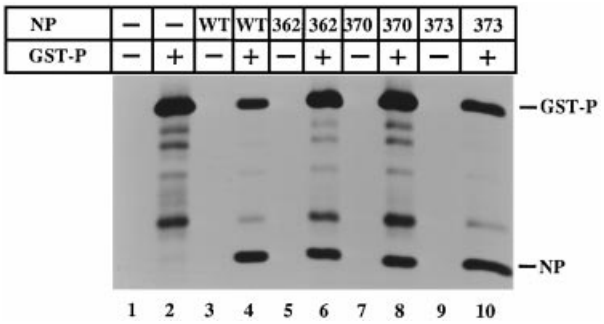

Fig. 3. Cobinding of the mutant NP and GST–P proteins to glutathione–Sepharose beads. VVT7-infected A549 cells were transfected with the mutant NP plasmids alone or together with GST–P plasmid, or with the GST–P plasmid alone as indicated. The cells were labelled with Tran^{35}S-label overnight, cell extracts prepared, incubated with glutathione–Sepharose beads and the bound proteins analysed by SDS–PAGE as described in Methods. The positions of the NP and GST–P proteins are indicated.

8). A time-course experiment showed that NP364 was stable only for about 1·5 h following the pulse (data not shown). The instability of this mutant has limited detailed characterization of this protein; however, in one subsequent experiment an extract harvested after a 1·5 h chase was used.

Activity of the NP mutants in RNA replication *in vitro*

The stable mutants, NP362, NP370 and NP373, were assayed for biological activity in supporting DI-H genome replication *in vitro*. Cytoplasmic extracts of cells expressing each of the NP proteins together with the L and P polymerase proteins were prepared in duplicate and incubated with purified dd DI-H in the presence of [α-^{32}P]CTP. The nuclease-resistant replication products were then banded on CsCl gradients, and the RNA extracted and analysed by gel electrophoresis. This assay measures one round of RNA synthesis and encapsidation from the template (Curran *et al.*, 1993). *In vitro* DI-H RNA replication occurred with the wt NP protein (Fig. 2B, lanes 3 and 4), and no replication occurred in the absence of viral proteins (Fig. 2B, lanes 1 and 2) as expected. Replication with both NP362 and NP370 was significantly inhibited (by 95–98%) (Fig. 2A, B, lanes 5–8), while in contrast, the level of replication with NP373 was more than 2-fold greater (Fig. 2B, lanes 9 and 10) than that of wt NP protein. Immunoblot analysis on a portion of the cell extracts showed that the NP and P proteins were all similarly expressed (Fig. 2C, lanes 2–9), showing that NP protein was not limiting in the inactive mutants. Thus the conserved Phe at aa 362, and Lys and Asp at aa 370 and 371, were all essential for the biological function of NP, while the nonconserved Glu and Lys at aa 373 and 375 in NP373 were not. Since disruption of any one of the protein–protein or protein–RNA interactions required for RNA replication could be responsible for the defective RNA synthesis phenotypes, the individual interactions of each NP mutant were tested.

NP_0–P complex formation is preserved in the NP mutants

An essential protein–protein interaction needed for RNA replication is the formation of the NP_0–P complex which is used for encapsidation of newly synthesized RNA (Horikami *et al.*, 1992). Using a fusion protein (GST–P) between glutathione S-transferase (GST) and the P protein, the formation of an NP_0–P complex can be measured by the cobinding of the NP protein with the GST–P protein to glutathione beads (Myers

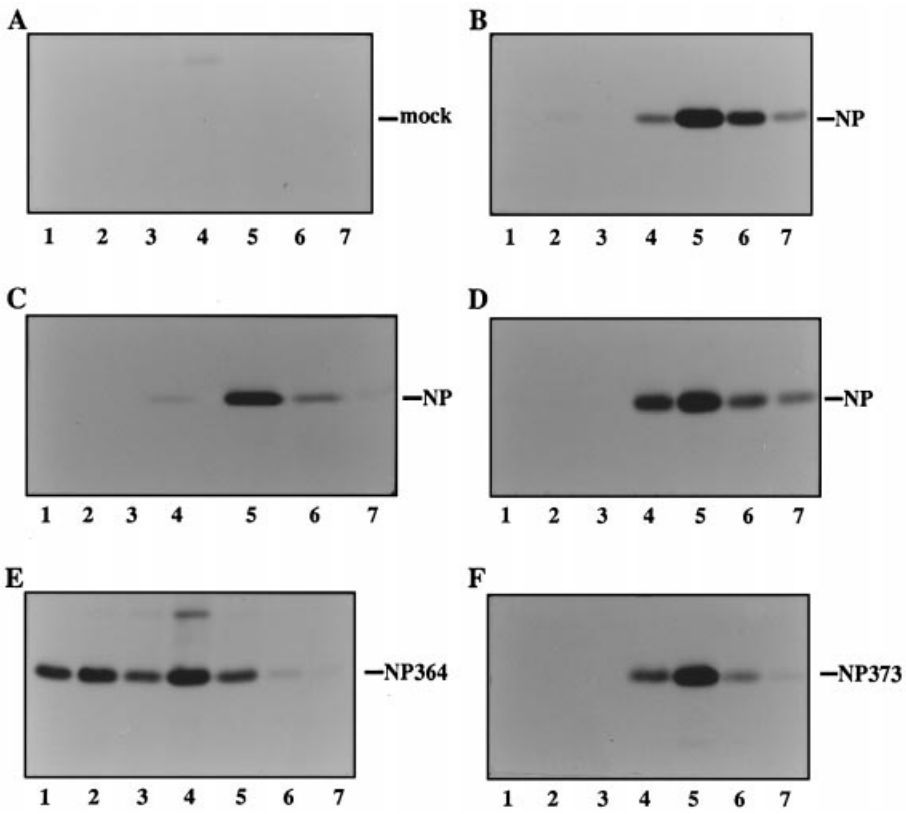

Fig. 4. CsCl step-gradient centrifugation of self-assembled wt and mutant NP proteins. VVT7-infected A549 cells were transfected with no plasmids (mock), wt NP, NP364 or NP373 plasmids as indicated. At 5·5 h p.t. the cells expressing NP or NP364 were pulsed with Tran^{35}S-label for 30 min and extracts prepared immediately (B) or following a 1·5 h (C, E) or 18 h (D) chase. Alternatively, mock-transfected and cells expressing NP373 were labelled overnight (A, F). Extracts were analysed separately on 5 ml CsCl step-gradients, samples of fractions were immunoprecipitated with α-SV antibody and analysed by SDS–PAGE. Sedimentation was from left to right and the positions of the wt and mutant NP proteins are indicated.

& Moyer, 1997). Radiolabelled mutant NP proteins were expressed alone or together with GST–P from plasmids, and immunoprecipitation of a portion of the cell extracts showed that each of the proteins was expressed (data not shown). As a positive control for bead-binding, the GST–P protein expressed alone bound to the beads (Fig. 3, lane 2), where the additional bands are apparently truncated or proteolysis products of GST–P that retained the GST moiety and thus bound beads. The wt NP and each of the mutants all cobound to beads in the presence of GST–P (Fig. 3, even-numbered lanes), but not in the absence of GST–P (Fig. 3, odd-numbered lanes). We previously showed that NP does not bind beads when coexpressed with GST, so complex formation is specific for the P portion of the fusion protein (Myers & Moyer, 1997). These data show that each NP mutant, even the ones defective for RNA replication, formed a complex with the P protein. In addition, NP370 was shown to cosediment with P protein as a soluble complex on a glycerol gradient (data not shown).

Self-assembly is altered in the NP mutants

The NP protein was previously shown to self-assemble into nucleocapsid-like particles which were identical in density and morphology, although not in length, to authentic nucleocapsids from virus-infected cells (Buchholz *et al*., 1993). To evaluate the ability of the mutant NP proteins to self-assemble, cell extracts expressing each protein were initially examined by banding on either step (Fig. 4) or linear (Fig. 5) CsCl gradients. Since the NP364 protein was unstable (Fig. 1) and the time required for self-assembly of the wt NP protein had not been established, pulse–chase assembly experiments were performed. Wt NP protein was expressed in triplicate, the cells were pulsed with Tran^{35}S-label and cytoplasmic cell extracts were prepared immediately (pulse) or following a 1·5 or 18 h chase. The proteins were banded on separate CsCl step-gradients, immunoprecipitated, and separated by SDS–PAGE as described in Methods. The step gradients are less precise in differentiating intermediates with close densities, but allow an overall distribution to be determined. In the mock extract, a few VVT7 proteins reacted nonspecifically with the α-SV antibody (Fig. 4A) which are seen also in Fig. 4(E). NP protein labelled just in the pulse was found primarily in fraction 5 at a density of 1·30 g/cm^3 (Fig. 4B), a density expected for ribonucleoprotein particles (nucleocapsids), demonstrating that self-assembly was quite rapid. As expected, the self-assembled

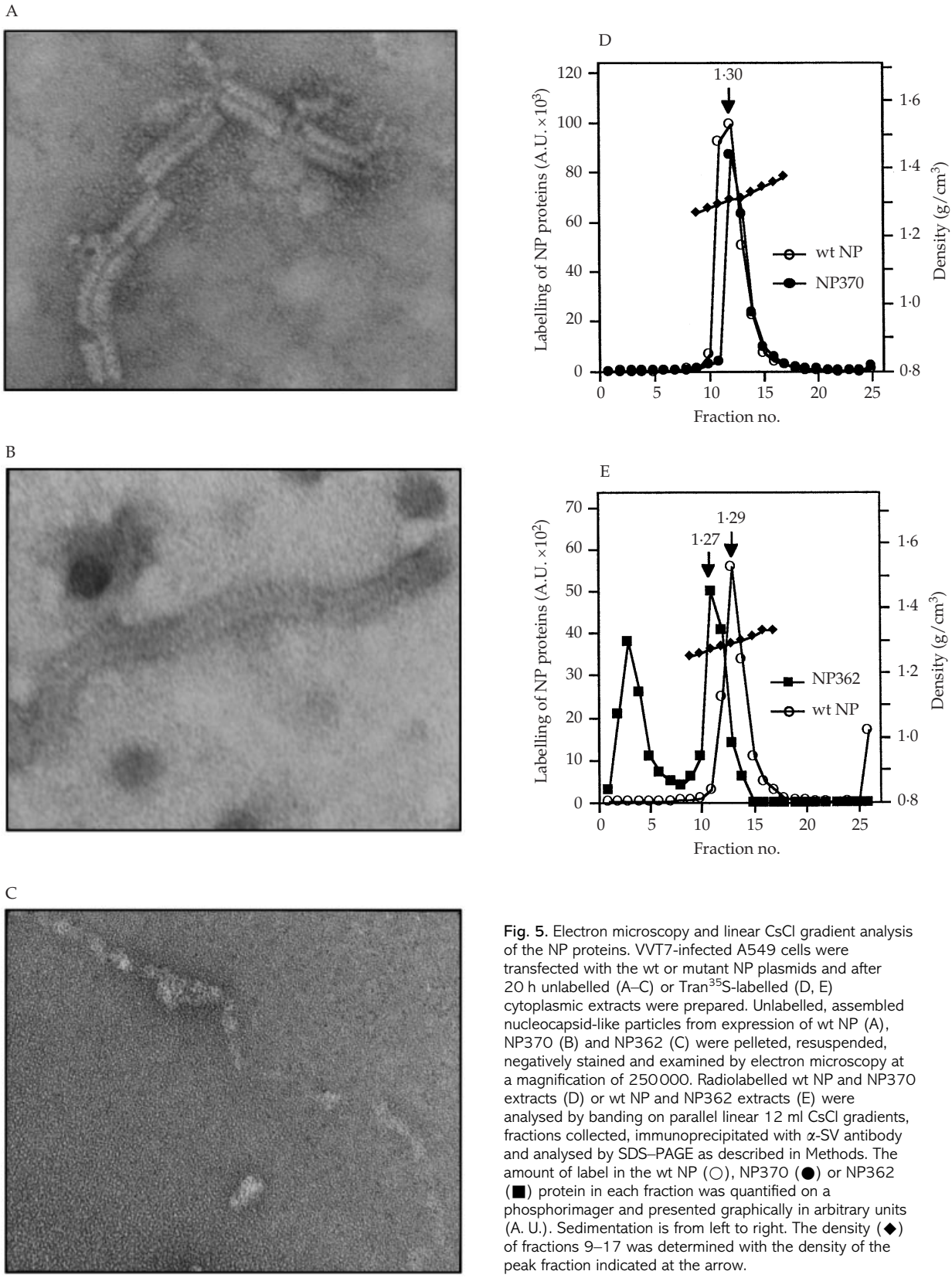

Fig. 5. Electron microscopy and linear CsCl gradient analysis of the NP proteins. VVT7-infected A549 cells were transfected with the wt or mutant NP plasmids and after 20 h unlabelled (A–C) or Tran^{35}S-labelled (D, E) cytoplasmic extracts were prepared. Unlabelled, assembled nucleocapsid-like particles from expression of wt NP (A), NP370 (B) and NP362 (C) were pelleted, resuspended, negatively stained and examined by electron microscopy at a magnification of 250000. Radiolabelled wt NP and NP370 extracts (D) or wt NP and NP362 extracts (E) were analysed by banding on parallel linear 12 ml CsCl gradients, fractions collected, immunoprecipitated with α-SV antibody and analysed by SDS–PAGE as described in Methods. The amount of label in the wt NP (○), NP370 (●) or NP362 (■) protein in each fraction was quantified on a phosphorimager and presented graphically in arbitrary units (A. U.). Sedimentation is from left to right. The density (◆) of fractions 9–17 was determined with the density of the peak fraction indicated at the arrow.

NP protein was stable for both the 1·5 h and 18 h chase (Figs 4 C and 5 D, respectively). The NP364 protein, pulsed and then chased for just 1·5 h, in contrast to wt NP, was found throughout fractions 1–5 (Fig. 4 E), suggesting that it was not able to form organized particles, although some limited NP–NP interaction apparently did occur since there was a portion of faster sedimenting protein. Like self-assembled wt NP, NP373 was found primarily in fraction 5 (Fig. 4 F), demonstrating that this mutant also formed nucleocapsid-like particles. This was expected since this mutant exhibited wt biological activity (Fig. 2).

To analyse the structure of the inactive mutants, NP362 and NP370, as well as wt NP, each was expressed alone and the assembled particles were pelleted through glycerol. The particles were negatively stained and examined by electron microscopy (EM). The NP370 protein self-assembled into nucleocapsid-like particles similar in morphology to self-assembled wt NP nucleocapsid-like particles (Fig. 5 B and 5 A, respectively) and to nucleocapsids found in extracts of Sendai virus-infected cells (data not shown). In contrast, the NP362 protein formed unusual oligomers (Fig. 5 C) which were smaller in diameter and not uniform, exhibiting more globular or aggregated arrays of protein. The lengths of the individual NP362 oligomers were heterogeneous, as was also seen in wt NP nucleocapsid-like particles (Fig. 5 A; Buchholz *et al*., 1993, Spehner *et al*., 1991). Clearly, NP362 has an abnormal NP–NP interaction which readily explains the inability of this mutant to support RNA replication.

Previous reports examining the expression of Sendai virus NP and MV N in mammalian cells suggested that the self-assembled nucleocapsid-like particles contained RNA (Buchholz *et al*., 1993; Spehner *et al*., 1991, 1997). In contrast, MV N nucleocapsid-like particles synthesized in insect cells did not appear to contain RNA as determined by their lighter buoyant density (Fooks *et al*., 1993). Based on the structures seen by electron microscopy we would predict that the NP370 particles, but not the NP362 oligomers, are associated with RNA. To test this prediction radiolabelled wt and mutant NP proteins were expressed separately and analysed by banding on linear CsCl gradients which allows a definitive separation of possible assembled forms. The proteins in each gradient fraction were immunoprecipitated, separated by SDS–PAGE, and the NP bands quantified and represented graphically with respect to density as described in Methods.

Both wt NP and NP370 nucleocapsid-like particles had densities of 1·29–1·30 g/cm^3 (Fig. 5 D, E), similar to the density we previously observed for authentic Sendai virus nucleocapsids (Chandrika *et al*., 1995 *b*). Sendai virus nucleocapsids have been reported to band at densities ranging from 1·29 to 1·33 g/cm^3 (Carlsen *et al*., 1985; Chandrika *et al*., 1995 *b*; Buchholz *et al*., 1993), with the differences probably reflecting variations associated with gradient collection and density measurement. Since the NP370 nucleocapsid-like particles banded at the density of ribonucleoprotein particles, the data suggest that they contain RNA. We have shown, in addition, that the viral P–L complex binds (via P protein) to the self-assembled NP370 particles (data not shown), indicating that the polymerase-binding site is maintained on these particles. In contrast, the NP362 oligomers banded at a buoyant density of 1·27 g/cm^3 (Fig. 5 E), reproducibly lighter than wt NP suggesting that they are not associated with RNA (Blumberg *et al*., 1983; Howard & Wertz, 1989). The population of NP362 molecules at the top of the gradient (fractions 2–4) most likely represents monomeric protein that has not reached equilibrium under these centrifugation conditions.

Discussion

The NP protein has been identified as a component of the NP_0–P, NP–NP and NP–RNA complexes, all of which are essential for *in vitro* RNA replication (Horikami *et al*., 1992; Buchholz *et al*., 1993; Curran *et al*., 1993). Deletion analysis of NP by Buchholz *et al*. (1993) showed that aa 1–400 were all required for all these interactions, and thus individual domains could not be identified. Subsequently, it was shown that sequences for the nucleocapsid to function specifically as a template for genome amplification encompassed aa 114–129 and aa 400–524 of NP (Curran *et al*., 1993; Myers & Moyer, 1997). The latter domain includes the P-binding site on the nucleocapsid through which the P–L polymerase complex is bound to the template (Buchholz *et al*., 1994). A highly conserved region from aa 258–357 was shown directly to contribute to the NP–NP interaction (Myers *et al*., 1997). In this study we have analysed the effect of site-directed mutations in a conserved region from aa 362–375 (Miyahara *et al*., 1992) of the NP protein on protein–protein interactions and *in vitro* RNA replication. Phe at aa 362 is conserved between 12 of 13 paramyxovirus NP proteins. Leu at 364 is conserved between 10 of 13 NP proteins and Gly at aa 365 is conserved in all 13. Charge is highly conserved at aa 370 and 371. A spectrum of defects was observed with the analysis of mutations in these amino acids.

The mutant NP364 with L364A and G365A failed to self-assemble (Fig. 4). This failure appears to be the result of improper folding since the protein is degraded within the cell (Fig. 1). While it is thought to be less likely that alanine substitutions will result in unstable proteins (Cunningham & Wells, 1989; Bass *et al*., 1991), they certainly have been observed elsewhere as well (Diamond & Kirkegaard, 1994; Bass *et al*., 1991). It is interesting, however, that one NP deletion mutant which removed the entire region from aa 359–384, encompassing NP364, was stable, although it still did not self-assemble (Buchholz *et al*., 1993).

These studies provide evidence that residues of NP needed for protein–protein interactions can be dissected from those required for activity in replication *in vitro*. Two mutants, NP362 and NP370, both of which were inactive in RNA

replication (Fig. 2), were clearly distinguishable in their other properties. Although NP370 failed to support replication, the NP_0–P interaction appeared normal (Fig. 3) and we found that self-assembled NP370 banded in CsCl gradients as a ribonucleoprotein particle, which upon EM analysis appeared normal (Fig. 5). These particles have, therefore, nonspecifically encapsidated cellular RNA, as was previously observed for wt NP (Buchholz *et al.*, 1993). Based on these findings, we propose that NP370 is defective in the specific initiation of encapsidation of Sendai virus RNA. The initiation of encapsidation of viral RNA probably requires the recognition of a specific leader RNA sequence as was shown for VSV (Blumberg *et al.*, 1983; Moyer *et al.*, 1991; Smallwood & Moyer, 1993). One possibility for RNA binding is that it is NP self-assembly which creates the RNA-binding site. In the case of NP370, however, the putative RNA-binding and NP–NP functions appear to be separable by mutation. We have not ruled out, however, that the defect in RNA replication for NP370 occurs at a subsequent, presently undefined step, perhaps during elongation.

The mutant NP362 protein also formed an NP_0–P complex (Fig. 3), but when expressed alone NP362 yielded unusual oligomers which were abnormal by EM and appeared to be devoid of RNA (Fig. 5). We propose that in this inactive mutant the substitution of Ala for Phe at aa 362 has disrupted both the specific (Sendai virus encapsidation) and the nonspecific (cellular RNA) NP–RNA interactions. It is possible that the altered NP–NP interaction of NP362 is responsible for its inability to bind any RNA. Based on these data, we propose that aa 362–371 of NP constitutes part of both an RNA-binding region and an NP–NP interaction site.

Although the NP protein, like the RNA-binding protein of *E. coli* ColE1 plasmid-encoded Rop protein, does not show any structural or sequence homology to known RNA-binding domains (RBD) (Predki *et al.*, 1995; Burd & Dreyfuss, 1994), amino acids contained within this conserved sequence of the NP protein have been shown to be required for other protein–RNA interactions. For example, site-directed mutagenesis of several RNA-binding proteins, including the *E. coli* Rop and rho proteins and the U1 small nuclear ribonucleoprotein (U1 snRNP), has identified charged, aromatic and hydrophobic residues that are involved in RNA binding (Burd & Dreyfuss, 1994; Predki *et al.*, 1995). For the Rop and rho proteins specific Phe residues are essential for binding to RNA and in Rop hydrophilic residues flanking Phe were also required (Brennan & Platt, 1991; Predki *et al.*, 1995). Photocross-linking studies on the C1/C2 RBD as well as the A1 RBD have also identified specific Phe residues that were UV cross-linked to RNA (Amrute *et al.*, 1994).

The predicted secondary structure of the Sendai virus NP protein in this region suggests the presence of two alpha-helical regions from aa 358–373 and 377–383 connected by a beta turn (Morgan *et al.*, 1984). This structure is reminiscent of a similar feature of the Rop protein revealed in the crystal structure (Predki *et al.*, 1995), where two helix–turn–helix monomers (helix 1/2 and 1′/2′) participate to form a four-helix bundle. All of the mutations in the Rop protein which disrupted RNA binding were located on one face of the protein (1/1′). By analogy, for NP the RNA binding and/or NP–NP domains may lie between aa 358–372 with the putative second helix (aa 377–383) not directly involved in these functions. Further mutagenesis within this overall region can address this hypothesis.

In conclusion, targeting conserved amino acids for site-directed mutagenesis has proved to be a fruitful approach that can now be applied to further delineate regions of the NP protein required for genome replication. Mutagenesis of the NP protein has shown that, although the NP–RNA and self-assembly domains may overlap, they can be separated genetically and that separate regions exist for these interactions and for the NP_0–P and P/L–NC binding domains.

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