

Interactions of viral matrix protein and nucleoprotein with the host cell cytoskeletal actin in influenza viral infection

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To understand the role of host cell cytoskeleton in regulating the assembly and budding of the externally enveloped viruses, we have studied the interactions between the cytoskeleton and viral proteins in the influenza A virus-infected Madin-Darby canine kidney (MDCK) cells, employing immunofluorescence microscopy and biochemical techniques. The Triton X-100-insoluble cytoskeletons prepared from the infected cells contained at least two viral proteins, viz. matrix protein (MP) and nucleoprotein (NP), the quantities of which considerably reduced (at least by 60%) upon including 1 M sodium chloride or 2.5 M urea in Triton X-100 (2%). Also, an extraction of these cytoskeletons in presence of the reagents that solubilized the cytoskeletal actin resulted in a release of actin together with NP and MP. Besides, these viral proteins were observed to codistribute with actin in both the cytochalasin E-treated and untreated infected cells. These results demonstrate that NP and MP ionically interact with cytoskeletal actin in the influenza virus-infected MDCK cells.

THE cytoskeleton, a structure experimentally defined as a cellular fraction remaining after cell treatment with non-ionic detergents¹, e.g. Triton X-100, is a cytoplasmic network composed of microfilaments, intermediate filaments and microtubules. Out of these, the microfilaments in particular have been shown to form stable contacts with the cytoplasmic face of the plasma membrane². These contacts of the microfilaments, which mainly consist of actin and actin-binding proteins, with the overlying membrane bilayer control not only the membrane mechanical stability and deformability but also the lateral redistribution of the cell surface components^{2,3}.

Externally enveloped viruses, such as influenza viruses, consist of a nucleocapsid core surrounded by a lipid bilayer. These viruses acquire their lipid bilayer as they bud from the host cell plasma membrane at areas enriched in viral envelope proteins⁴. It may therefore be argued that to form the budding sites on the plasma membrane and also to assemble at these sites the proteins that are integral to viral envelope, the virus must first modify the host cell cytoskeleton interactions with the

overlying plasma membrane bilayer. However, little is known as to how the virus alters these interactions in the infected cells.

To understand the role of the host cell cytoskeleton in assembly and budding of the externally enveloped viruses, several investigators have attempted to analyse the cytoskeleton-viral proteins interactions in the virus-infected cells⁵. The viral matrix protein (MP) and nucleoprotein (NP) have been shown to interact with the cytoskeletons of the cells that are infected with paramyxoviruses⁶⁻⁸. Also, interactions of MPs of these viruses with F-actin have been demonstrated *in vitro*⁹. Further, cytoskeletal actin has been reported to interact with HIV-1 Gag protein both *in vitro* and *in vivo*^{10,11}. Moreover, actin has been detected in mouse mammary tumour virus and HIV-1-purified virions¹¹⁻¹³. Furthermore, interactions of MP with the host cell cytoskeleton have been observed in influenza viral infections^{14,15}. To further examine these interactions at the molecular level, we analysed the associations of viral proteins with cytoskeletons of the Madin-Darby canine kidney (MDCK) cells that were infected with influenza A virus. Results of these studies strongly suggest that influenza viral NP and MP interact with cytoskeletal actin of the virus-infected MDCK cells.

Materials and methods

Cells, virus and growth conditions

MDCK cells obtained from National Facility for Animal Tissue and Cell Culture, Pune, India (presently National Centre for Cell Science) were maintained in Eagle's minimum essential medium (MEM) supplemented with 290 µg/ml L-glutamine, 70 µg/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum. Influenza virus type A(H3N2) 8912370 strain (similar to A/Sichuan/60/89 strain) was obtained from National Institute of Virology, Pune, India, as lyophilized allantoic fluid. The virus was multiplied in the allantoic cavity of 9-10 days old embryonated chicken eggs. The infected allantoic fluid was harvested and stored in liquid nitrogen¹⁶.

Infection of MDCK cells with influenza A virus

2×10^6 MDCK cells were seeded per well in a 6-well culture plate and grown to confluency. The medium was removed, and the cell monolayer washed two times with Hank's balanced salt solution (HBSS). The frozen aliquot of allantoic fluid was diluted with phosphate-buffered saline (PBS), and then added (0.5 ml/well) to the MDCK cells at 5–10 pfu/cell after treating with trypsin (10 µg/ml). The cells were incubated for 1 h at 37°C. To the control cells was added virus-free PBS (0.5 ml/well), and the cell monolayers incubated under identical conditions. After completion of the incubation, the supernatant was aspirated off, and the cells washed two times with HBSS to remove the unbound virus particles. To the washed cells was added the minimum essential medium (1 ml/well) supplemented with 2% fetal calf serum and trypsin (10 µg/ml). The cells were incubated for the desired period of time at 37°C.

Metabolic labeling of proteins in virus-infected cells

A confluent monolayer of MDCK cells in a 6-well cell culture plate was infected with influenza A virus as described above. After a desired period of time, the medium was removed and the cells washed two times with HBSS. To the washed cells was added L-[³⁵S]-methionine in methionine-free minimum essential medium (0.5 ml/well) at a concentration of 10 µCi/ml. The mixture was incubated at 37°C for 30 min.

In pulse-chase experiments, the L-[³⁵S]-methionine (20 µCi/ml) labeling was carried out for 15 min and then chased in Eagle's MEM supplemented with 2% fetal calf serum, 10 µg/ml trypsin and 10 mM methionine for 0, 30, 60, 90, 120 and 180 min. The cells were harvested and fractionated into Triton X-100 soluble and insoluble fractions.

Isolation of Triton X-100 insoluble cytoskeletons from cultured MDCK cells

The cells were harvested and washed with PBS. The washed cells were resuspended in 100 µl of PBS. To it was added an equal volume of CSK buffer (50 mM Tris, 10 mM EDTA, 100 µg/ml PMSF, 10 µM leupeptin and 1 µM pepstatin, pH 7.4) containing 4% Triton X-100. The mixture was incubated for 10 min at 22°C followed by another 10 min on ice. The cell lysate thus obtained was layered onto 30% sucrose column (1.0 ml) in centrifuge tubes containing 24 mM HEPES, 600 mM KCl, 0.5 mM DTT and 0.5 mM EDTA (pH 7.0). The tubes were centrifuged at 26,000 g for 30 min at 22°C. The pellet was washed with CSK buffer, once after including in it 2% Triton X-100 and then without Triton X-100.

Generation of polyclonal antibodies against cytoskeleton-associated NP and MP

Cytoskeleton-associated NP and MP were isolated by the preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The polyclonal sera against these proteins were raised in rabbits. The antibody titre was determined by enzyme-linked immunosorbent assay (ELISA).

Isolation of infected cell cytoskeleton in the presence of higher concentrations of urea, NaCl and of Triton X-100

Confluent monolayers of MDCK cells were infected with influenza A virus, and then pulse labeled with 10 µCi/ml L-[³⁵S]-methionine at 4 h after the infection. The cells were harvested and the cytoskeletons isolated using CSK buffer containing 2% Triton X-100 and 2.5 M urea, 2% Triton X-100 and 1 M NaCl or 5% Triton X-100.

Treatment of infected cell cytoskeleton pellet with guanidine hydrochloride and ammonium sulphate

The virus-infected cells were pulse labeled with 10 µCi/ml L-[³⁵S]-methionine at 4 or 8 h after the infection, and the cytoskeletons isolated. After suspending the cytoskeleton pellet in 20 µl of CSK buffer, to it was added 20 µl of guanidine hydrochloride (GuHCl) solution (1.5 M GuHCl, 1 M sodium acetate, 1 mM CaCl₂, 20 mM Tris and 1 mM ATP, pH 7.5). To the control cytoskeleton pellet was added 40 µl of GuHCl-free buffer. The mixtures were incubated for 30 min on ice, and then centrifuged at 15,000 g for 10 min at 4°C in a microfuge. The supernatant was removed and the pellet resuspended in 50 µl of the sample buffer.

Treatment of the infected cell cytoskeleton with ammonium sulphate was carried out following the published procedure¹⁷.

SDS-PAGE and immunoblotting

Protein compositions in cell lysate and cytoskeleton pellet were analysed by SDS-PAGE using 5% acrylamide as stacking and 10% (or 8%) acrylamide as running gels. The protein bands were visualized by staining the gels with Coomassie blue. In case of ³⁵S-labeled cells, the ³⁵S-labeled protein bands were visualized by fluorography followed by radioautography¹⁸. Densitometric scanning of one-dimensional radioautograms and Coomassie-blue-stained gels was carried out on a Phar-

macia ultrascan XL. Peak area of each band was calculated for comparative analysis of radioautograms. Comparison between the Coomassie blue-stained gels was done by calculating the ratio of the peak areas of actin and vimentin/keratin bands.

The protein bands were first resolved by SDS-PAGE and then transblotted on the nitrocellulose membranes at 110 mA for 1.0 to 1.5 h (ref. 19). It was incubated with 3% BSA solution in Tris-buffered saline overnight at 4°C to block the free sites, washed and incubated with rabbit antiserum (1 : 500 dilution) for 1 h at 22°C. This was followed by washing with Tris-buffered saline containing 0.05% Tween-20. The washed membranes were incubated with anti rabbit-IgG-peroxidase conjugate for 1 h at 22°C and then developed with diaminobenzidine.

Immunofluorescence

Intracellular localization of NP and MP was studied by indirect immunofluorescence microscopy. MDCK cells (about 10^5 cells/cover slip) were grown on 18 mm glass coverslips and then infected with 5–10 pfu/cell of influenza A virus. The cells were incubated in MEM containing 2% fetal calf serum and trypsin (10 µg/ml) at

37°C. The medium was removed, and the cells washed twice with PBS. The washed cells were fixed with 3% paraformaldehyde for 20 min at 22°C. The fixed cells were washed three times with PBS and permeabilized with 0.2% Triton X-100 for 5 min at 22°C. The cells were washed again with PBS, and incubated with rabbit antiserum (1 : 50 dilution) for 1 h at 22°C. After washing, the cells were incubated with FITC-labeled anti-rabbit IgG at 1 : 300 dilution for 1 h at 22°C. The incubated cells were washed again three times with PBS under gentle shaking.

For F-actin staining, the coverslip was incubated with phalloidin-rhodamine at a dilution of 1 : 100 in PBS for 30 min at 22°C. It was washed extensively with PBS and mounted in 20% glycerol on glass slides, and then viewed under a fluorescence microscope.

In some experiments, cytochalasin E (10 µg/ml) was added to the culture medium at 8 h postinfection, and the cells incubated for 3 h at 37°C. The washed cells were first labeled with antibodies and then with phalloidin-rhodamine, as described above.

Results

The cultured MDCK cells, with or without influenza A

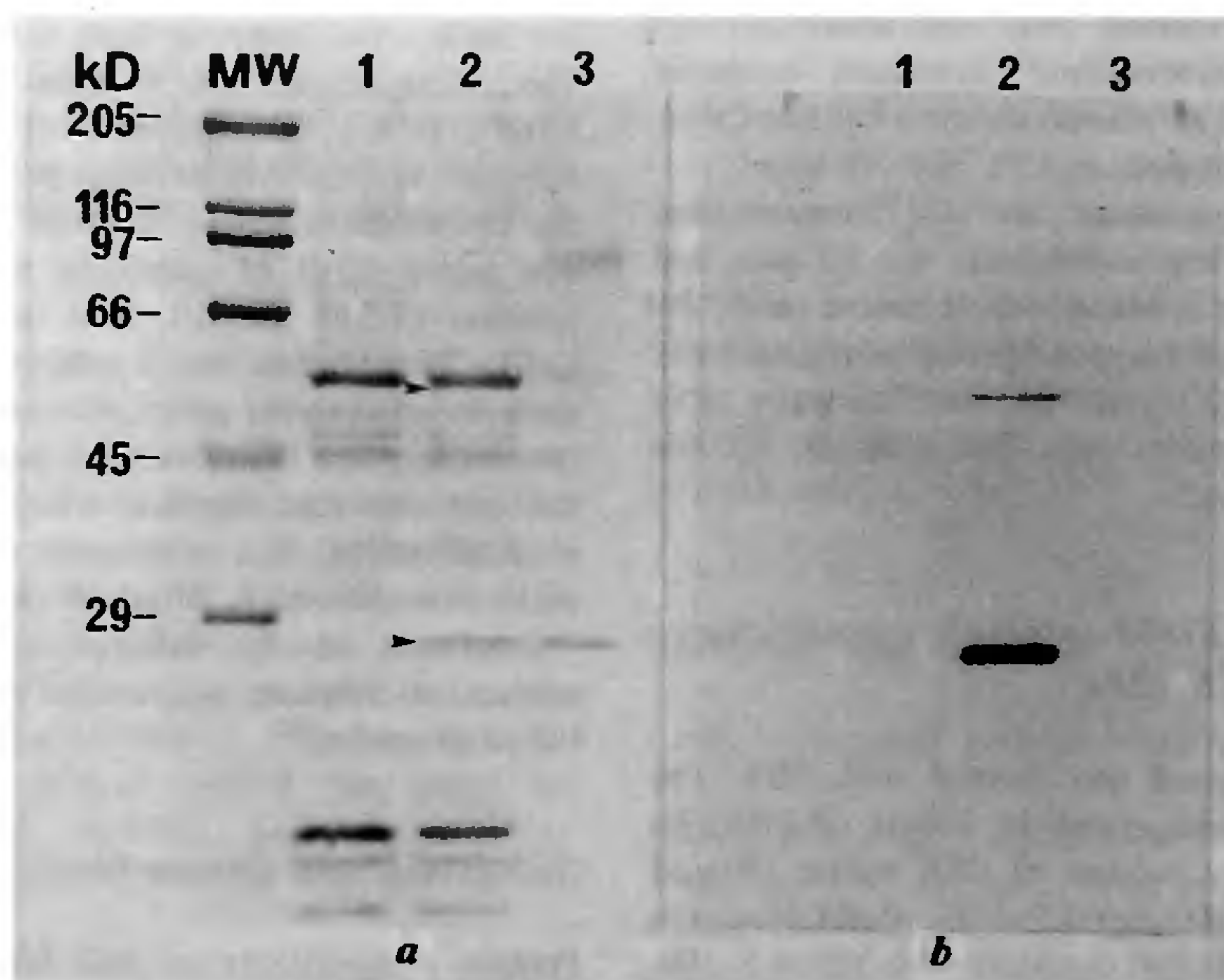


Figure 1. Viral proteins in Triton X-100 insoluble cytoskeleton prepared from the influenza A virus-infected MDCK cells. MDCK cells were infected with influenza A virus and at 8 h postinfection, labeled with L-[35 S]-methionine for 30 min. The Triton X-100 insoluble cytoskeleton was prepared and analysed by SDS-PAGE. *a*, Coomassie blue stained gel; *b*, radioautogram of the same gel. Lane 1, cytoskeleton prepared from normal uninfected MDCK cells; lane 2, cytoskeleton prepared from the influenza A virus-infected MDCK cells; lane 3, purified virus; MW, molecular weight markers. Note that two viral proteins, viz. NP (56 kDa) and MP (27 kDa) were coextracted with the cytoskeleton in the virus-infected MDCK cells.

virus infection, were treated with 2% Triton X-100. The cell lysates were loaded over 30% sucrose and centrifuged to remove any triton-insoluble lipids²⁰. The cytoskeleton pellet thus obtained was washed and then analysed by SDS-PAGE. Figure 1 shows that cytoskeletons obtained from the influenza A virus-infected cells contained at least two new protein bands of about 27 kDa and 56 kDa molecular mass which were completely absent in the normal uninfected cells. These two new proteins were later identified as nucleoprotein and matrix protein of influenza A virus by Western blotting and immunofluorescence analysis (data not shown), using polyclonal antisera.

Pulse-chase experiments were carried out to determine the kinetics by which NP and MP acquire the Triton X-100 insolubility. Cells were pulse-labeled for 15 min at 8 h postinfection, and chased for 0, 30, 60, 90, 120 and 180 min. The cells were harvested and treated with 2% Triton X-100. Triton X-100 soluble and insoluble fractions were analysed by SDS-PAGE (Figure 2 *a,b*). About 35% of both NP and MP became Triton X-100 insoluble immediately after their synthesis. There was gradual increase in the Triton X-100 insolubility of MP with chase time (Figure 3). But no change was observed in the Triton X-100 insolubility of NP at least up to 180 min (Figure 3).

These results indicate that influenza viral NP and MP associate with the host cell cytoskeleton in the infected cells. To analyse the nature of these associations, we measured the amounts of these proteins in the cytoskeletons that were isolated in the presence of high concentrations of Triton X-100, sodium chloride or urea. Table 1 shows that the amounts of NP and MP associated with the cytoskeletons were not much altered by increasing the concentration of Triton X-100 from 2% to 5%. However, these amounts were considerably reduced if 1 M sodium chloride or 2.5 M urea was included in 2% Triton X-100. From these results, we inferred that the observed association between the host cell cytoskeleton and viral proteins could primarily be ionic in nature. Since high ionic strength buffers, besides disrupting the ionic interactions, also dissociate actin from the cytoskeletons²¹, we further speculated that the reduced amounts of NP and MP associated with the cytoskeletons that were isolated in the presence of 1 M sodium chloride could perhaps be due to the association of these proteins with the host cell cytoskeletal actin. To examine the validity of this speculation, we extracted the isolated cytoskeletons under the conditions that have earlier been reported to solubilize cytoskeletal actin^{17,22}. It was argued that if NP and MP were associated with

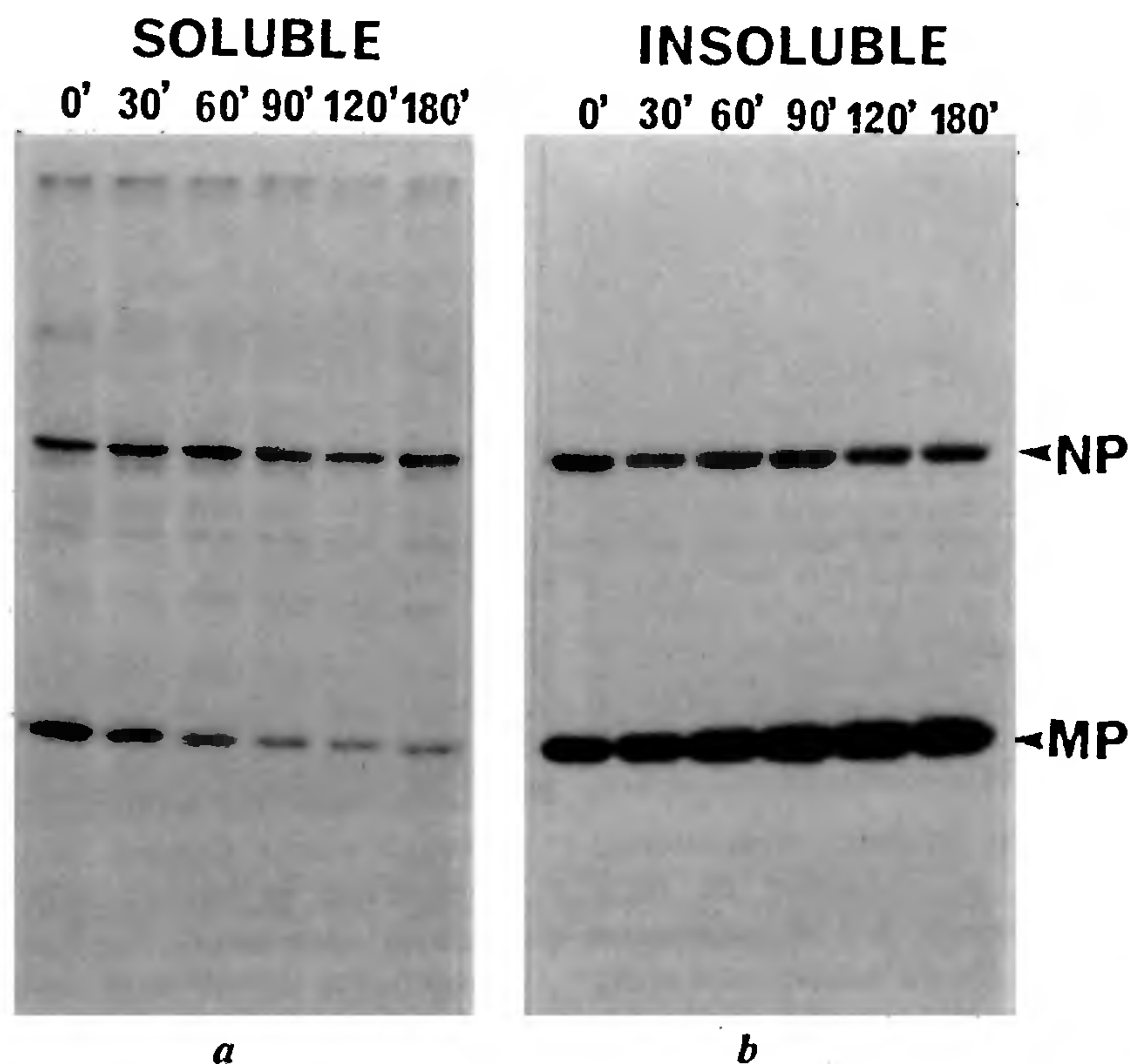


Figure 2. Time-dependent Triton X-100 insolubility of NP and MP. Influenza A virus-infected MDCK cells were pulse labeled at 8 h postinfection for 15 min with L-[³⁵S]-methionine and incubated in chase medium for 0, 30, 60, 90, 120 and 180 min. Cells were treated with Triton X-100 and fractionated into soluble (*a*) and insoluble (*b*) fractions. Proteins were analysed by SDS-PAGE.

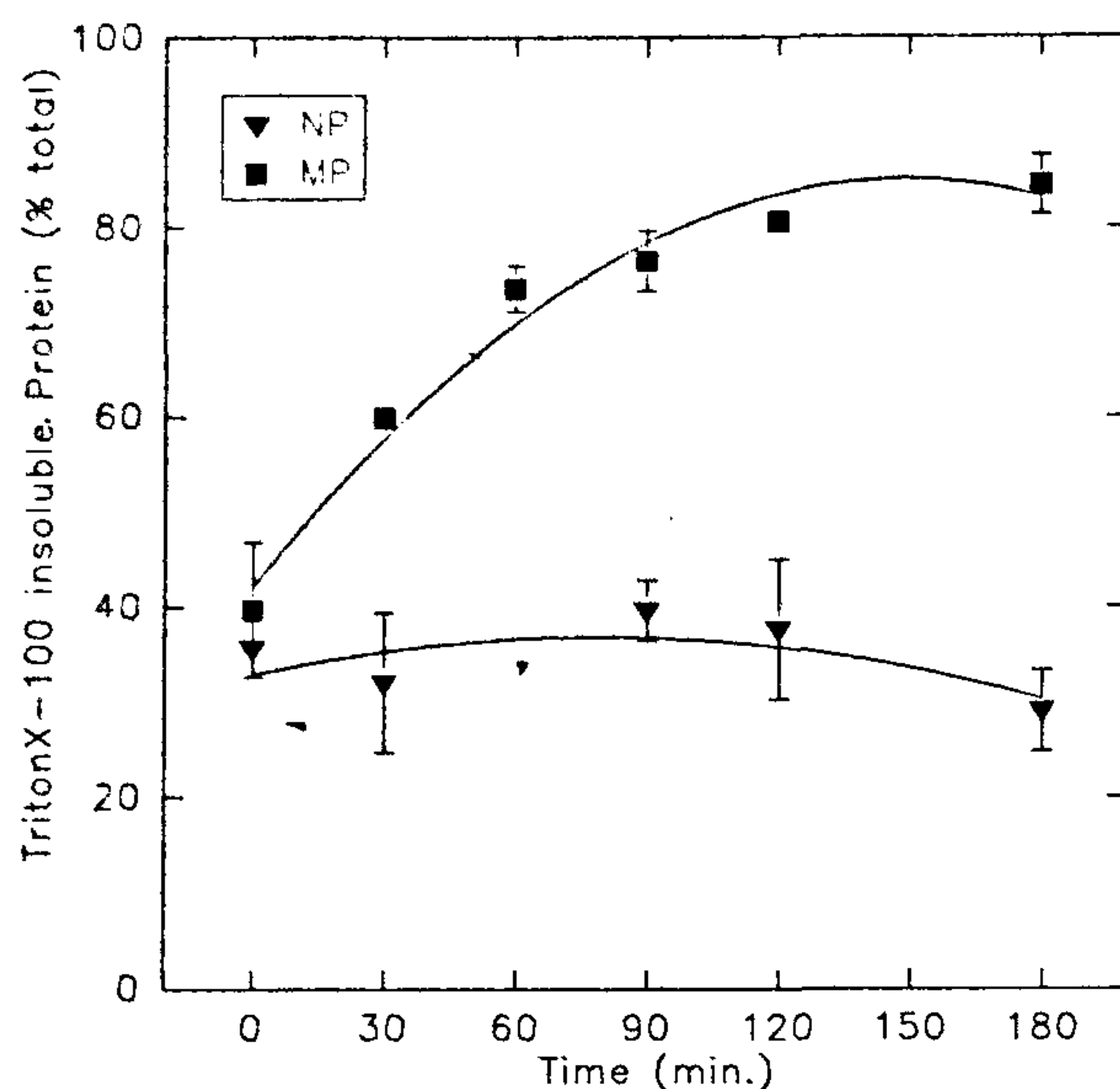


Figure 3. Densitometric quantitation of the Triton X-100 insoluble NP and MP in influenza A virus-infected MDCK cells.

Table 1. Effect of the high salt and urea concentrations on the retention of the viral proteins in the triton-insoluble cytoskeletons prepared from the influenza A virus-infected MDCK cells

Treatment	Viral proteins	Retention in cytoskeleton ^a (%)
Triton X-100 (2%)	NP	100
	MP	100
Triton X-100 (5%)	NP	92.2 ± 1.4
	MP	91.5 ± 8.5
Triton X-100 (2%) + NaCl (1 M)	NP	41.7 ± 6.2
	MP	39.2 ± 9.8
Triton X-100 (2%) + Urea (2.5 M)	NP	25.8 ± 2.3
	MP	36.6 ± 1.3

^aPer cent retentions of MP and NP were calculated by assuming the amount of NP and MP associated with the cytoskeletons isolated using 2% Triton X-100 as 100%. The cells were pulse labeled with L-[³⁵S]-methionine at 4 h postinfection. Cytoskeletons from the ³⁵S-labeled cells were isolated and then analysed by SDS-PAGE, as described in Materials and Methods. The gels were radioautographed and the relative amounts of various proteins determined by densitometry. Values shown are means of three experiments ± standard deviation.

the F-actin component of the influenza virus-infected MDCK cells cytoskeletons, an extraction of these cytoskeletons in the presence of 0.25 M ammonium sulphate¹⁷ or 0.75 M GuHCl²² should release actin along with NP and MP. Table 2 shows that indeed, considerable amounts of actin, MP and NP could be released from the cytoskeletons of the infected cells by extracting them under the above conditions.

The intracellular localization of NP and MP in influenza A virus infected MDCK cells was determined by indirect immunofluorescence. At the 8 h postinfection both NP

and MP showed similar cellular distribution with a distinct morphology. Both the NP and MP were predominantly cytoplasmic at 8 h postinfection (Figure 4 a, c). The pattern of MP was diffused and homogeneous throughout the cytoplasm (Figure 4 c), whereas NP exhibited granular staining pattern in the cytoplasm (Figure 4 a). Both NP and MP showed a characteristic staining pattern at the leading edges of the cells (arrow heads, Figure 4 a, c) which was present in all the influenza A virus-infected cells. On the other hand, in phalloidin-rhodamine stained cells these leading edges were also rich in the actin filaments (arrow heads, Figure 4 b, d), which indicated the codistribution of these two viral proteins with the actin filaments. To further confirm the colocalization of the two proteins with the actin filaments, influenza A virus-infected cells were treated with cytochalasin E (or cytochalasin B).

Cytochalasins bind to the barbed ends of the actin filaments, which inhibit both the association and dissociation of actin monomers at the ends²³⁻²⁶. It has been reported that an incubation of mammalian cells with cytochalasins leads to the formation of membrane blebs and cell retraction due to disruption of the actin filaments^{27,28}. In agreement with these findings, we observed an extensive modification of the influenza A virus-infected cells after their incubation with cytochalasin E for 3 h. This treatment led not only to the formation of membrane blebs and cell retraction but also an extensive aggregation of actin, as revealed by phalloidin-rhodamine staining (Figure 5 b, d). Also MP in cytochalasin E-treated influenza A virus-infected cells showed a morphological alteration changing from homogenous to clumping distribution (Figure 5 c). Further, similar changes were observed in the NP distribution (Figure 5 a). These results strongly indicated that NP and MP are colocalized with actin in the virus-infected cells.

Discussion

Earlier studies with paramyxoviruses, viz. Newcastle disease virus and sendai virus, have reported that nucleoproteins and matrix proteins of these viruses associate with the cytoskeletons of the host cells^{6,7}. Although matrix proteins of these viruses have been demonstrated to interact with F-actin under *in vitro* condition⁹, Sanderson *et al.*⁸ have failed to observe any codistribution of MP with actin filaments in the sendai virus-infected BHK cells. However, immunofluorescence analysis employing monoclonal antibody has shown codistribution of influenza viral MP with actin filaments in the virus-infected CV-1 cells¹⁴. The present study further extends these observations by demonstrating that viral NP and MP ionically interact with cytoskeletal actin in the influenza virus-infected MDCK cells.

Table 2. Release of viral proteins and host cell actin from the influenza A virus-infected MDCK cell cytoskeleton upon their treatments with ammonium sulphate or GuHCl

Treatment	Time post infection (h)	Protein release (%)	Retention in cytoskeleton ^a (%)		
			Actin	NP	MP
(NH ₄) ₂ SO ₄ (0.25 M)	4	33.3 ± 2.3	56.0 ± 6.3	53.2 ± 9.5	75.6 ± 9.8
	8	35.0 ± 0.6	55.7 ± 11.3	54.9 ± 8.4	72.2 ± 9.2
GuHCl (0.75 M)	4	32.5 ± 3.5	45.5 ± 6.1	59.1 ± 6.8	73.6 ± 8.4
	8	28.8 ± 0.2	46.4 ± 7.6	59.3 ± 7.3	59.6 ± 4.3

^aPer cent retentions of NP and MP were calculated as given in Table 1. The per cent retention of actin was calculated by comparing the vimentin/keratin band-to-actin band ratios calculated from the Coomassie blue-stained gels before and after the treatments. Values shown are means of four experiments ± standard deviations.

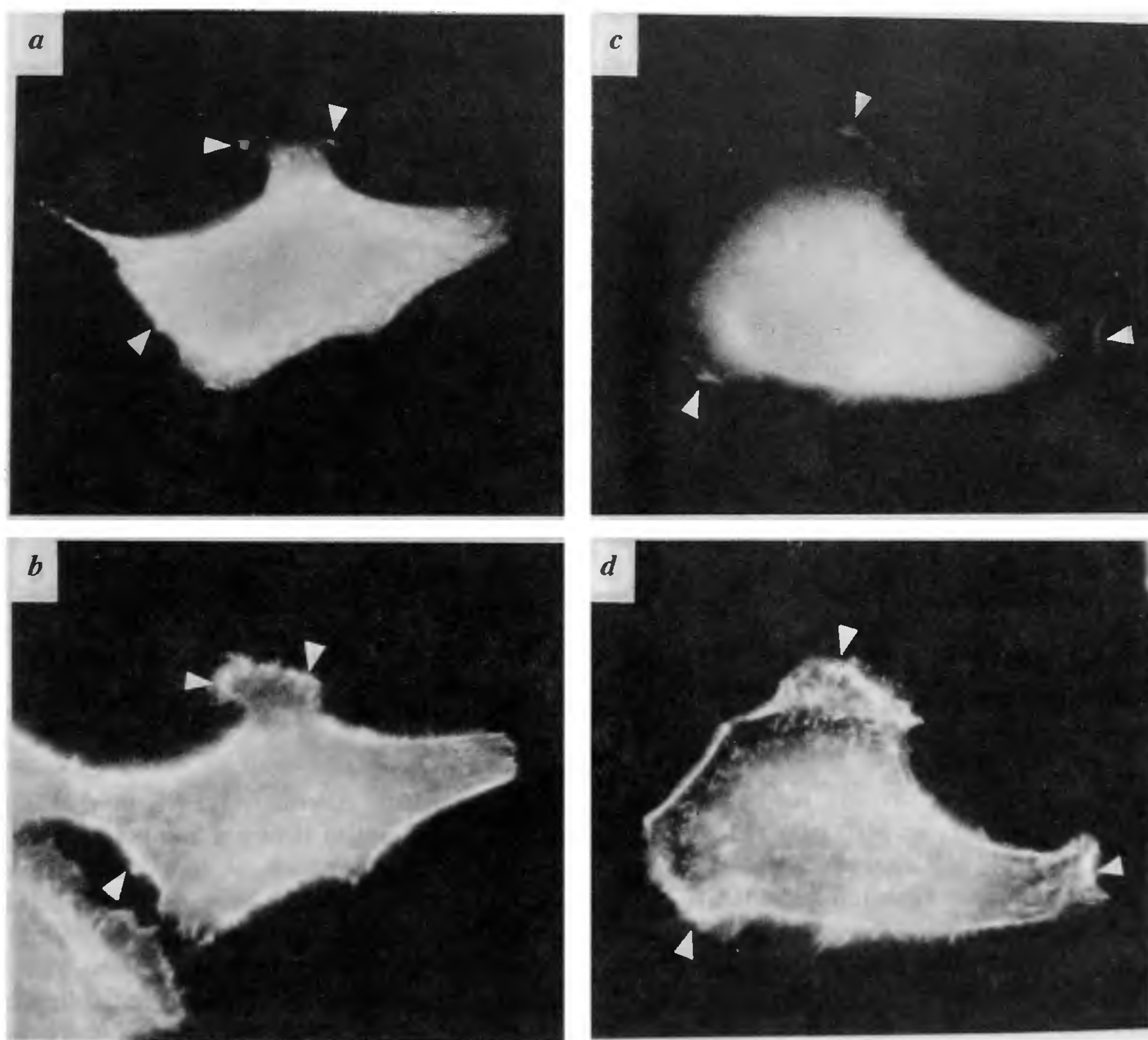


Figure 4. Intracellular localization of NP, MP and actin filaments in influenza A virus-infected MDCK cells. Eight hour postinfected MDCK cells were fixed, permeabilized and doubly stained with anti-NP (*a*), anti-MP (*b*) and phalloidin-rhodamine (*b*, *d*). Note the distribution of NP (arrowheads, *a*) and MP (arrowheads, *c*) at the periphery of the cells which corresponds to distribution of actin filaments at the periphery of the phalloidin-rhodamine stained cells (arrowheads, *b*, *d*).

It is not clear whether NP and MP independently bind the host cell cytoskeleton. But based on their recent observation that expression of MP alone, using vac/17 expression vector, in the HeLa-T4 cells does not lead to its Triton X-100 insolubility, Zhang and Lamb¹⁵ have

speculated that perhaps coexpression of another influenza virus protein is required for MP to become Triton X-100 insoluble. We suggest that the binding of MP with the cytoskeleton may perhaps be induced by its prior association with NP. That NP and MP have a tendency to

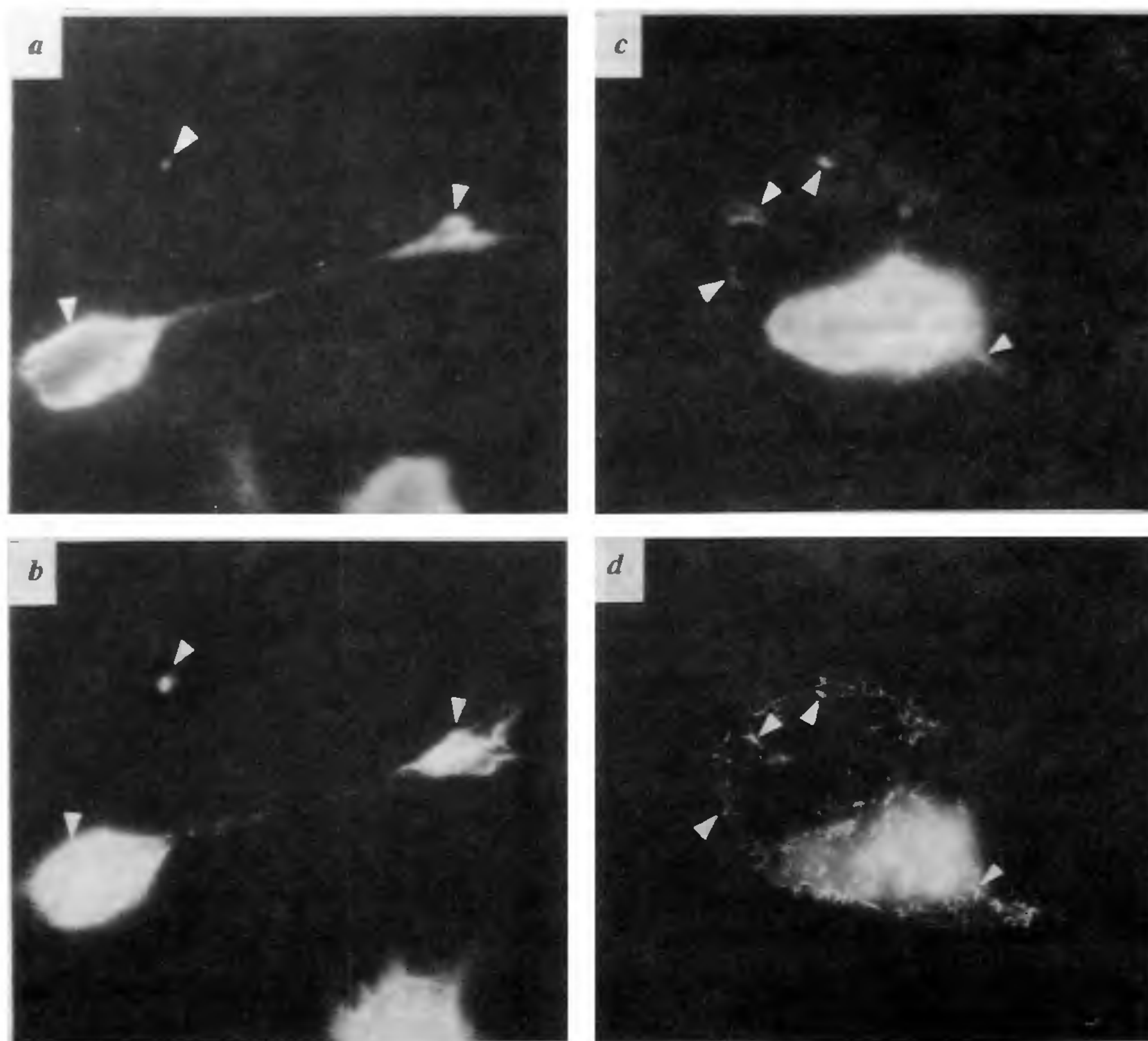


Figure 5. Intracellular distribution of NP, MP and actin filaments in influenza A virus-infected MDCK cells after cytochalasin E treatment. Eight hour postinfected cells were treated with cytochalasin E (10 µg/ml) for 3 h, and fixed and stained as in Figure 4. Note an extensive modification of the actin filaments (*b, d*) and corresponding changes in distribution of NP (*a*) and MP (*c*). Arrowheads show the codistribution of NP (*a*) and MP (*c*) with the actin filaments (*b, d*).

associate with each other is strongly supported by the earlier studies of Patterson *et al.*²⁹ who observed co-distribution of NP and MP in both the nucleus and cytoplasm of the influenza virus-infected MDCK cells. Also, it finds support from our present findings that solubilization of actin or disruption of actin filaments invariably leads to the release of both MP and NP from the cytoskeletons.

The influenza viral MP plays a central role in the virus assembly. This protein is required not only for exporting the viral ribonucleoproteins (vRNPs) out of the nucleus but also for preventing the re-entry of the exported out vRNPs back into the nucleus³⁰. This function of MP may be suitably explained by envisaging that MP after entering into the nucleus forms a complex with vRNPs through its association with NP and then this complex migrates to the cytosol where it binds the cytoskeleton through MP to prevent its re-entry back

into the nucleus. Alternatively, NP may associate with MP in the cytosol to induce a binding between MP and cytoskeletal actin, which in turn could serve as the capture sites for vRNPs being exported out from the nucleus. It would therefore seem that the binding between the host cell cytoskeletal actin and the viral proteins may prove useful to the virus by providing it with a mechanism which can prevent the re-entry of vRNPs back into the nucleus. Also, this binding may have been required for completion of the virus assembly as well as motility during virus budding³¹.

Apart from its role in the nuclear export of vRNPs, MP is believed to decorate the cytoplasmic face of that portion of the host cell plasma membrane which serves as the site for virus budding and subsequently as the viral membrane³⁰. Since the budding sites contain the viral proteins that are integral to the viral envelope, it may be argued that assembly of these proteins in the

host cell membrane bilayer should occur only after its dissociation from the underlying cytoskeleton^{2,3}. As only up to 80% of MP and 40% of NP were observed to become Triton X-100 insoluble during the pulse-chase experiments, we speculate that this dissociation is perhaps induced by the binding of remainder MP to the membrane. It is, however, difficult to ascertain whether all the above functions are performed by a single form of MP or there are more than one form of this protein to accomplish different functions.

It has recently been shown that expression of MP in the absence of any other viral protein in the HeLa cells renders it totally soluble in Triton X-100 (refs 15, 32). But even under these conditions, a significant fraction of this protein tightly associated with the host cell plasma membrane. This may be taken to suggest that the membrane-binding and cytoskeleton-binding forms of MP could perhaps represent two different structural forms of this protein. More studies are clearly required to understand the role of viral proteins in modulating the structure and interactions of the host cell cytoskeleton in the virus-infected cells.

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