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VISUALIZATION OF CYTOSKELETAL ELEMENTS AND ASSOCIATED RETROVIRAL ANTIGENS BY IMMUNOGOLD TRANSMISSION ELECTRON MICROSCOPY OF DETERGENT EXTRACTED CELLS

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Abstract

Several investigators have reported an association between the cytoskeleton and viral antigens. In our laboratory, biochemical immunofluorescence and immuno-gold electron microscopy studies were conducted on TX-100 extracted NIH/3T3 cells infected with Moloney-murine leukemia virus. Cytochalasin B treatment causes reversible microfilament disruption and a concomitant decrease in virus production. No effect on microtubules was seen. Immunogold electron microscopy reveals an association between cytoskeletal actin and the viral antigens gp70 and p15E. The results of these immunocytological and biochemical studies indicate that the cytoskeleton may play an integral role in transport and processing of viral gene-envelope products.

KEY WORDS: Cytoskeleton, TX-100, immunofluorescence, protein A-Gold, murine leukemia virus, anti-tubulin, anti-actin, anti-gp70, anti-p15E, NIH/3T3 cells.

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Introduction

The eukaryotic cytoskeleton is comprised of a complex, highly organized network of interconnected filaments (4). The major components are microfilaments, intermediate filaments, and microtubules which collectively regulate cell structure and function (3,4). Detailed studies on the architecture of the cytoskeleton have been made possible by the discovery that extraction of cells with non-ionic detergents such as Triton X-100 (TX-100) removes the plasma membrane and cytosol, leaving cytoskeletal structures intact (16). Studies with detergent-extracted cells have provided much biochemical and morphological information on the cytoskeletal network (1,17). Additionally, this method has proven to be extremely valuable in studying cytoskeleton-associated proteins and their function in regulating cell activities (2,5,6,12,14).

Exposed cytoskeletal elements can be examined using either transmission or high-resolution scanning electron microscopy. Development of protein A-colloidal gold as a high affinity probe for immunoglobulins has provided a major advancement in immunocytology. Immunogold labeling has been used to effectively visualize numerous cellular markers at the transmission electron microscopic (TEM) level, including cytoskeletal elements and associated protein (10,14,15). Mecham et al., (14) employed this technique to present the first clear-cut evidence for binding association of calmodulin (CAM) with cytoskeletal microfilaments (MF) and suggested a functional correlation between CAM-MF binding and extension of the cytoskeleton network.

We have been interested in the role of the cytoskeleton in the processing and/or transport of retroviral envelope (env) antigens and have recently presented evidence that the cellular pathology resulting from certain retrovirus infections may be related to the interaction of viral glycoprotein with elements of the cell cytoskeleton (unpublished abstract). Evidence for an association between retroviral antigens and cytoskeletal elements comes from detection of actin in purified MuLV particles (8) and from the observation that disruption of

microtubules reduced production of infectious MuLV with concomitant accumulation of virions within the cytoplasm (18). In addition, Soong and Tompkins (in preparation) have shown that the gPr80^{env} precursor of the MoMuLV accumulates in the cytoskeleton-rich fraction in cells treated with CB or monensin.

Immunofluorescence studies of extracted NIH/3T3 cells infected with Moloney-murine leukemia virus (Mo-MuLV) have indicated an association between cytoskeletal actin and the env viral antigens gp70 and p15E (unpublished data). These two viral antigens are disulfide-linked subunits produced proteolytically from a glycosylated precursor protein PrENV and comprise the MuLV env gene end products. These end products are transported through the cytosol and inserted into the plasma membrane. Previous studies have indicated that the PrENV precursor is found predominantly in smooth and rough endoplasmic reticulum, but the gp70 is only expressed on the external cell membrane surface (7). The precursor processing events and transport mechanism are not known.

While evidence for the involvement of the cytoskeleton in transport and processing of retroviral env glycoprotein is circumstantial, it appears that the gPr 80^{env} precursor is transported from the Golgi to the surface in association with the cytoskeleton in much the same manner as other excreted cellular glycoproteins (17).

In this paper we employed immunofluorescence and protein A-colloidal gold probes and antibodies against cytoskeletal elements and viral env antigens to study the relationship between MuLV env proteins and cell cytoskeleton structures. The results of these studies provide additional evidence that Mo-MuLV env proteins are bound to the cytoskeleton at some stage of their maturation.

Materials and Methods

Cell and Virus

NIH/3T3 cells obtained from Dr. S. Aaronson (NIH) were infected with Moloney murine leukemia virus (Mo-MuLV). Fifteen F cells, a murine sarcoma-positive, leukemia-negative (S^+L^-) cell line described previously (20), were used for infectivity assay. All cell lines were grown at 37 C in Eagle's minimum essential medium (MEM) supplemented with 10 percent fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. The cells were shown to be productively infected with Mo-MuLV by reverse transcriptase and infectivity assays (20).

Immuno-Fluorescence Microscopy

Mo-MuLV-infected NIH/3T3 cells were seeded on coverslips with 1 x 10^5 cells/coverslip. After 12 h of growth, cells were treated with cytochalasin B (5 µg/ml) for 16 h at 36 C, then pretreated for 2 to 3 minutes with 5 mM taxol (MSC 125973, obtained from NIH) in extraction buffer (10 mM PIPES, 100 mM potassium chloride, 3 mM magnesium chloride, and 300 mM sucrose at pH 6.8) to preserve microtubules.

Cells were then extracted with 1 percent TX-100 in extraction buffer for 3 to 4 minutes, followed by a 10 second rinse in the same buffer without the detergent. The glass adherent cytoskeleton shells were fixed in methanol: phosphate buffered saline (PBS; 1:1) for 5 minutes followed by methanol for 5 minutes at -20 C. Ten microliters of appropriate monoclonal antibodies (antitubulin 1:500, anti-actin 1:100, anti-gp70 1:100, and anti-p15E 1:10) diluted in PBS with 1 percent bovine serum albumin (BSA) were used to react with the methanol-fixed cytoskeletons for 30 minutes at 37 C.

Monoclonal anti-actin and anti-tubulin were obtained from Amersham. Monoclonal anti-gp70 and anti-p15E were obtained from NEN. Fluorescein conjugated goat anti-mouse or rabbit anti-mouse Ig serum (Miles) preabsorbed with TX-100 extracted NIH/3T3 cells at 37 C for 45 minutes and clarified at 15,000 g for 15 minutes were used at a final dilution of 1:30 as secondary antibody. After incubation at 37 C for 30 minutes with the secondary antibody, coverslips were washed 3 times in PBS with 1 percent BSA then briefly washed in distilled water before being inverted over a drop of PBS-glycerol (1:9) on a glass microscope slide.

Slides were viewed and photographed with a Zeiss fluorescence microscope. Photographs were taken on Kodak Ektachrome 400 color slides.

Immunogold-Transmission Electron Microscopy

Gold grids (200 mesh) were coated and adhered to glass coverslips using 0.2 percent formvar in chloroform. The coverslips were seeded with cells prepared as already described for immunofluorescence at a density of 3 x 10^5 cells/ml.

Following incubation for 16 h at 37 C and washing in calcium-free PBS (pH 7.2), cells were extracted as described below.

Anti-actin for immunogold studies was prepared in rabbits against actin isolated from chicken back muscle (Miles Yeda, Ltd.). The anti-tubulin was a murine IgG monoclonal (Amersham). The anti-gp70 and p15E were monoclonals obtained from New England Nuclear. All steps were at room temperature unless otherwise noted.

The buffer system of Schliwa and Van Blerkom (19) was used for extraction. This consisted of freshly prepared 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$. 6 H_2O at pH 6.9 (PHEM) as the stock buffer. From this stock, stabilization buffer and extraction buffer were each prepared immediately before using in the following proportions: stabilization buffer consisted of 16 parts PHEM, 4 parts dH_2O, 0.02 parts TAXOL (10 mg/ml, DMSO), and extraction buffer consisted of 16 parts PHEM, 4 parts 10 percent TX-100.

Each coverslip with adhered grids was incubated in 300 µl of stabilization buffer for 3 minutes. Taxol promotes microtubule assembly for increased stabilization. After removing the supernatant, the cells were incubated with 300 µl of extraction buffer for 10 minutes to

remove detergent-soluble components. A gentle rinse with PBS was followed by a mild preincubation step with a 1:10 dilution of modified Karnovsky's fixative in PHEM buffer for 10 minutes. After rinsing with PBS, the grids were removed from the coverslips and placed on an 8 µl drop of antibody for 30 minutes. The grids were again rinsed gently with PBS and then placed on an 8 µl drop of colloidal gold-protein A (5 nm or 15 nm obtained from Janssen Life Sciences Products, Beerse, Belgium) for an additional 20 minutes and then fixed for 20 minutes or overnight in a 0.1 M phosphate-buffered 2 percent glutaraldehyde solution, pH 7.2. The cells were then rinsed in distilled water and treated for 10 minutes in an aqueous 0.1 percent osmium tetroxide solution. After another brief distilled water rinse, the cells were stained for 10 minutes with an aqueous 1 percent uranyl acetate solution.

The cells were put through a final distilled water rinse and dehydrated through an ascending ethanol series starting with 50 percent ethanol. Each step was repeated twice for 2 to 3 minutes. The final three 5-minute rinses were in absolute ethanol maintained in a Linde 3A molecular sieve. The grids were critical point dried in a Samdri dryer, lightly carbon coated, and then stored in a desiccator. Grids were examined at 100 kV with a JEOL 100CX transmission electron microscope.

Results

Immunofluorescence localization of MuLV env proteins on the cell cytoskeleton.

To explore the association of env polypeptides with the cytoskeleton, we extracted Mo-MuLV-infected NIH/3T3 cells with non-ionic detergent, TX-100, in a buffer system that has been shown to stabilize the cytoskeletal structures, and examined their reactions with monoclonal anti-gp70 and anti-p15E by indirect immunofluorescence. Figures 1-3 depict the reactions of anti-actin (microfilaments; Mf), anti-tubulin (microtubules; Mt), and anti-gp70 with TX-100 extracted cytoskeletons. Anti-actin reveals well delineated microfilaments arranged as linear stress fibers in the central cytoskeleton and in some cases what appear to be actin aggregates, particularly in the nuclear region (Fig. 1).

Reaction of cells with anti-tubulin shows a wavy pattern of microtubules which for the most part appear to radiate out from a central organizing center in the vicinity of the nucleus (Fig. 2). Anti-gp70 revealed a speckled staining pattern in the cytoplasmic region (Fig. 3). A similar reaction was observed with anti-p15E (data not presented). Uninfected NIH/3T3 cytoskeletons showed no apparent reaction with anti-gp70 (data not presented).

Effect of cytochalasin B (CB) on maturation of MuLV.

The above observations suggest that MuLV antigens gp70 and p15E or the env precursor protein $Pr80^{env}$ are associated with the cell cytoskeleton at some stage of virus maturation.

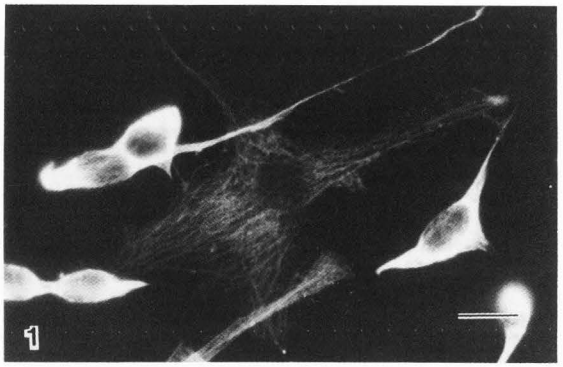

Figure 1: Extracted cell stained with monclonal anti-actin, fluorescein anti-mouse. Bar = 5 µm.

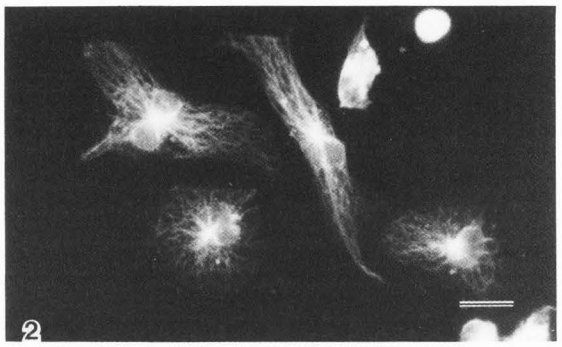

Figure 2: Extracted cell stained with monoclonal anti-tubulin, fluorescein anti-mouse. Bar = 10 µm.

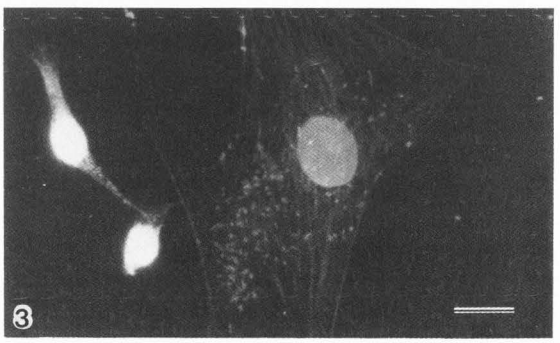

Figure 3: Extracted, infected NIH/3T3 cell stained with monoclonal anti-gp70, fluorescein anti-mouse. Bar = 5 µm.

In this experiment, we examined the effect of CB, a Mf disrupting drug, on the production of MuLV by NIH/3T3 cells, on the processing of $Pr80^{env}$, and on the distribution of intracellular viral env antigens on the cytoskeleton.

Moloney-MuLV-infected NIH/3T3 cells were incubated with 5 µg/ml CB for 6 h, 12 h, and 16 h, and the supernatants were assayed for virus by measuring infectivity and reverse transcriptase (RT) activity. After treatment

with CB for 6 h, infectious virus production decreased about 35 percent. Twelve to 16 h of treatment with CB reduced the virus yield by 90 to 95 percent.

The decrease in virus production could also be seen by measuring the reverse transcriptase activity in the supernatant. There was only about 10 percent decrease in reverse transcriptase activity compared to the 35 percent decrease in infectivity after 6 h treatment with CB. Longer treatment with CB, e.g., 12 and 16 h, reduced RT yield by 21 percent and 35 percent, respectively.

Immunofluorescence visualization of microfilaments (Mf) of MuLV infected cells treated with CB demonstrated a marked disruption of the stress fibers (Fig. 4), whereas the distribution of microtubules (Mt) was similar to untreated cells. Figure 5 depicts the reactions of CB-treated cytoskeleton with anti-gp70, which is similar in pattern to that with anti-actin. Further, following the removal of CB from cell cultures, both the anti-actin and anti-gp70 staining pattern returns to the normal untreated state as depicted in Fig. 1 and Fig. 3. This recovery of the structure of the microfilaments correlated with recovery of the cells' ability to produce infectious virus.

Localization of MuLV gp70 on the cell cytoskeleton by immunogold labeling.

The above studies indicate that the MuLV env proteins may be associated with cell microfilaments (Mf) as a necessary requisite for transport and processing. In an attempt to confirm this association, we grew cells on gold grids and extracted with TX-100 to reveal the cytoskeleton network as described by Mecham et al. (14). Cells were reacted with anti-actin, anti-tubulin, or anti-gp70 and then with protein A-tagged colloidal gold. The gold particles were visualized by transmission electron microscopy. Preliminary observation (Fig. 6) suggests that anti-gp70 binds to the 4 to 5 nm Mf but not to the larger 20 to 24 nm Mt of MuLV infected cells. Figures 7 and 8 show well delineated 4 to 5 nm Mf reacting with anti-actin (Fig. 7) and 20 to 24 nm Mt reacting with anti-tubulin (Fig. 8), respectively.

No appreciable colloidal gold was associated with cytoskeletal elements from uninfected 3T3 cells reacted with anti-gp 70 (Fig. 9). While these data suggest that anti-gp70 reacts with the 4 to 5 nm Mf, we cannot exclude the possibility that there is also some binding to the intermediate filaments (10 to 15 nm). Some gold particles appear to be associated with structures of slightly greater diameter than the Mf.

Discussion

The eukaryotic cytoskeleton is comprised of a complex, highly organized network of interconnected filaments (3,4). The major components are microfilaments, intermediate filaments, and microtubules which collectively regulate cell structure and function (3,4). Detailed studies on the architecture of the cytoskeleton have been made possible by the discovery that extraction of cells with nonionic detergents such as TX-100 removes the plasma membrane and cytosol, leaving cytoskeletal structures intact (16). Detergent extracted cells have provided biochemical and morphological information on the cytoskeletal network (1,19).

An association between viral antigens and cytoskeletal components has been indicated (3,9,13). In this paper, we employ various immunocytological and biochemical methods in combination with pharmacological agents to demonstrate an association of Mo-MuLV env antigens gp70 and p15E with the microfilaments of infected cells.

TX-100 extraction of Mo-MuLV infected, adherent NIH/3T3 cells leaves an intact cytoskeletal network of distinct microtubules (Mt) and microfilaments (Mf) as observed by indirect immunofluorescence with anti-tubulin and anti-actin, respectively. Reaction of extracted cells with anti-gp70 or anti-p15E revealed a speckled fluorescence, indicating association of p15E and gp70 or the $Pr80^{env}$ precursor with the cytoskeleton.

Further evidence that MuLV env proteins are bound to the cytoskeleton comes from studies with cytochalasin B (CB), a drug which disrupts Mf and several Mf dependent processes, including secretion (11). CB treatment caused a reduction in infectious virus and reverse transcriptase activity in cell supernatants. Coincident with this was a topographically similar alteration of anti-actin and anti-gp70 immunofluorescence staining patterns, whereas anti-tubulin staining was unaltered. The validity of this extraction procedure for preservation of microtubules and microfilaments was confirmed by transmission electron microscopy of immunogold-labeled cells. Association of MuLV gp70 with the actin containing Mf was confirmed by immunogold tagging of cytoskeletal elements of TX-100 extracted cells. Anti-gp70 reacted with the 4 to 5 nm diameter Mf but not the 20 to 24 nm diameter Mt. This apparent association of env proteins with cellular Mf is consistent with reports of actin found in purified retrovirus particles (8). SDS-PAGE analysis of TX-100 extracted and iodinated cytoskeletons demonstrate $gPr80^{env}$ but not gp70 or p15E in the cytoskeleton fraction of MuLV infected cells (Soong and Tompkins, unpublished observation). Moreover, treatment of MuLV infected cells with CB prevents processing of $gPr80^{env}$ to p15E and gp70 with an accumulation of the env precursor in the cytoskeleton rich fraction (Soong and Tompkins, in preparation). These observations suggest that the env precursor $gPr80^{env}$ binds to the cytoskeleton at some stage prior to its processing and insertion into the plasma membrane.

These studies clearly indicate that immunogold-labeling and TEM can be powerful tools to study subcellular architecture as well as function.

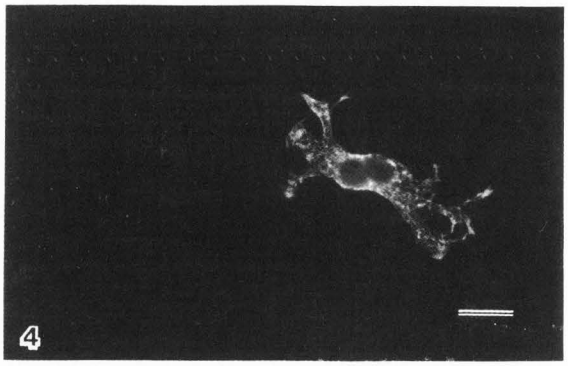

Figure 4: Cytochalasin-B treated, extracted cell stained with monoclonal anti-actin, fluorescein anti-mouse. Bar = 10 μm.

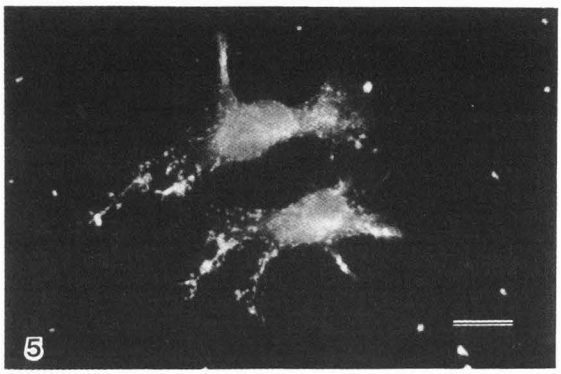

Figure 5: Cytochalasin-B treated, extracted cell stained with monoclonal anti-gp70, fluorescein anti-mouse. Bar = 5 μm.

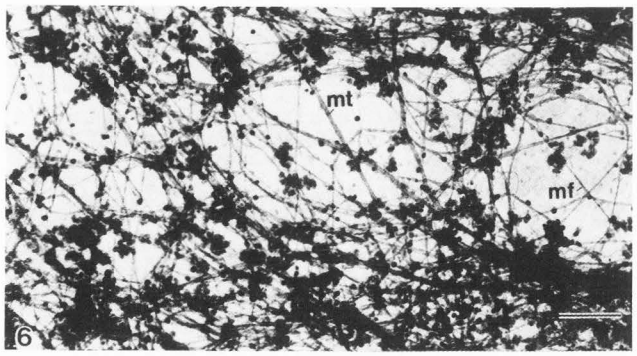

Figure 6: Extracted, infected NIH/3T3 cell stained with monoclonal anti-gp 70, 15 nm colloidal gold. (Abbreviations used: Mt, microtubule; Mf, microfilament). Bar = 0.2 μm.

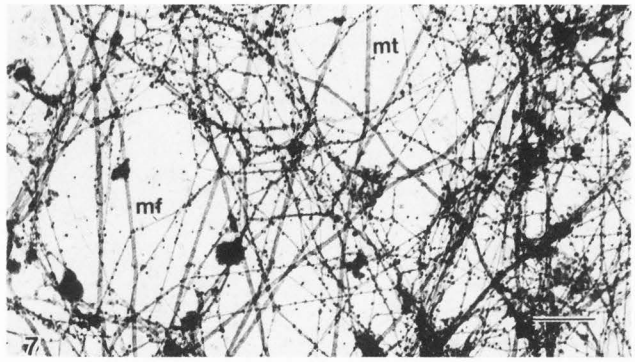

Figure 7: Extracted cell stained with anti-actin, 5 nm colloidal gold. (For abbreviations, see Figure 6). Bar = 0.2 μm.

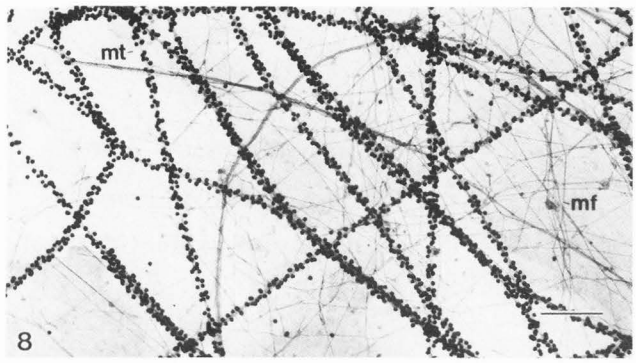

Figure 8: Extracted cell stained with monoclonal anti-tubulin, 15 nm colloidal gold. (For abbreviations, see Figure 6). Bar = 0.2 μm.

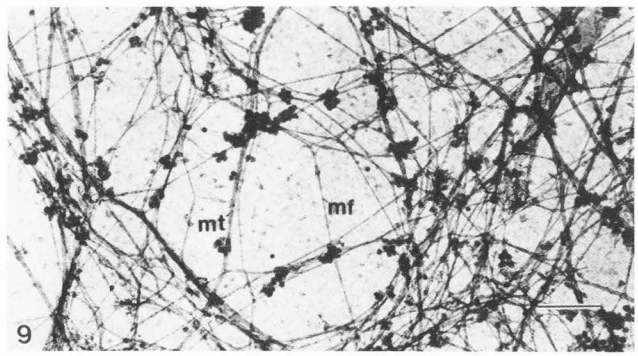

Figure 9: Extracted uninfected cell stained with anti-gp70, 15 nm colloidal gold. (For abbreviations, see Figure 6). Bar = 0.2 μm.

Acknowledgement

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Discussion with Reviewers

J. O. Mecham: Why did the authors use monoclonal anti-actin for the immunofluorescent studies and polyclonal anti-actin for the immunogold studies?

Authors: The monoclonal anti-actin was an IgM which does not react well with protein A-Au. The polyclonal anti-actin contains some IgG which binds more specifically to protein A-Au.

J. O. Mecham: After removal of CB do virus infected cells resume normal production of virus and reverse transcriptase?

Authors: Yes, this data is the subject of a separate publication (in preparation).

J. O. Mecham: What are the effects of 16 hour exposure with CB upon viability of cells? Is the decrease in virus production a reflection of disruption of the microfilament network or a reflection of cell viability?

Authors: The viability of cells is apparently not harmed by CB treatment since they appear to recover their normal distribution of antigen. They synthesize, and secrete virus normally.

J. O. Mecham: Can the immunogold staining pattern with anti gp70 be eliminated upon treatment of infected cells with CB?

Authors: We did not look at the phenomenon, but based on the fluorescence data, the answer would be yes.

J. O. Mecham: Have the authors examined the relative amount of gp70 and p15E associated with TX-100 soluble fraction versus the cytoskeleton by radioimmune assay or some other sensitive bioassay? This might help determine the significance of the observed association of viral proteins with the cell cytoskeleton that the authors have reported.

Authors: We have not done this. However, we have studied the relative amounts of $gPr80^{env}$ in the soluble and insoluble fraction after CB treatment by SDS-Page and found a change only in the insoluble fraction. This data is the subject of a separate publication.

J. O. Mecham: Have the authors examined the degree of intermediate filament involvement in determining the architecture of the cytoskeleton of 3T3 cells? This might shed some light upon the contribution of these elements to binding the viral proteins discussed in this paper.

T. D. Allen: Do you have any information as to the interaction between intermediate filaments and viral components?

Authors: We have not investigated the role of intermediate filaments and viral protein. However, there are some preliminary reports in the literature indicating that some interaction may occur.

R. Albrecht: The cells in Figure 4 appear to be less spread and perhaps abnormal in shape. Is this also due to the cytochalasin treatment?

Authors: Yes, the disruption of the microfilament network due to CB treatment would lead to an alteration in the cells' shape.

R. Albrecht: Has the association of viral proteins with cytoskeletal elements been observed in unextracted cells either by immunofluorescence or immuno-gold staining of whole cells or sectioned material? This would eliminate the possibility of nonspecific association of the viral proteins with cytoskeletal elements as a result of the extraction procedure.

Authors: We are currently developing techniques to investigate this association in intact sectioned cells and should have results within the year.

R. Albrecht: Do the authors know the concentration of the gold beads used in their labelling suspension?

Authors: No, the gold probes are used as supplied from Janssen.