Monoclonal Antibody-Aided Characterization of Cellular p220 in Uninfected and Poliovirus-Infected HeLa Cells: Subcellular Distribution and Identification of Conformers

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A monoclonal antibody directed against the M_r -220,000 subunit (p220) of the mRNA cap-binding complex has been prepared and used to analyze the sucrose gradient sedimentation and subcellular location of p220 and its poliovirus-induced cleavage products. The antibody reacted with p220 on immunoblots of cell lysates from uninfected cells, but only with several smaller polypeptides, the p220 cleavage products, in cell lysates from poliovirus-infected cells. The sedimentation of p220 antigens from uninfected or infected cells was analyzed by immunoblot and by enzyme-linked immunosorbent assay (ELISA) of sucrose gradient fractions. The results indicate that antibody reactivity was partially influenced by antigen conformation. Major forms of intact p220 and cleaved p220 were identified by immunoblot, and these had similar sedimentation properties. ELISA analysis of the same gradient fractions detected only uncleaved p220; p220 cleavage products were not recognized. Furthermore, the antibody recognized two forms of native uncleaved p220, one of which appeared to bind antibody with greater affinity. This result suggested the existence of conformational variants of p220. The differential reactivity of the antibody for cleaved versus uncleaved p220 served as a useful control during indirect immunofluorescence analysis to determine the subcellular distribution of p220 antigens. The distribution of p220 in uninfected cells was mainly cytoplasmic, but some nuclear antigens were also apparent. After poliovirus infection only the nuclear pattern remained. Disappearance of the cytoplasmic pattern confirmed the inability of the antibody to react with native p220 cleavage products. The cytoplasmic pattern also disappeared after human rhinovirus 14 infection, but not after mengovirus infection, results which correlated with the ability of human rhinovirus 14 and the inability of mengovirus to induce the cleavage of p220. The results demonstrate that p220 is not likely to be associated with the cytoskeleton and hint at the possibility of a partially nuclear location.

The inhibition of host cell protein synthesis caused by poliovirus infection has been correlated with the cleavage of a cellular M_r -220,000 polypeptide (p220) (12). p220 is a subunit of the protein synthesis initiation factor complex eIF-4F, which recognizes and binds to the mRNA cap structure (7, 15). Although p220 is part of eIF-4F, its role within the complex is still unknown. Cap binding appears to be the function of the p28 subunit (cap-binding protein [CBP]), the M_r 26,000 to 28,000 polypeptide (31) which can be chemically cross-linked to mRNA cap structures *in vitro* (34). The cap-binding property of CBP isolated from infected cells remains unaltered, however (16, 21), and no structural changes are detectable by two-dimensional gel electrophoretic analysis (3). Only a complex containing CBP along with other polypeptides can restore the ability of infected-cell lysates to translate capped mRNA (36). The translation-stimulating activity of the complex containing CBP also copurifies with p220 (D. Etchison and S. Milburn, *Mol. Cell. Biochem.*, in press); thus, it is likely that p220 plays an essential role in the cap recognition function of eIF-4F.

Important evidence for the direct involvement of p220 cleavage in host cell shutoff was recently obtained by the construction of a poliovirus carrying a mutation in polypeptide 2A which is temperature sensitive for both inhibition of host cell translation and ability to induce cleavage of p220 (1). Polypeptide 2A has now been confirmed to be the viral product responsible for inducing p220 cleavage, but it appar-

ently does so indirectly, possibly by activating a host enzyme (18a). Despite these advances in our understanding of the mechanism of virus-induced cleavage of p220, little is known about the mechanism of inhibition of translation which appears to result from that cleavage.

Several possible mechanisms have been suggested to explain how p220 cleavage might affect translation. Cleavage may result in the disruption or increased lability of a cap-binding complex (CBC), rendering it incapable of recognizing or utilizing capped mRNA. Evidence for disruption of a CBP-associated complex in infected cells has been presented previously (17). A rapidly sedimenting CBP complex found in crude initiation factors from uninfected cells was not found in infected cell preparations, while a slowly sedimenting complex was found in both infected and uninfected cell preparations. Analyses of these complexes for p220 were not conducted. Another role that p220 might play is that of a structural link between the translational machinery and the cell cytoskeleton. Immunofluorescence studies with monoclonal antibodies directed against CBPs have indicated that these polypeptides might be structurally bound to cytoskeletal elements (35, 39), which in turn have been reported to be associated with the translational apparatus (2, 4, 18, 23). The association of p220 with the cytoskeleton as a putative structural link has not been examined previously.

To study both the sedimentation and subcellular distribution of p220 before and after poliovirus infection, we have developed a monoclonal antibody directed against it. In this report we describe a conformation-dependent reactivity of

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the antibody which has permitted the identification of two p220-containing complexes on sucrose gradients and serves as a control for identifying the subcellular location of the p220 antigen by immunofluorescence. We show that the p220 subunit of the CBC is located mainly in the cytoplasm and that it does not appear to be associated with cytoskeletal structures.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in suspension culture in Joklik modified minimal essential medium supplemented with 6% calf serum and 2 mM glutamine. Poliovirus 1 (Mahoney strain) was grown in HeLa S3 cells according to previously published procedures (9). Human rhinovirus 14 (HRV-14) and its host HeLa H1 cell line (24) were obtained from Shay Fout and Roland Rueckert (University of Wisconsin, Madison). HeLa H1 cells were grown in monolayer culture in minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4). Mengovirus was originally obtained from John J. Holland (University of California, San Diego) and was also propagated on HeLa H1 cells. Sp2/O-Ag14 cells were obtained from the Salk Institute hybridoma bank (La Jolla, Calif.) and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 µg of gentamicin per ml, and 20 U of mycostatin per ml.

Purification of antigens. A CBC was purified by procedures described elsewhere (Etchison and Milburn, in press). Briefly, a ribosomal salt wash-40% ammonium sulfate-precipitated fraction (RSW-A) from HeLa S3 cells was prepared and applied to a 10 to 30% sucrose gradient in buffer A 100 (10 mM Tris, pH 7.4, 2 mM EDTA, 5% glycerol, 5 mM β-mercaptoethanol, 100 mM potassium chloride). After centrifugation the gradient was fractionated, and each fraction was analyzed for the presence of CBC activity and p220 antigen. Fractions containing both were pooled and chromatographed on DEAE-Sephacel. Column fractions containing both antigen and activity were pooled and designated CBC-DE. This preparation was used to immunize mice for the production of monoclonal antibodies, and it was used as the primary enzyme-linked immunosorbent assay (ELISA) screening antigen. Part of the CBC-DE preparation was further purified by chromatography on phosphocellulose P11. Pooled fractions containing p220 antigen and CBC activity, designated CBC-PC, were used as a secondary ELISA screening antigen.

High-salt-exposed eIF-3 was prepared for use as an immunogen and ELISA screening antigen as follows. RSW-A from uninfected Hela cells was sedimented on 10 to 30% sucrose gradients in buffer A 500 (500 mM KCl). eIF-3 was detected by immunoblot with an eIF-3/CBC antiserum as previously described (12), and fractions which contained eIF-3 antigens but not p220 antigens were pooled and concentrated by vacuum dialysis.

Immunization procedures and preparation of hybridoma cells. Approximately 25 µg of CBC-DE (100 µl) was mixed with an equal volume of Freund complete adjuvant and injected into each BALB/c mouse in several sites: popliteal nodes, footpads, and the abdominal cavity. Subsequent immunizations were done intraperitoneally with 25 µg of the immunogen mixed with an equal volume of Freund incomplete adjuvant. The immunization schedule was as follows: 3

months, 2 months, 1 month, 2 weeks, 1 week, 4 days, and 2 days prior to sacrificing the animals. Mouse spleen cells were fused with Sp2/O-Ag14 myeloma cells by the procedures of Goding (13). Hybridomas were screened by ELISA against the immunizing antigen, and positive cultures were cloned at least twice by limiting dilution. Antibody was collected as ascites fluid and stored at -70°C. Frozen cell stocks were established, and when recovery was necessary the cells were recloned. Hybridoma cells were maintained in the medium described for growth of myeloma cells supplemented with hypoxanthine and thymidine. No effort was made to wean cells from hypoxanthine and thymidine.

For development of monoclonal antibodies directed against eIF-3 polypeptides, mice were injected with 25 µg of high-salt-exposed eIF-3 in Freund complete adjuvant followed by 25 µg in incomplete adjuvant on the same schedule described for CBC immunization. Hybridoma production and ELISA screening were also identical except that the ELISA screening antigen used was the immunogen. Growth media from cultures which tested positive for production of antibody against eIF-3 were further tested for immunoblot reactivity against the immunogen as well as against cell lysate from uninfected and poliovirus-infected cells.

ELISA procedure. Polyvinyl plates were coated overnight with 50 µl of antigen diluted to 20 µg/ml in phosphate-buffered saline (PBS; 0.02 M potassium phosphate, pH 7.4, 150 mM sodium chloride). The plates were washed with 0.05% Triton X-100 and 0.02% sodium dodecyl sulfate (SDS), in PBS and blocked by incubation with 1% bovine serum albumin (BSA) in PBS. After incubation with the antibody solution, bound antibodies were detected by addition of goat anti-mouse immunoglobulin G (IgG) peroxidase conjugate (Cooper Biomedical, Malverne, Pa.). The orthophenylenediamine (1 mg/ml) substrate was prepared in 100 mM sodium citrate, (pH 5.0)-0.1% BSA-0.015% hydrogen peroxide. Color development was stopped by the addition of 10% sulfuric acid.

Purification and radioiodination of monoclonal antibodies. Antibodies were purified by DEAE chromatography by a modification of the procedure described by Laurent et al. (20). After 50% ammonium sulfate precipitation, the immunoglobulin fraction of ascites fluid was chromatographed on DEAE-Trisacryl developed with a gradient of 25 to 250 mM NaCl in 25 mM Tris, pH 8.8. The antibody peak was identified by ELISA against CBC-DE. Antibody-containing column fractions were pooled and concentrated, if necessary, to 1 to 5 µg/ml.

The DEAE-purified 25AC2 antibodies were iodinated by a modification of the procedure of Greenwood et al. (14). Ten micrograms of antibody in 50 µl of 25 mM Tris (pH 8.8)-50 mM NaCl was added to a Reacti-vial containing 1 mCi of sodium iodide (New England Nuclear Corp., Boston, Mass.) together with 50 mM potassium phosphate, pH 7.4. Thirty microliters of freshly prepared chloramine T was added to a final concentration of 50 mM. Within 30 to 45 s after the chloramine T was added, the reaction was stopped by the addition of 50 µl of saturated L-tyrosine. Two hundred microliters of 0.5% BSA-PBS was added, and the reaction mixture was applied to a 5-ml Sephadex G-50 column which had been preequilibrated with 0.5% BSA-PBS. The excluded radioactive material was pooled and made 0.02% in sodium azide and stored at 4°C. For immunoblots, this preparation was applied as a solution containing 2×10^5 to 5×10^5 cpm/ml of blotting buffer (approximately 0.01 µg of antibody per ml).

Both the chloramine T concentration and the time of

exposure to antibody were found to be critical in this procedure. Higher concentrations of chloramine T (80 $\mu\text{g/ml}$) and exposures of 2 min completely destroyed the antigen-combining activity of the anti-p220 antibodies. Instead, these preparations contained radiolabeled antibodies which were capable of reacting with a 36-kilodalton (kDa) band on immunoblots of infected and uninfected cell lysates. This band only appeared when high concentrations of antibody (approximately 0.5 $\mu\text{g/ml}$) were used and was a minor band or failed to appear at all on immunoblots done with antibody preparations labeled under conditions which preserved anti-p220 activity. The 36-kDa-reactive antibody is presumed to be an endogenous mouse antibody which is more resistant to the iodination procedure than the monoclonal antibody produced by the hybridoma cells.

Sucrose gradient sedimentation and immunoblot analysis. Postmitochondrial supernatants (S10) and ribosomal salt wash-0 to 40% ammonium sulfate fractions (RSW-A) were prepared from uninfected or poliovirus-infected HeLa S3 cells as described previously (12; Etchison and Milburn, in press). Fourteen A_{280} units of RSW-A from poliovirus-infected or 6 A_{280} units of RSW-A from uninfected cells were loaded onto separate 13-ml 15 to 30% sucrose gradients in buffer A 100. The gradients were centrifuged for 22 h at 38,000 rpm in a Beckman SW40 rotor, and 21 fractions were collected for each. Fifteen-microliter portions of the fractions from the poliovirus-infected RSW-A gradient or 30- μl portions of the fractions from the uninfected RSW-A gradient were applied to SDS-10% polyacrylamide gels. Proteins in the gels were electrophoretically transferred to nitrocellulose paper by a procedure which has been described previously (12), and immunoblots were prepared as indicated in the figure legends.

Indirect immunofluorescence. HeLa monolayer cells were grown to approximately 50 to 90% confluence on 12-mm circular glass cover slips in 24-well tissue culture plates. They were treated or infected as indicated, then washed with PBS, fixed, and permeabilized with acetone at -20°C for 5 min, and air dried. Where indicated, cells were infected with 500 PFU of virus per cell in 200 μl of medium without serum per well. After 30 min, 1 ml of medium plus serum was added, and infected cells were fixed 4 h after infection. Fixed cells on cover slips were incubated for 1 h at room temperature or overnight at 4°C with antibody diluted in 200 μl of BSA in PBS containing 0.02% sodium azide (BSA-PBS). They were then washed four times with BSA-PBS and further incubated with a 1/100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Antibodies Incorporated, Davis, Calif.) for 1 h at room temperature. After being washed five times with BSA-PBS, the cover slips were mounted onto a drop (5 μl) of 2% *n*-propyl gallate in 70% glycerol-PBS on microscope slides. The slides were viewed by epiillumination under the appropriate fluorescent filter. The fluorescent images were photographed on Kodak Ektachrome colored slide film with a Nikon camera with an automatic light meter. Figures are black and white photographic reproductions of the color slides.

Antibodies. Anticytokeratin monoclonal (mouse) antibodies were a gift of Paul Rossiter and Robert Cardiff. The preparation and characterization of these antibodies have been described (5). The antibody used in these studies is designated UCD/AB10.11 and reacts with cytokeratins 8 and 18. Antivimentin monoclonal (mouse) antibodies were purchased from Labsystems (Helsinki, Finland).

The monoclonal antibodies developed during this study, 25AC2 and 20FD5, were identified as IgG and IgM, respec-

FIG. 1. Immunoblot analysis with 25AC2 monoclonal antibodies. Uninfected (UNINF) and poliovirus-infected (INF) S10 cell lysates (0.2 A_{280} units of each) and 4 μg of the immunizing antigen (Im. Ag). CBC-DE, were loaded onto an SDS-10% polyacrylamide slab gel. After electrophoresis, the proteins were transferred to nitrocellulose paper, and the immunoblot was prepared by incubation with the following series of antibodies. The first antibody was applied by incubation with a 1/10,000 dilution of growth medium from 25AC2 hybridoma cells. The second antibody was applied by incubation with a 1/5,000 dilution of goat anti-mouse IgG, IgA, and IgM antisera (Cooper Biomedical, Malverne, Pa.), and the final antibody applied was an iodinated rabbit anti-goat IgG antibody (5×10^5 cpm/ml of blotting buffer).

tively, by ELISA with a series of typing antibodies. Both antibodies contain kappa light chains.

RESULTS

Preparation of hybridomas secreting antibodies directed against p220. To develop monoclonal antibodies directed against p220, a translationally active CBC was partially purified from HeLa cells for use as an immunogen (Etchison and Milburn, in press). Hybridomas were prepared as described in Materials and Methods and screened by ELISA against the immunogen. Growth medium from one culture reacted with preparations containing p220, but not with either purified eIF-4A or CBP. This culture and the anti-p220 antibodies produced by these cells will be referred to by the original culture well designation, 25AC2. Positive-testing growth medium was also analyzed by immunoblot against the immunogen as well as crude cell lysates from uninfected and poliovirus-infected cells. Figure 1 shows immunoblot results for growth medium from twice-cloned 25AC2 cells. The antibody reacted with an M_r 220,000 polypeptide (or set of polypeptides) in the immunogen and in the uninfected cell lysate, but only with the smaller cleavage products present in poliovirus-infected cell lysates. The antigen is therefore identical to the polypeptide, which is cleaved after poliovirus infection. Furthermore, since the cleavage products all have the same antigenic epitope, it is possible that they are the result of structural modifications of a single polypeptide.

Immunoblot and ELISA detection of p220 in sucrose gradient fractions of crude initiation factors. Previous results had

FIG. 2. Immunoblot and ELISA analysis of crude initiation factors from uninfected and poliovirus-infected cells with the anti-p220 monoclonal antibody. RSW-A fractions from uninfected and poliovirus-infected HeLa S3 cells were prepared, fractionated by sucrose gradient sedimentation, and analyzed by immunoblot as described in Materials and Methods. The immunoblots were prepared by incubation with iodinated 25AC2 monoclonal antibodies. Control lanes are RSW from poliovirus-infected (PSW) and uninfected (HSW) HeLa cells. The gel positions of p220 and the cleavage products (CP) are indicated. For ELISA analysis, 25 μ l of each gradient fraction was applied together with 25 μ l of PBS to wells of a microtiter plate and incubated overnight at 4°C. The primary antibody was applied as a 1/1,000 dilution of ascites fluid from mice primed with 25AC2 hybridoma cells, and bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies as described in Materials and Methods. Color development was quantified spectrophotometrically by an automated ELISA plate reader. For comparison, the same procedure was followed with a 1/1,000 dilution of ascites fluid from mice primed with anti-p170-eIF-3 hybridoma cells, and the two graphs for each gradient are superimposed. Symbols: ●, anti-p170-eIF-3 (20FD5) reaction; ×, anti-p220 (25AC2) reaction. Fractions of the gradient of RSW-A from uninfected cells (A and C) and infected cells (B and D).

indicated that p220 present in crude initiation factor preparations cosediments with eIF-3 in the presence of buffers of low ionic strength (100 mM salt) (12, 37). Furthermore, after poliovirus infection the association of CBP with eIF-3 appears to be abolished (17). To analyze the sedimentation of the p220 antigen recognized by monoclonal antibody 25AC2 before and after infection, two different detection methods were used. Figure 2 shows immunoblot and ELISA analyses of fractions of two sucrose gradients of RSW-A from uninfected and poliovirus-infected cells. The two gradients were run in parallel and fractionated identically. Autoradiograms of the immunoblots reacted with the directly iodinated monoclonal antibody are shown (Fig. 2A and B). This method detects SDS-denatured antigen, and the intensity of the radiolabeled band should indicate the relative quantity of antigen present in each fraction. In the gradient of RSW-A from uninfected cells (panel A), intact p220 was detected in a broad region, from fraction 9 to fraction 17. Fraction 15 appeared to contain the greatest amount of antigen. In the gradient of RSW-A from infected cells (panel B), cleaved p220 antigen was detected in a narrower region of the gradient, from fraction 15 to fraction 17, with fraction 16 containing the greatest amount of antigen. Although uncleaved p220 appeared to trail more than cleaved p220, this may simply be due to differences in the amount of antigen present in these two gradients or to differences in the sensitivity of the antibody for the two forms of antigen. Since the sedimentation of cleaved and uncleaved p220 antigen differed only slightly, as indicated by the relative positions of the peak fractions, it is likely that p220 is part of

a complex with other polypeptides and that cleavage does not significantly alter the structure of the complex. Evidence that some of the p220 cleavage products remained associated with a CBC that can be isolated by cap analog affinity chromatography was presented earlier by Lee et al. (21). Here we have shown that cleavage of p220 does not dramatically affect the sedimentation of the major form of a p220-associated complex.

p220 antigen present in the same sucrose gradient fractions analyzed by immunoblot was also detected by measuring color development in an ELISA reaction (Fig. 2C and D). This method detects non-SDS-denatured antigen bound to microtiter plates. The antibody reacted with nondenatured intact p220 (panel C), but the antigen sedimentation pattern determined by the ELISA detection method was different from that of the immunoblot method. By ELISA there appeared to be two forms of p220. One form peaked in fraction 9 (the rapidly sedimenting form), and the other peaked in fraction 15 (the slowly sedimenting form). The slowly sedimenting form was the major form of p220 detected by immunoblot, while the rapidly sedimenting form presumably represented a conformational variant of p220 in which the antigenic epitope was more exposed for antibody binding. In similar sucrose gradient analyses of other RSW-A preparations, the slowly sedimenting p220 form detected by ELISA often appeared to be severalfold more abundant than the slowly sedimenting form, yet by immunoblot it was still the minor form. The reason for this variability is not known. By contrast, when sucrose gradient fractions of RSW-A from poliovirus-infected cells were analyzed by

FIG. 3. Indirect immunofluorescence pattern of anti-p220 monoclonal antibody in uninfected and poliovirus-infected cells. HeLa monolayer cells were grown to near confluency on cover slips in 24-well tissue culture plates. One set of wells was infected with poliovirus, and 4 h after infection the cover slips were processed for immunofluorescence as described in Materials and Methods. The air-dried slides were incubated with a 1/200 dilution of DEAE-purified 25AC2 antibodies in 200 μ l of BSA-PBS followed by a 1/100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibodies and processed for microscopy as described in Materials and Methods. (A and C) Uninfected cells (A, $\times 1,000$; C, $\times 500$); (B and D) cells infected for 4 h (B, $\times 1,000$) or 2 h (D, $\times 630$).

ELISA, neither form of cleaved p220 was detected (Fig. 2D). The antibody apparently was unable to react with the native nondenatured form of cleaved p220 and could only react with the SDS-denatured form of the cleavage products. For comparison, a second antigen was also detected by ELISA with a monoclonal antibody (20FD5) directed against the eIF-3 subunit p170 (immunoblot reactivity not shown). This control demonstrated that sedimentation in the two gradients was virtually identical and that each gradient contained similar amounts of initiation factors.

Cellular distribution of p220-related antigens in uninfected and poliovirus-infected cells. Because monoclonal antibody reactivities can be identified with precision, they are an important tool for determining antigen distribution by immunofluorescence studies of intact cells. Since the function of p220 is not yet known, information about its subcellular location might conceivably aid in the search for its function. In particular, we hoped to learn whether this subunit of the CBC was partitioned in the cytoskeletal subfraction of the cell. Indirect immunofluorescence was therefore used to determine the cellular distribution of the antigen recognized by 25AC2 antibodies. Figure 3 shows the immunofluorescence pattern in HeLa monolayer cells. The cells were permeabilized and fixed by treating with acetone at -20°C . Several other fixation procedures designed to fix microfilaments and microtubules (39) were tested, but all gave identical results. Since the acetone fixation procedure does not fix either microtubules or microfilaments, the antigen is not likely to be associated with either cytoskeletal element. In uninfected cells (Fig. 3A) the antigen pattern was largely cytoplasmic and did not appear to be fibriform. There was also a fainter pattern over the nucleus, and the nuclear pattern often included "speckles." After poliovirus infection (Fig. 3B), the cytoplasmic pattern faded significantly or disappeared, as shown, so that only the nuclear speckles

remained. Figure 3D shows a reduced magnification of infected cells 2 h after infection. In some cells the cytoplasmic pattern had largely disappeared, while in other cells there was still some cytoplasmic pattern, but the cytoplasm appeared to be more vacuolated than that of uninfected cells. The reduction or disappearance of the cytoplasmic pattern correlated well with the previous finding that 25AC2 antibodies were unable to react with non-SDS-denatured p220 cleavage products, and therefore the cytoplasmic pattern of uninfected cells was indeed that of p220 and not due to some nonspecific reactivity. There was virtually no background staining by the fluorescein-conjugated antibodies alone on a control slide (not shown).

One of the characteristics of the 25AC2 immunofluorescence pattern in uninfected cells was increased intensity of fluorescence in cells undergoing division. Figure 3C shows a photograph which was exposed for such a cell, presumably in anaphase, in which the cytoplasm fluoresced intensely and the streak formed by the separating chromosomes was highly visible. Two cells which were in the final stages of division were much more faintly fluorescent. Also present in the field were normal nondividing cells, but these are not visible in this exposure. Exposure for the nondividing cells resulted in overexposure of the dividing cells. This immunofluorescence pattern suggested a possible redistribution of p220 antigen during cell division. The increased intensity of 25AC2 antibody-directed immunofluorescence in dividing cells was not seen in the immunofluorescence patterns of the other antibodies used in this study.

p220 antigen distribution in HRV-14 and mengovirus-infected cells. Other picornaviruses which are capable of inducing cleavage of cellular p220 include HRV-14 (10) and coxsackievirus (unpublished data). Not every picornavirus effects this cleavage, however, since the mouse picornavirus encephalomyocarditis virus (EMC) does not (27). To inves-

FIG. 4. Indirect immunofluorescence of (A) HRV-14- and (B) mengovirus-infected cells with the anti-p220 monoclonal antibody. HeLa cells on 12-mm cover slips were infected with HRV-14 or mengovirus as described in Materials and Methods. Four hours after infection the cover slips were removed from the wells and processed for immunofluorescence. Magnification, $\times 630$.

to investigate the effect of infection by picornaviruses other than poliovirus on the p220 immunofluorescence pattern, HeLa cells infected with HRV-14 and with mengovirus, a close relative of EMC, were processed for anti-p220 immunofluorescence analysis by the methods described in the preceding section. The p220 immunofluorescence pattern of HRV-14-infected cells was similar to the poliovirus-infected pattern shown previously (Fig. 4). In this photograph there is a faint overall background fluorescence which was within the range of variability of the pattern seen in poliovirus-infected cells, but there was no obvious demarcation between the nucleus and the cytoplasm, a characteristic which is typical of uninfected cells. Like the pattern seen with poliovirus-infected cells, there were speckles of fluorescence over the nuclei. The p220 immunofluorescence pattern of mengovirus-infected cells, by contrast, was more typical of the pattern of uninfected cells in that the cytoplasmic pattern

was still present and was distinct from the nucleus. Interestingly, the cytoplasmic pattern accentuated the appearance of cytopathology. The cytoplasm appeared to be highly vacuolated and the nuclei appeared to be crenated. Although there were no nuclear speckles, there was still a faint pattern of fluorescence over the nucleus.

Comparison of indirect immunofluorescence patterns of cytoskeletal and eIF-3-associated antigens in uninfected and poliovirus-infected cells. Because of previous reports that a 50-kDa subunit of the CBC might be associated with the cell cytoskeleton (39), it was of interest to determine whether the p220 subunit might be similarly distributed. Since the p220 immunofluorescence pattern was the same whether or not microtubules or microfilaments were preserved during the fixation procedure, only an intermediate filament association seemed likely. HeLa cells contain two types of intermediate filaments, cytokeratins (types 7, 8, 17, and 18) and vimentin (26). Mouse monoclonal antibodies directed against cytokeratins 8 and 18 vimentin were obtained for use in determining the immunofluorescence patterns of these cytoskeleton subunits under conditions identical to those used for the p220 antibody.

The cytokeratin immunofluorescence pattern in uninfected and infected cells was distinctly fibriform and did not resemble that of p220, and there was no significant change in the pattern after infection (Fig. 5A and B). Since the cytokeratin intermediate filaments are mixed polymers of the various cytokeratin subunits, the pattern which arose by

FIG. 5. Indirect immunofluorescence with antibodies directed against cytokeratin, vimentin, or the p170-eIF-3 subunit. Uninfected HeLa monolayer cells or cells infected with poliovirus were acetone fixed on cover slips. One cover slip containing infected cells and one containing uninfected cells were independently incubated with one of the following: a 1/500 dilution of anticytokeratin antibodies, a 1/100 dilution of antivimentin antibodies, or a 1/500 dilution of anti-p170-eIF-3 antibodies. Processing of cover slips for immunofluorescence and a description of the antibodies is presented in Materials and Methods. Magnification, $\times 510$.

reaction with antibodies directed against a subset of the cytokeratins should be representative of the entire population of cytokeratin subtypes.

The immunofluorescence pattern produced by the antivimentin antibody, like the p220 pattern, was not fibriform (Fig. 5C and D). However, one aspect of this vimentin pattern that was not shared by the p220 pattern was a more intense fluorescence, or capping, around one portion of the nucleus. The p220 pattern did not show this capping distribution. Interestingly, there appeared to be a slight increase in total fluorescence due to antivimentin antibodies in infected cells compared with uninfected cells. This was most evident when viewing slides under low magnification (4 $\times$), but it can be seen to some degree by comparing Fig. 5C and D. This increase may be related to the increase in cytoskeletal structures seen after poliovirus infection by electron microscopy (22).

For further comparison, an antibody directed against the p170 subunit of the protein synthesis initiation factor complex eIF-3 was used for indirect immunofluorescence. This antibody (20FD5) was the same one used for the ELISA analysis of sucrose gradients shown in Fig. 2. p170-eIF-3 distribution was entirely cytoplasmic (Fig. 5E and F). There was no trace of fluorescence in the nucleus. A solely cytoplasmic distribution might be expected of a protein which is involved only in the initiation of protein synthesis. Furthermore, there was no significant change in antigen distribution after infection. By comparison, p220 appeared to be more widely distributed, due to its apparent nuclear as well as cytoplasmic location, than p170-eIF-3.

Among the hybridomas prepared from the CBC-immunized mice, one high-titer antibody was found which reacted with p220-containing preparations by ELISA and produced a fibriform immunofluorescence pattern (Fig. 6). The immunoblot reactivity, however, indicated that the antibody reacted with a large number of polypeptides present in the total cell lysate, as well as p220 present in the immunogen (very weakly by immunoblot). Furthermore, it reacted with several polypeptides which appeared to be unique to the poliovirus-infected cell lysate (arrows). This antibody presumably reacted with a determinant common to a variety of polypeptides. When used at a fairly high dilution (1/5,000) for indirect immunofluorescence, it reacted most strongly with a cytoskeletal protein. This pattern was not altered by infection but was altered by treatment with sodium orthovanadate (data not shown), a reagent which has been found to disrupt intermediate filaments (38). At higher concentrations of this antibody the immunofluorescence pattern became more complex and included a diffuse cytoplasmic pattern as well as a more complex cytoskeletal pattern. The existence of such antibodies, which react with determinants common to a large number of polypeptides, indicates that immunofluorescence results must be interpreted with care and may provide an explanation of apparently contradictory results reported in other studies.

DISCUSSION

At present, the only structural defect which has been detected in poliovirus-infected cells which might account for the virus-induced inhibition of host cell mRNA translation is cleavage of the p220 subunit of the CBC. CBCs containing p220 appear to be required for translation of capped mRNA *in vitro* (8, 15; Etchison and Milburn, *in press*), yet the actual function of p220 during translation remains unknown. This report describes the characterization of a monoclonal anti-

body which reacts with p220. This antibody has been used to locate p220 in whole cells by immunofluorescence and to detect p220-containing complexes on sucrose gradients by immunoblot and ELISA. We have found evidence for the existence of more than one conformational form of p220 in initiation factor preparations and have demonstrated a differential reactivity with uncleaved and cleaved p220. We have also found the subcellular distribution of p220 to be mainly cytoplasmic but possibly also nuclear. There is no evidence for an association of this subunit of the CBC with cytoskeletal structures.

Comparison of the immunoblot and ELISA analyses of p220-related antigens in sucrose gradients of crude initiation factors from uninfected and poliovirus-infected cells demonstrated that the reaction of the anti-p220 antibody for antigen was partly conformation dependent. The antibody reacted with both native and SDS-denatured intact p220 by ELISA and immunoblot, respectively, but only with SDS-denatured cleavage products on immunoblots. These results suggest that the antigenic epitope which is normally exposed on the intact p220 molecule may become folded into an inaccessible region of the secondary structure of the cleaved p220 polypeptide. Alternatively, the epitope on the cleavage products might be blocked by an interaction with another protein. The antibody also reacted with two conformers of p220. One of these conformers sedimented more rapidly than the other, possibly as a complex with eIF-3. Although the rapidly sedimenting form represented a minor amount of p220 as detected by immunoblot, the antibody reacted more readily with it, presumably because the antigenic epitope was more

FIG. 6. Immunoblot and indirect immunofluorescence analysis with monoclonal antibody 26ED3. Immunoblot and immunofluorescence procedures were identical to those described in the legends to Fig. 1 and 3, with the following exceptions. The first antibody to be applied to the immunoblot was a 1/5,000 dilution of ascites fluid obtained from mice injected with the 26ED3 hybridoma cells. For immunofluorescence the cover slip of acetone-fixed uninfected cells was first incubated in a 1/5,000 dilution of 26ED3 ascites fluid. Magnification, $\times 630$.

exposed for antibody binding as a result of changes in secondary structure due to interaction with other polypeptides.

These results can be correlated with previously published results demonstrating that the CBP which is detected by a cross-linking reaction is found in two forms in crude initiation factors from uninfected cells (17). One of these forms of CBP appeared to cosediment with eIF-3 antigens. It is quite possible that the two p220 forms identified in this report and the two CBP forms identified earlier are part of the same two complexes. In the earlier report CBP was found exclusively in a slowly sedimenting form in factors from infected cells. Although we were unable to detect native cleavage products by ELISA with this antibody, there was no apparent antigen detectable by immunoblot in the rapidly sedimenting region of the gradient. Thus, it is possible that the rapidly sedimenting p220 complex is present only in uninfected cells. To confirm this it will be necessary to develop additional monoclonal antibodies which react with the native form of the cleavage products.

One problem that was partially resolved here is the identity of the multiple cleavage product polypeptides. Since these polypeptides were previously detected by polyclonal antisera (12), it was unclear whether they represented different or similar polypeptides. The presence of an identical antigenic epitope on at least three cleavage product polypeptides as revealed by immunoblot reactivity with the monoclonal antibody of Fig. 1 and 2 indicates that they all share common sequences. Whether they represent a nested set of polypeptides or are differentially modified by carbohydrate, phosphorylation, or other posttranslational modifications must still be determined. Intact p220 also appears to be a set of multiple bands (10); thus, each cleavage product polypeptide may be derived by cleavage at the same amino acid sequence of a precursor molecule, which itself represents one of a nested set or otherwise structurally modified polypeptides.

The lack of an apparent association of p220 with the cytoskeleton may appear to be in contradiction to a previous report that antibodies to another CBC polypeptide (p50) react with cell cytoskeleton (35, 39). One explanation for the previous results might be the preparation of an antibody which, although raised against CBC, cross-reacts with a cytoskeletal polypeptide. We have shown that one of the anti-p220 monoclonal antibodies that we prepared resulted in a distinctly cytoskeletonlike immunofluorescence pattern but proved to be of fairly nonspecific reactivity by immunoblot analysis. In spite of the high degree of specificity of reactivity provided by monospecific antibodies, reaction with polypeptides which are unrelated to the antigen but may be carrying similar epitopes can occur (19, 28). Alternatively, the 50-kDa polypeptide with which the antibody reacted might have been a cytoskeleton subunit which contaminated the CBC preparation.

A second explanation for the apparently contradictory results is the possibility that p220 is more widely distributed than eIF-4A, the p50 subunit of the CBC. It is possible that eIF-4A is associated with the cytoskeleton, while p220 has both cytoskeletal and cytoplasmic distribution. Our immunofluorescence results might not detect a minor population of p220 molecules bound to cytoskeletal filaments. It is also possible that p220 and eIF-4A are only transiently associated during translation *in vivo*. We and others have shown that eIF-4A may be separated from eIF-4F without affecting the ability of the CBC to function *in vitro* (30; Etchison and Milburn, *in press*). Furthermore, eIF-4A can be purified as a

separate initiation factor, and only a minor portion of the cell's complement of eIF-4A appears to be associated with eIF-4F. It might not be surprising, therefore, if p220 and eIF-4A were not found in the same cellular location.

The appearance of a nuclear immunofluorescence pattern which did not disappear after poliovirus infection was surprising. At first it appeared that this might be due to antibody cross-reactivity, because cleaved p220 was not recognized by the antigen. However, one could imagine that a nuclear location for some of the p220 might protect it from a protease activity located in the cytoplasm. The finding of CBPs in the nucleus is not new (29). Several polypeptides present in the nucleus were identified by photoaffinity labeling to a cap analog. This approach would not be expected to identify p220, however, because this subunit of the CBC does not appear to cross-link to cap structures. Continuing efforts to identify the location of p220 by immunoblot of various cell subfractions suggest that a small amount of antigen remains associated with nuclei (unpublished data). To rule out non-specific trapping, however, it will be necessary to compare the cofractionation of some defined nuclear and cytoplasmic antigens with the p220 antigen under various fractionation conditions.

Earlier, we outlined two possible mechanisms by which p220 cleavage might inhibit host cell translation; disruption of a CBC structure or release of the complex from a functional association with the cell cytoskeleton. Results of studies with a monoclonal antibody to identify p220 antigens indicated that the p220-containing complex in uninfected cells was not associated with the cytoskeleton in the first place. An effect on complex structure or its association with other initiation factors is a more likely possibility.

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