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Cross-Linking of a Polyomavirus Attachment Protein to Its Mouse Kidney Cell Receptor†

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We used photoaffinity cross-linking with the heterobifunctional cross-linker *N*-hydroxysuccinimidyl 4-azidobenzoate (HSAB) to covalently link polyomavirus to a mouse kidney cell surface component. The virus-HSAB combination was adsorbed to the cells and then cross-linked and isolated in monopinocytotic vesicles from the cells after endocytosis. The cross-linked product was identified on sodium dodecyl sulfate-polyacrylamide gels by the presence of a new band carrying ^{125}I-labeled virion protein with a higher molecular mass than the normal virion protein bands. A single new band, with an apparent molecular mass of 120 kilodaltons (120 kDa), was identified by this procedure. This band was formed only in the presence of the HSAB cross-linker when virions were bound to the cells. The band also copurified with cross-linked virions when virion-containing vesicles were treated with detergent to remove the cell membrane. Antibody treatments that blocked up to 100% of virus binding and internalization also blocked cross-linking, as measured by the formation of the 120-kDa band. The 120-kDa band was characterized by preparation of antibody against the excised band from the gel. This antibody was shown to have the expected dual specificity for polyomavirus VP1 sequences and plasma membrane proteins, as analyzed on Western blots. The anti-120-kDa antibody was also shown by immunofluorescence to bind to the surface of mouse kidney cells. These data have demonstrated that molecules of possible biological significance in the binding of polyomavirus to mouse kidney cells have been cross-linked and that cell surface molecules have been identified that may be characterized further for possible receptor function in polyomavirus attachment.

The entry of polyomavirus into mouse kidney cells (MKC) takes place by receptor-mediated endocytosis, in which the virion particle is enclosed by the cell membrane to form monopinocytotic vesicles (16). These vesicles are transported to the nucleus, where the infecting virions cross the nuclear membranes and initiate productive infection (21). Attachment of polyomavirus to MKC has been characterized by binding studies, which demonstrated that polyoma virions and empty capsids utilize different receptors (6). It has also been shown that isoelectric species D, E, and F of polyomavirus VP1 are the virus attachment proteins (5), but nothing is known about the cellular receptor for polyomavirus. Thus, our knowledge of the details of early events of polyomavirus infection is still limited.

As an initial approach to the study of polyomavirus attachment and entry, we isolated the virion-containing monopinocytotic vesicles from the cytoplasm of infected MKCs (16). These vesicles contained individual virions surrounded by plasma membrane that serves as an enriched source of receptor molecules that are recognized by polyomavirus on the MKC surface. The technique of photoaffinity cross-linking was chosen to continue this investigation of the cell receptors for polyomavirus because of the widespread success of the technique in similar systems involving receptor-mediated endocytosis of hormones and other ligands (11, 20). The specificity attainable by this technique is particularly important with ligands such as polyomavirus that have relatively few (about 10,000) receptors per cell (5). Thus, specific cross-linking should enable us to identify a cell surface component involved in virus binding. This component then could be analyzed in the isolated monopinocytotic vesicles, as well as the virions recovered from the vesicles. In this work, we report the application of these techniques in the polyomavirus-MKC system as a further step in identifying the molecules involved in virus attachment.

MATERIALS AND METHODS

Cell and virus propagation. Primary cultures of MKCs were prepared as described previously (10, 26). Wild-type small-plaque polyomavirus was used to infect MKCs. Infected cultures were maintained in serum-free Dulbecco modified Eagle medium (23).

Radioactive labeling of virus. Polyomavirus was purified from infected cell lysates as described previously (7, 23). Purified virus was labeled in vitro with ^{125}I with chloramine T by the method of Frost and Bourgaux (15) by using excess tyrosine instead of sodium metabisulfite to quench the reaction.

Photoaffinity cross-linking of polyomavirus. The heterobifunctional reagent *N*-hydroxysuccinimidyl 4-azidobenzoate (HSAB) (Pierce Chemical Co.) was prepared fresh for each use as a 1 mM solution in 100% dimethyl sulfoxide. This stock solution, as well as all subsequently prepared solutions containing HSAB, was protected from light to prevent premature formation of the nitrene radical from the azidobenzoate portion of the molecule. Purified, ^{125}I-labeled polyomavirus, which was dialyzed against 0.01 M sodium phosphate (pH 7.5), was mixed with the HSAB solution to obtain a final concentration of 50 μM HSAB. This mixture was mixed by inversion at room temperature (25°C) for 45 min to allow reaction of the HSAB with the virus. The reaction was then quenched by the addition of 1 M arginine hydrochloride (pH 7.5) to a final concentration of 0.05 M.

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The mixture was then mixed for an additional 30 min at room temperature to quench the unreacted HSAB.

Plates (100-mm diameter) of washed primary MKCs were cooled on ice. The derivatized virus preparation (virus-HSAB) was diluted with phosphate-buffered saline (PBS) to allow an infection volume of 0.7 ml per plate. This mixture was then added to the plates, and the virus was allowed to adsorb for 1 h at 0°C with rotation of the plates at 20-min intervals. All operations, mixing, dilutions, and adsorption were performed in near darkness.

Completion of the cross-linking was achieved by removing the covers from the plates and exposing the MKCs to longwave UV light from a Mineralight model UVSL-25 UV lamp for 15 min at a distance of 30 cm. During the irradiation, the plates were kept at 0°C on ice. Afterwards, the plates could be exposed to room light without the cross-linking being affected.

Isolation of monopinocytotic vesicles. Infection was initiated on the cells immediately after the last step of cross-linking. The plates were incubated at 37°C for 1 h, followed by treatment with receptor-destroying enzyme, cell fractionation, and isolation of virus-containing vesicles on the 15 to 45% sucrose gradient as described previously (16).

Recovery of cross-linked virus from isolated vesicles. Detergent treatment of isolated vesicles to recover cross-linked virus was accomplished by conditions which were more stringent than reported previously (16) but which still preserved the integrity of the virions, as judged by sedimentation properties. The fractions of vesicle material from the 15 to 45% sucrose gradient were pooled in a Beckman SW50.1 centrifuge tube and diluted to 5 ml with 0.01 M Tris hydrochloride (pH 7.4) containing 0.15 M NaCl and 0.01% Triton X-100. The vesicles were pelleted for 90 min at 33,000 rpm in the Beckman SW50.1 rotor. The vesicles were resuspended in 1 ml of the same dilution buffer that contained a final concentration of 0.2% Nonidet P-40. This suspension was mixed by inversion overnight (18 h) at 4°C. After the Nonidet P-40 treatment, the suspension was placed directly on a 10 to 30% sucrose gradient containing 0.01 M Tris hydrochloride (pH 7.4), 0.15 M NaCl, and 0.01% Triton X-100 and centrifuged for 45 min at 33,000 rpm in the Beckman SW50.1 rotor. The virus band was pooled, diluted to 5 ml with 0.01 M Tris hydrochloride (pH 7.4) containing 0.15 M NaCl and 0.01% Triton X-100, and pelleted for 90 min at 33,000 rpm in the Beckman SW50.1 rotor. The cross-linked virus thus recovered and concentrated was free of contaminating membranes and was used for analysis by gel electrophoresis.

Electrophoresis. Preparations of cross-linked virus and other samples were treated and analyzed on a 5 to 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gradient slab gel system as described previously (16).

Immunoblots. Protein samples analyzed on the SDS-PAGE gradient gels were transferred to nitrocellulose sheets by the method of Bittner et al. (4) by using 10% methanol in the transfer buffer. The sheet was blocked for 16 to 18 h in PBS, pH 7.2, containing 0.05% Tween 20, as described by Batteiger et al. (3). The ^{125}I-labeled immunoglobulin G (IgG) fractions of prepared antibodies were then added in fresh PBS-Tween 20 and incubated with the nitrocellulose sheet for another 16 to 18 h. The labeled antibody solution was removed, and the nitrocellulose sheet was washed ten times with an equivalent volume of PBS-Tween 20; one washing lasted 12 to 14 h. Then the sheet was dried, and an autoradiogram was prepared to identify bands to which the ^{125}I-labeled IgG bound on the sheet.

Whole-cell digestion to recover virus. Experiments using only 10^7 MKCs (i.e., one 100-mm-diameter plate) necessitated a direct method for recovering bound or internalized virus or both. After being washed and then treated with receptor-destroying enzyme (16), the MKCs were scraped from the plates, pelleted, and treated with 0.5% Nonidet P-40–0.1% (wt/vol) Zwittergent 3-14 (Calbiochem-Behring) in 0.01 M Tris hydrochloride, pH 7.5, containing 0.15 M NaCl. This suspension was rocked overnight (18 h) at room temperature (25°C) to disrupt the cells, with the virions remaining intact. After a low-speed centrifugation for 10 min at 8,500 rpm in a Sorvall HB-4 rotor, the supernatant was centrifuged for 2 h at 33,000 rpm in the SW50.1 rotor to pellet the released virus. This pelleted virus was used directly for SDS-PAGE analysis.

Plasma membrane preparation. The procedure for preparation of plasma membranes was adapted from the method of Esko et al. (14). MKCs were washed, scraped into PBS, and pelleted. They were then lysed by dropwise addition of the resuspended cell pellet into cold, stirred 1 mM Tris hydrochloride, pH 7.4, by using 1 ml of Tris hydrochloride per 100-mm-diameter plate of cells used. After 1 h of stirring on ice, the lysed cell suspension was pelleted by centrifugation for 20 min at 8,500 rpm in the HB-4 rotor. The pelleted material was resuspended in an equal volume of 10 mM Tris hydrochloride, pH 7.4, and centrifuged again at 5,000 rpm in an HB-4 rotor for 10 min to pellet unbroken cells and large organelles. Then the supernatant was centrifuged at 8,500 rpm in an HB-4 rotor for 40 min to pellet the crude membranes. This crude membrane pellet was resuspended in 10 mM Tris hydrochloride and layered on a four-step sucrose gradient consisting of 0.5 ml of 60% sucrose, 1.0 ml of 45% sucrose, 2.0 ml of 31% sucrose, and 1.0 ml of 16% sucrose, each solution having 10 mM Tris hydrochloride. This gradient was centrifuged for 2 h at 33,000 rpm in the SW50.1 rotor, and the upper band at the 31% sucrose–16% sucrose interface was collected. The collected material was diluted with 10 mM Tris hydrochloride in another SW50.1 centrifuge tube and pelleted for 1 h at 33,000 rpm in the SW50.1 rotor. This plasma membrane pellet was resuspended and used in immunoblot experiments. The protease inhibitors aprotinin, phenylmethylsulfonyl fluoride, *n*-α-*p*-tosyl-1-lysine chloromethyl ketone (TLCK), L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK) (all from Sigma Chemical Co.), and leupeptin (all at 10 μg/ml) were used throughout the cell lysis and plasma membrane isolation procedures.

Preparation of the anti-120-kDa antibody. HSAB cross-linking was carried out with purified, unlabeled polyomavirus in a relatively high multiplicity of infection (>20 μg of virus protein per 100-mm-diameter MKC plate) experiment. A small amount (10×10^6 cpm) of ^{125}I-labeled virus was included as a marker during the isolation to identify the cross-linked virus. Recovered cross-linked virus was applied to the SDS-PAGE gradient system, and an autoradiogram was prepared from the wet gel. The autoradiogram was used as a guide, and the 120-kilodalton (120-kDa) band was excised from the gel. This gel material was homogenized with complete Freund adjuvant and PBS and was injected subcutaneously into New Zealand White rabbits. Subsequent antigen preparations were identical except for the use of incomplete Freund adjuvant. The IgG fraction was obtained from serum by sodium sulfate precipitation and dialysis to remove excess salt. Then the dialyzed material from 10 ml of serum was applied to a 5-ml-bed-volume column of Affi-Gel Blue (Bio-Rad Laboratories) and eluted with 0.01 M Tris hydrochloride, pH 8.0, containing

0.028 M NaCl. The determinations of the IgG fraction eluted in the void volume of the column and of protein were made on pooled fractions. Preimmune IgG was prepared as described above.

Immunofluorescence. Cover slips of MKCs were washed, and the MKCs were fixed with 1% formaldehyde for 30 min at 4°C. After being washed, the MKCs were treated with goat anti-rabbit serum containing Evans Blue to reduce nonspecific adsorption and enhance background contrast. Then rabbit anti-120-kDa IgG or preimmune IgG was added, followed by goat anti-rabbit serum conjugated with fluorescein isothiocyanate. The cells were washed several times after each addition. Cells thus stained were viewed with a Leitz Wetzlar Ortholux UV microscope.

RESULTS

Cross-linking of polyomavirus to the surface of MKCs. Polyomavirus was labeled with ^{125}I and then was reacted with the cross-linker HSAB in darkness. The ^{125}I-labeled virus-HSAB mixture was allowed to bind to MKCs in darkness at 0°C, followed by completion of the cross-linking by irradiation with UV light with the virus bound to the cell surface. After internalization was allowed to occur at 37°C, monopinocytotic vesicles containing polyomavirus were isolated from the cells by using sucrose gradients (16). The sedimentation patterns of vesicles containing polyomavirus without cross-linking (panel A) and vesicles containing polyomavirus with HSAB cross-linking (panel B) are shown in Fig. 1. Both profiles show vesicle peaks I and II in the sucrose gradient; these peaks represent the characteristic banding pattern of virion-containing vesicles routinely observed and reported previously (16). Thus, the formation of covalent cross-links between the virus and the cell surface apparently does not prevent the internalization and formation of monopinocytotic vesicles in polyomavirus infection.

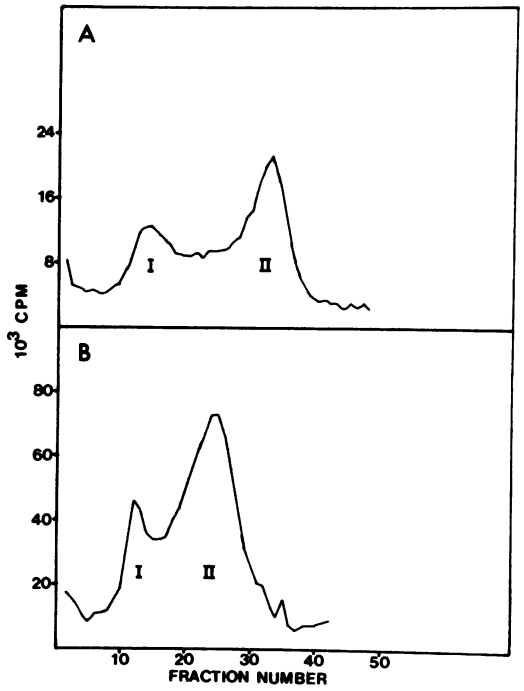

FIG. 1. Sedimentation patterns of vesicles containing ^{125}I-labeled polyomavirus in the presence and absence of the HSAB cross-linker. The cytoplasmic fraction, as described previously (16), containing either 7×10^5 cpm of ^{125}I-labeled virus (A) or 15.4×10^5 cpm of ^{125}I-labeled virus (B), was applied to a 15 to 45% sucrose gradient containing 0.01 M Tris hydrochloride (pH 7.4), 0.15 M NaCl, and 0.01% Triton X-100 and was centrifuged for 16 h at 32,000 rpm in an SW41 rotor at 4°C. (A) No HSAB cross-linker was present. (B) The purified ^{125}I-labeled virus used for infection was derivatized with HSAB and then cross-linked to the cell surface before endocytosis. Sedimentation is from right to left in the figure.

Identification of the cross-linked products from the polyomavirus-cell surface union was achieved with SDS-PAGE. Our analysis involved a comparison of preparations of vesicles containing ^{125}I-labeled virus with and without HSAB cross-linking to determine whether any new labeled bands had appeared as a result of the cross-linking. Such a new band necessarily would contain a virus protein component by virtue of having ^{125}I-label but would also contain an MKC component as a result of cross-linking that would impart different migration properties to the product.

The autoradiogram prepared from this gel analysis is shown in Fig. 2. The normal pattern of virus protein bands was demonstrated by purified, ^{125}I-labeled polyomavirus (Fig. 2, lane B). Isolated vesicle samples containing cross-linked ^{125}I-labeled virus from peaks I and II (Fig. 2, lanes D and E, respectively), however, showed an extra band in the 120-kDa region. This band was designated the "120-kDa band." When similar vesicle samples, prepared in the absence of HSAB but in otherwise identical conditions, were analyzed, only the normal virus protein pattern was observed (Fig. 2, lanes F and G). The dimer band ("D," about 90 kDa) has been shown to contain only VP1 sequences by peptide mapping (19) and immunological studies (7). This band is routinely observed to increase up to 10-fold in intensity upon virus binding (see Table 1) and is not a result of cross-linking.

These results indicated that HSAB cross-linking must occur for isolated vesicles to contain the 120-kDa band. An additional control was performed in which ^{125}I-labeled virus was reacted with HSAB and bound to the cell as in the previous samples, but no UV radiation was used to complete the cross-link. Again, no 120-kDa band was observed in the resulting autoradiogram (data not shown). We also established that the 120-kDa band was not formed as a result of the cross-linking of one virion protein with another virion protein. Virions labeled with ^{125}I were reacted with HSAB and then irradiated with UV light without exposure to MKCs. Only the normal virus protein pattern was observed by this procedure (Fig. 2, lane C). Thus, HSAB cross-linking resulted in the formation of the 120-kDa band that represented a covalent linkage between a virion protein and a component from the MKC surface.

It was essential to determine whether the MKC-derived component of the 120-kDa band remained with the virion during removal of the vesicle membrane from the cross-linked virus. In a separate experiment from the one described for Fig. 2, lanes A to G, isolated monopinocytotic vesicles containing cross-linked polyomavirus were incubated with 0.2% Nonidet P-40 for 18 h, followed by banding of the released virus on a sucrose gradient. This treatment allowed recovery of free virions that had been cross-linked. The recovered, ^{125}I-labeled cross-linked virus from vesicle peaks I and II (Fig. 2, lanes H and I, respectively) also showed the presence of the 120-kDa band, with relative intensity quite similar to that of the virion proteins. Thus, the 120-kDa band was observed in samples that had been treated with detergent to remove the membranes and thus appears to have purified with the virus through this procedure.

Preparation and characterization of the antibody against the

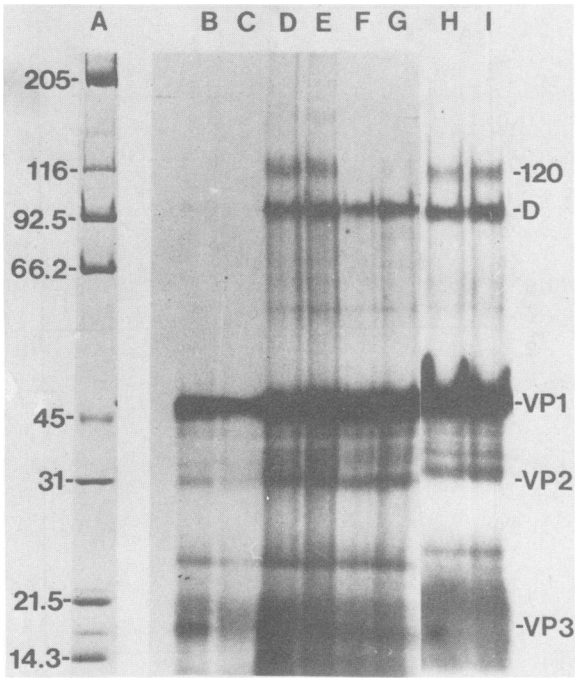

FIG. 2. SDS-PAGE of HSAB cross-linked and un-cross-linked ^{125}I-labeled polyomavirus contained in isolated monopinocytotic vesicles and cross-linked virus recovered from vesicles. Vesicle samples containing virus were prepared from pooled fractions of a sucrose gradient (15 to 45%) (16). Recovered cross-linked virus was prepared by detergent treatment of vesicles as described in the text. Equivalent counts per minute (1.1×10^5) were applied to a 5 to 15% gradient gel as described previously (16). This figure is a composite of two separate gels: A to G and H and I. Lane A is a Coomassie blue stain, and lanes B through I are the autoradiograms prepared from the gels. Lane A, Molecular mass markers: lysozyme (14,300), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase *b* (92,500), β-galactosidase (116,000), and myosin (205,000); lane B, purified ^{125}I-labeled polyoma virions; lane C, purified ^{125}I-labeled polyoma virions derivatized with HSAB and irradiated with UV light; lane D, monopinocytotic vesicles containing polyoma virions from peak I (Fig. 1B) prepared by infection with HSAB cross-linked virus; lane E, monopinocytotic vesicles containing polyoma virions from peak II (Fig. 1B) prepared by infection with HSAB cross-linked virus; lane F, monopinocytotic vesicles containing polyoma virions from peak I (Fig. 1A) prepared by infection with virus without HSAB cross-linking; lane G, monopinocytotic vesicles containing polyoma virions from peak II (Fig. 1A) prepared by infection with virus without HSAB cross-linking; lane H, free, cross-linked polyoma virions recovered from the monopinocytotic vesicles in peak I (Fig. 1B); lane I, free, cross-linked polyoma virions recovered from the monopinocytotic vesicles in peak II (Fig. 1B). The band designated 120 is the 120-kDa cross-linked band. D, Dimer of VP1 (90 kDa).

120-kDa band. With the autoradiograms as markers, the 120-kDa band was excised from the gel and used as the immunizing antigen in rabbits. The resulting antibody preparation was then purified to IgG and characterized by immunoblot analysis. We expected two specificities for this antibody: one for the polyomavirus protein portion of the 120-kDa band and one for the MKC component (presumed to be a cell surface receptor protein). To confirm this latter expectation, we prepared MKC plasma membranes from uninfected MKCs for analysis on the immunoblots. Thus, recognition of any band(s) on the blot of the plasma membranes by the anti-120-kDa antibody would provide evidence for the cell surface nature of the MKC component of the cross-linked product. The polyoma protein component of the cross-linked band could be confirmed by probing unlabeled virus proteins on the blot as well. The results of this immunoblot analysis are shown in Fig. 3. The nitrocellulose sheets containing the transferred proteins were probed with ^{125}I-labeled anti-120-kDa IgG. The autoradiogram prepared from the nitrocellulose sheet is shown in lanes A and B, and Coomassie blue-stained samples are shown in lanes C and D (Fig. 3). Lane A, with only purified polyomavirus, demonstrated bands in three regions: dimer (90 kDa), VP1 (45 kDa), and the 16- and 18-kDa fragments of VP1 (7, 24) (Fig. 3). These are all VP1 sequences (19), confirming our prediction that the 120-kDa band contained a VP1 sequence. Since no VP2 or VP3 bands (35 and 23 kDa, respectively) were recognized by the antibody, we concluded that VP1 is the only viral protein sequence represented in the 120-kDa cross-linked product. Lane B, containing isolated plasma membranes, shows the presence of four major membrane bands that are recognized by the anti-120-kDa antibody, with apparent molecular masses of approximately 31, 28, 27 and 24 kDa (Fig. 3). Four minor bands also were observed in the 50- to 100-kDa region. Perhaps a common determinant present on more than one protein in the plasma membrane is part of the cell component of the cross-linked product, or the

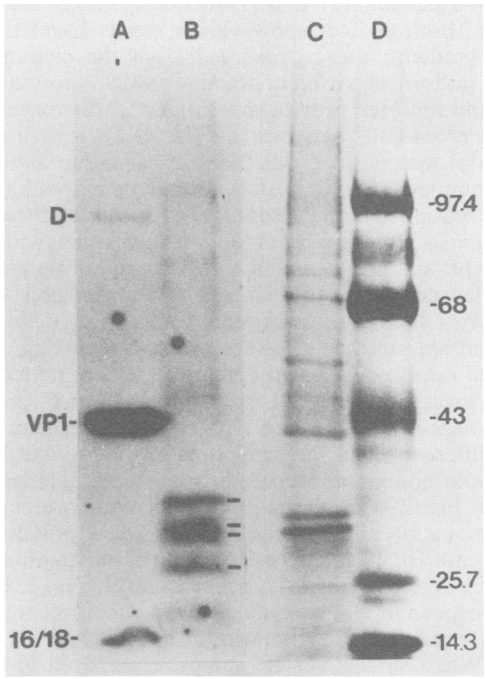

FIG. 3. Immunoblot of polyomavirus and MKC plasma membrane proteins with the anti-120-kDa antibody. After transfer from the SDS-PAGE gel, the polyomavirus and MKC plasma membrane protein bands were probed with 10×10^6 cpm of ^{125}I-labeled anti-120-kDa IgG. The autoradiogram prepared from the blot is shown in lanes A and B, and Coomassie blue-stained samples are shown in lanes C and D. Lane A, Purified polyomavirus (5 μg); lane B, MKC plasma membrane preparation; lane C, Coomassie blue-stained MKC plasma membrane preparation; lane D, Coomassie blue-stained molecular mass markers (from the bottom of the gel): lysozyme (14,300), α-chymotrypsinogen (25,700), ovalbumin (43,000), bovine serum albumin (68,000), and phosphorylase *b* (97,400). D, Dimer of VP1 (90 kDa); 16/18, 16- and 18-kDa fragments of VP1.

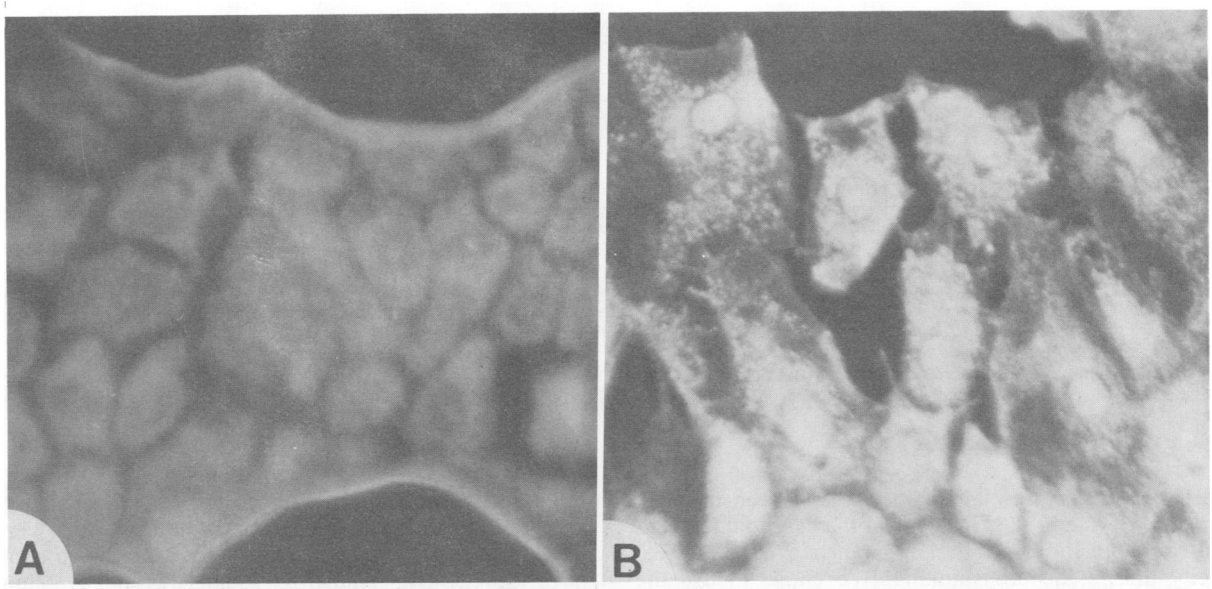

FIG. 4. Immunofluorescence of MKC surface with the anti-120-kDa antibody. Confluent layers of primary MKCs were fixed in 1% formaldehyde in PBS and incubated with the anti-120-kDa IgG fraction, then with fluorescein-conjugated goat anti-rabbit serum. (A) Photomicrograph of a field of MKCs that received preimmune IgG treatment, indicating background fluorescence. (B) Photomicrograph of a field of MKCs that were treated with the anti-120-kDa IgG, showing distinct areas of fluorescence on the cell surface.

four bands may represent immunologically similar subunits of the same membrane protein. Many other plasma membrane bands were not recognized by the anti-120-kDa antibody, as shown in the Coomassie blue-stained plasma membrane sample in lane C (Fig. 3). Labeled preimmune IgG did not recognize any of the plasma membrane bands in similar immunoblots (data not shown). Thus, the anti-120-kDa antibody had a specificity for MKC membranes, as well as for polyomavirus VP1. This confirmed our expectations of the dual nature of the 120-kDa cross-linked product as a covalent linkage between virus protein and an MKC surface protein formed only when polyomavirus was bound to the MKC in the presence of cross-linker.

Further characterization of the anti-120-kDa antibody involved the fluorescent antibody technique to determine whether the antibody could bind the surface of MKCs. Formaldehyde-fixed MKCs were treated with the anti-120-kDa IgG and then were stained with fluorescein-conjugated goat anti-rabbit serum. The results are shown in Fig. 4. Panel A shows the MKCs treated with preimmune IgG, resulting in little background fluorescence; panel B shows the MKCs treated with the anti-120-kDa antibody, resulting in definite fluorescence on the cell surface (Fig. 4). The patching phenomenon that can be seen on the MKCs in panel B indicates a possible distribution pattern for determinants on the cell surface that are recognized by the anti-120-kDa antibody (Fig. 4). Thus, the anti-120-kDa antibody was able to bind the MKC surface, further demonstrating that the 120-kDa cross-linked band contained a component derived from the plasma membrane. It is also significant that the anti-120-kDa antibody recognized nondenatured determinants on the cell surface, since the antibody was raised to an SDS-denatured 120-kDa-band antigen.

Effect of monoclonal antibody treatments of virus on infection and cross-linking. Previous studies on monoclonal antibodies against polyomavirus VP1 characterized two groups of monoclonals—those directed against isoelectric species B and C and those directed against isoelectric species D, E, and F (22). Of these, one monoclonal (D3) against the structural species B and C and one monoclonal (E7) against the virus attachment proteins D, E, and F were chosen to study the effect on virus infection and cross-linking. The monoclonal E7 demonstrated a 100% neutralization activity, and the monoclonal D3 showed an 18% neutralization activity (22). Binding was measured as the amount of labeled virus attached to the MKCs, and internalization was measured as the amount of labeled virus contained in peaks I and II of the sucrose gradient, as in Fig. 1. The degree of dimer formation and cross-linking (cross-linking was determined by the formation of the 120-kDa band) was estimated by measurement of the 120-kDa, dimer, and VP1 band intensities by densitometry. A whole-cell digest of a single MKC plate was used to recover all bound or internalized virus to preserve the stoichiometry of the bands derived from the cross-linked samples. The results of these experiments are summarized in Table 1.

With the bound but untreated virus as a 100% value for binding and internalization, the monoclonal D3 could prevent only about 10% of either virus binding or internalization, whereas the monoclonal E7 blocked nearly 90% of virus binding and more than 99% of internalization. However, analysis of the dimer and 120-kDa bands for the cross-linked samples demonstrated that D3 reduced the 120-kDa band, and thus cross-linking, to less than one-fourth of the level of untreated virus, and it reduced the dimer to less than one-half. The nearly complete prevention of internalization by E7 resulted in no detectable dimer or 120-kDa bands in the autoradiogram. Also, it was observed that dimer increased from a relatively low level in purified virus (a dimer:VP1 ratio of 3:100) to more than eight times as much upon binding to the MKC. This is expressed in Table 1 as the percentage of bound virus, increasing from 12 to 100%. This increase is limited to only about threefold, from 12 to 38%, in the D3-treated virus sample.

DISCUSSION

Although photoaffinity cross-linking has been used extensively with other ligands such as hormones and growth

TABLE 1. Effect of monoclonal antibody treatments of polyomavirus on binding, internalization, dimer formation, and cross-linking

Antibody treatment	Specificity	% Binding[a]	% Internalization[b]	Dimer:VP1 intensity (%)[c]	120-kDa band:VP1 intensity (%)[c]
Purified virus[d]				12	0
No treatment[e]		100	100	100	100
D3 monoclonal[f]	(VP1 species B and C)	93	91	38	23
E7 monoclonal[f]	(VP1 species D, E, and F)	11	0.6	0	0

[a] Expressed as the percentage of bound ^{125}I-labeled polyomavirus from antibody-treated samples as compared with the untreated sample.
[b] Expressed as the percentage of ^{125}I-labeled polyomavirus present in vesicle peaks I and II from antibody-treated samples as compared with the untreated sample.
[c] VP1, dimer, and 120-kDa band intensities were determined by densitometry of the autoradiogram of the gel from SDS-PAGE of the ^{125}I-labeled polyomavirus recovered from the whole-cell digest or of purified ^{125}I-labeled polyomavirus. The ratios of dimer to VP1 and 120-kDa band to VP1 band intensities were expressed as the percentage of antibody-treated sample (or purified virus) as compared with the untreated sample.
[d] ^{125}I-labeled polyomavirus not bound to MKC.
[e] ^{125}I-labeled polyomavirus not treated with antibody but bound to MKC.
[f] ^{125}I-labeled polyomavirus treated with 30 μg of IgG per ml for 1 h at 25°C, then bound to MKC.

factors (11, 18), very few applications have been made in virus-host cell systems (18, 30). One of the essential features of such an application is the maintenance of biological specificity during the cross-linking. We chose to use the heterobifunctional cross-linker in two stages, attaching the HSAB randomly on the virion and then allowing specific biological attachment, followed by completion of the cross-link with the photoreactive portion of the molecule. This approach has been used successfully in other systems, such as in the attachment and cross-linking of insulin (28, 29), thyrotropin (9), and platelet-derived growth factor (17) to their specific receptors.

The geometrical relationships among the virion, the cross-linker, and the cell surface component appeared to be crucial in forming the cross-link. In our efforts to observe new labeled bands similar to the 120-kDa band, cross-linking with reagents of different chain lengths was unsuccessful. Only the relatively short HSAB cross-linker (<0.5 nm) could form a detectable cross-linked product in the procedure used. This reflects the juxtaposition of the virion attachment protein and the cell surface component and increased our confidence that we were cross-linking virion and cell molecules of biological significance in polyomavirus-MKC interaction. The formation of the second covalent bond (and thus the 120-kDa band) by the photoreactive portion of the cross-linker could be accomplished by UV irradiation either before endocytosis at 37°C, as reported here, or after endocytosis (data not shown). Thus, the association of the virion and the cell surface component remained tight during internalization even without cross-linking, as would be expected for a ligand-receptor interaction. The continued proximity of the virion to the cell membrane in isolated vesicles was noted previously by electron microscopy (16).

The contribution of the virion to the cross-linked product was demonstrated easily by the presence of the ^{125}I label in the initial detection of the new band (Fig. 2). It was necessary to consider, however, that the 120-kDa band might have been generated by intra- or intervirion cross-linking, resulting in a new band composed of only virion protein sequences. The control experiment described in Fig. 2, lane C, demonstrated that, in the absence of the cell, there is no effect of the cross-linker at all upon virion protein patterns in the gel. The 120-kDa band could be formed only when all three—the virus, the cross-linker, and the cells—were present. The possibility of intervirion cross-linking was also excluded when the cross-linked virus was recovered from vesicles and analyzed by SDS-PAGE (Fig. 2). The banding of the recovered virion particles in the sucrose gradient at the same sedimentation rate as purified virions (data not shown) indicated that single cross-linked virions were isolated. These individual cross-linked virions were shown to have the 120-kDa band (Fig. 2, lanes H and I). Thus, the 120-kDa band was not derived from aggregates or even pairs of cross-linked virions.

The anti-120-kDa antibody preparation was shown (Fig. 3) to have specificity against both polyomavirus VP1 and plasma membrane proteins. As a polyclonal antibody preparation, the anti-120-kDa IgG may be a mixture of specific antibodies against different epitopes of the cross-linked molecule as injected in the denatured form from the SDS-PAGE band. Immunoprecipitation studies (data not shown) demonstrated the inability of the anti-120-kDa IgG to recognize intact polyoma virions. When the antibody was used to treat ^{125}I-labeled virus before infection of MKCs, the anti-120-kDa IgG had no effect upon virus binding, internalization, or cross-linking. Similarly, the preparation was shown to attach to the surfaces of MKCs by immunofluorescence (Fig. 4), but treatment of MKCs with anti-120-kDa IgG caused no reduction in the binding or internalization of ^{125}I-labeled virus compared with that of untreated MKCs (G. R. Griffith and R. A. Consigli, unpublished data). These observations can be accounted for by a consideration of the nature of the available epitopes of the immunizing antigen. If the cross-linked material does indeed represent the union of the virus attachment protein (a VP1 sequence) and the cellular receptor (or a part of that molecule), then the regions actually involved in recognition and virus attachment, therefore, would be the regions of those molecules that were cross-linked, according to the way the cross-linking was done. This would effectively mask these regions as epitopes in the production of antibody and result in observations very much like ours.

The four plasma membrane bands recognized by the anti-120-kDa IgG shown in Fig. 3 are relatively close in apparent molecular mass, in the 24- to 31-kDa range. These bands are immunologically related and may represent similar proteins with some sequences in common. If these bands exist as individual proteins on the MKC surface that are distinct from one another, however, it is unlikely that all of them would have been involved in the cross-linking. This would have resulted in the observation of many new bands from cross-linking, which was not the case. It should be mentioned that the recognition of several bands in plasma membrane preparations on the blot by anti-120-kDa IgG is not completely unexpected. Recent genetic studies have suggested that many cellular receptors with distinct func-

tions share quite similar primary protein structures (27). This is consistent with our earlier discussion of the inaccessibility of the biologically active region during production of antibody against the 120-kDa cross-linked molecule. Thus, antibody against nonspecific regions of the cell surface component in the 120-kDa band could recognize similar nonspecific regions on other cell surface molecules with receptor function for other ligands. The presence of carbohydrate moieties in the plasma membrane bands or the 120-kDa band has not been fully investigated, but preliminary experiments have not been able to detect such groups on these proteins.

The exact contribution of virion protein and cell surface protein to the 120-kDa band is not known at this time. However, some information exists concerning the VP1 component. It has been shown that polyomavirus VP1 can be chemically cleaved into an 18-kDa and a 29-kDa fragment (1, 2). Also, previous studies in our laboratory showed that when intact polyomavirus is labeled by the chloramine T procedure, it is the 18-kDa portion, not the 29-kDa portion, of VP1 protein that becomes labeled with ^{125}I (D. G. Anders and R. A. Consigli, unpublished data). Thus, the appearance of ^{125}I label in the 120-kDa band derived from virions labeled in this manner indicated that the 18-kDa portion was present in the 120-kDa band. This was confirmed by the recognition of the 18-kDa fragment by the antibody prepared against the 120-kDa band on the Western blot as shown in Fig. 3, lane A. It has been reported that this 18-kDa fragment of VP1 contains the region responsible for virus attachment (1).

The cell surface protein contribution to the 120-kDa molecule may be estimated to be a molecule of about 75-kDa apparent molecular mass, assuming that a single VP1 sequence is present. If either two VP1 sequences or the VP1 dimer is involved in cross-linking, the cell surface protein would be estimated to be 25 to 30 kDa. Any of the four bands identified in Fig. 3, lane B, could thus be the cross-linked cell surface component, resulting in an apparent combined molecular mass of around 120 kDa. A logical approach to the separation and size estimation of the two components is the use of a short-span (0.5 nm) cleavable cross-linker. However, nearly all of the cleavable heterobifunctional cross-linkers that are available are cleaved by thiol reagents, and SDS-PAGE of polyomavirus requires reduction for complete dissociation of the virions and entry of virion proteins into the gel. Thus, our attempts with these cleavable cross-linkers resulted in either the cleavage of the cross-link before it could be visualized or the inability of non-reducing gel conditions to resolve the proteins present (G. R. Griffith and R. A. Consigli, unpublished data).

The effect of two monoclonal antibodies against polyomavirus VP1 on virus binding and internalization is established in Table 1. The two columns on the right side of Table 1 show the ability of these treatments to selectively inhibit dimer formation or 120-kDa band formation, as indicated by the change in the intensity of these bands relative to the amount of VP1 present. The monoclonal E7, with a specificity for the virus attachment proteins (species D, E, and F), was able to completely prevent dimer and 120-kDa band formation as well as to nearly totally block binding and internalization. Thus, when virions cannot bind to the cell and internalize, cross-linking also does not occur, suggesting a direct relationship between virus binding and cross-linking. The most striking result listed in Table 1, however, is the ability of the monoclonal D3 to reduce the degree of cross-linking by more than 75% while still allowing over 90% binding and internalization. Although D3 has specificity for the structural species B and C, it does have

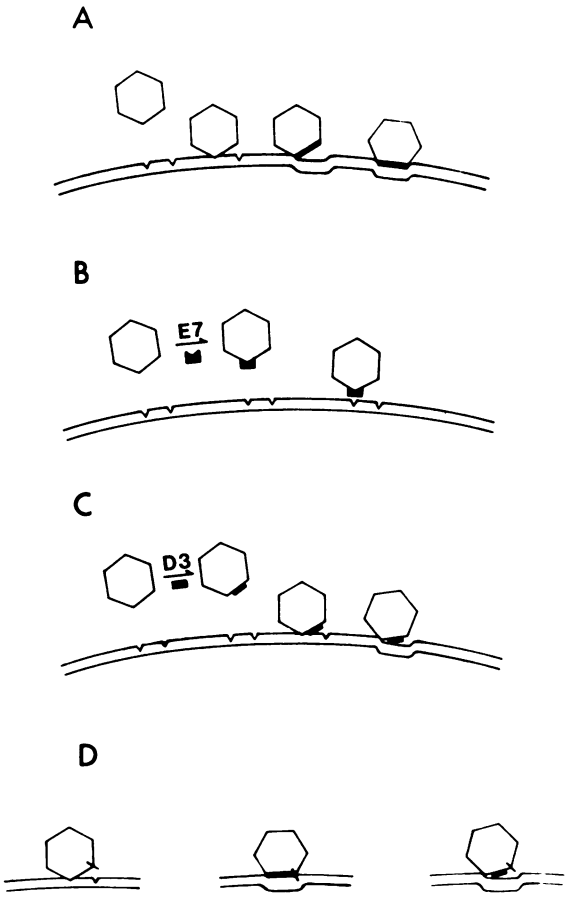

FIG. 5. Proposed two-step model for the attachment of polyomavirus to MKCs. (A) Normal two-step binding, first at a single vertex of the virion and then as a tight three-point attachment to the cell membrane, with an accompanying change in the conformation of the VP1 proteins of the virion face. (B) Treatment of virions with the monoclonal antibody E7, preventing all attachment. (C) Effect of treatment of the virions with the monoclonal antibody D3, allowing single-point attachment but preventing tight binding and the conformational change. (D) Formation of the HSAB cross-link, represented by the short bar at the vertex of the virion. Formation of this cross-link is blocked by the presence of D3 on the virion. See text for full explanation of the model.

some neutralization activity (22). Thus, this monoclonal apparently has a unique effect by binding a region of the structural VP1 molecules that is involved either directly or indirectly in cross-linking. Another significant observation from these measurements is the formation of new dimer molecules as a result of virus binding to MKCs. This increase has been found to be up to eight to ten times that of purified virus but only three times that of D3-treated virus.

These observations allow us to speculate on the possible mechanism of polyomavirus binding to the MKC receptor. Earlier, our laboratory reported a loose and a tight binding of the virus to the cell surface in electron microscopy studies (21). The loose binding, considered to be the attachment of a virion at a single vertex, precedes the tight binding, which is considered to be binding of the virion on a threefold axis, that is, with a face of the virion tightly held against the cell surface. Adsorption studies by Bolen and Consigli (6) revealed two types of binding for virions on MKCs. This suggests a two-step binding process in which the virus first binds loosely, inducing a second step in which the membrane

and the virion face move together to form a close, three-point contact. Thus, the monoclonal E7, binding to species D, E, and F, would block the first step, preventing all binding and internalization as well as cross-linking. It has been shown previously that species D, E, and F are the principal components isolated as a 5S species of capsomere preparations that are most likely located at the vertices of the virion (8).

The monoclonal D3, binding species B and C on the face of the virion, would not block the first step of loose binding, as shown in Table 1, but would prevent the second step. This could occur by a physical blocking of the virion face from becoming close enough to the cell surface to form the tight association. In addition, it is possible that D3 binding prevents a conformational change in the virion proteins that occurs during the second step. In responding to the attractive forces bringing the virion face near to the membrane, the VP1 molecules might change to an energetically more favorable conformation to allow the tight binding. Conformational changes in virion proteins have been observed in other virus systems (12, 13, 25). Such a conformational change also might explain the formation of new VP1 dimer molecules upon binding, as shown in Table 1, perhaps forming intermolecular disulfide links between two adjacent VP1 molecules that would be protected by a hydrophobic pocket. Thus, the effect of D3 binding on the second step would be to prevent new dimer formation and not to allow the virion face, containing three vertices, each composed primarily of species D, E, and F, to obtain the close proximity needed to form a cross-link to the cell receptor with the HSAB. The second step of polyomavirus binding to MKC, then, would involve the movement of the virion and the cell membrane into a close association and could induce a conformational change in the virion that allows new dimer formation, as well as completion of the cross-link to the cell receptor. This model is described graphically in Fig. 5.

We have shown that a cell surface component involved in binding can be cross-linked to polyomavirus VP1 by using antibody to the cross-linked product. The principal value of this dual-specificity antibody is in identification and not as much as in characterization of the cross-linked components in detail. As techniques are developed, the VP1 and the cell-derived components of the cross-linked product will be characterized both chemically and functionally.

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