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# The 32-Kilodalton Envelope Protein of Vaccinia Virus Synthesized in *Escherichia coli* Binds with Specificity to Cell Surfaces

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**The nature of interaction between vaccinia virus and the surface of host cells as the first step in virus infection is undefined. A 32-kDa virus envelope protein has been identified as a cell surface binding protein (J.-S. Maa, J. F. Rodriguez, and M. Esteban, J. Biol. Chem. 265:1569-1577, 1990). To carry out studies on the structure-function relationship of this protein, the 32-kDa protein was obtained from *Escherichia coli* cells harboring the expression plasmid pT7Ek32. The recombinant polypeptide was found to have structural properties similar to those of the native virus envelope protein. Binding studies of $^{125}$I-labeled 32-kDa protein to cultured cells of various origins revealed that the *E. coli*-produced 32-kDa protein exhibited selectivity, specificity, and saturability. Scatchard analysis indicated about $4.5 \times 10^4$ sites per cell with a high affinity ($K_d = 1.8 \times 10^{-9}$ M), suggesting interaction of the 32-kDa protein with a specific receptor. The availability of large quantities of the 32-kDa virus protein in bacteria will permit further structural and functional studies of this virus envelope protein and facilitate identification of the specific cell surface receptor.**

Adsorption of a virus to the surface of host cells represents the earliest stage in the infection of cells by viruses (9, 21). Attachment is mediated by the binding of viral attachment proteins to a molecule on the cell surface acting as a virus receptor (28, 32). Virus entry is thought to occur by direct fusion of the virus envelope with the plasma membrane, adsorptive or receptor-mediated endocytosis, or possibly direct translocation of virus particles across the plasma membrane (6, 23).

Vaccinia virus, a member of the orthopoxvirus genera, has contributed to one of the major successes in modern health care in its use as a vaccine to eradicate smallpox. The virus has potential as a vector for immunological studies and as live recombinant vaccine against many diseases of human and veterinary importance (24). However, very little is known about the nature of viral proteins involved in the uptake of vaccinia virus by animal cells. Both mechanisms, virus-induced cell fusion (1, 2) and endocytosis (7), have been shown to occur in cells infected with vaccinia virus, although the relative importance of fusion versus endocytosis for virus infection remains undefined. The entry of vaccinia virus into cells appears to be a complex process involving at least several viral proteins. On the basis of studies with specific neutralizing antibodies, it has been proposed that an abundant 54-kDa surface tubule protein may mediate entry of the virus (31), while a 14-kDa envelope protein has been implicated in virus penetration, possibly acting at the level of fusion of the virus envelope with the cell plasma membrane (13, 29). Studies with proteases and neutralizing antibodies have suggested that vaccinia virus envelope proteins of 59 and 34 kDa as well as an aggregate of 17- and 25-kDa proteins may have an essential role in the virus penetration; two other envelope proteins of 32 and 29 kDa, as well as a cleavage fragment of a 54-kDa protein, may be adsorption proteins (16, 27). These previous studies suggest that viral proteins involved in virus entry are localized on the envelope and that virus binding and subsequent entry can be dissociated into separate events.

Because vaccinia virus is a large and complex DNA virus and it contains about 100 different polypeptides in the virion (11, 26), studies on the identification of viral genes encoding proteins involved in virus entry have been difficult to approach. We have identified a 32-kDa envelope protein which binds selectively to the surface of cultured cells of different origins (22). A correlation between binding of the 32-kDa protein and ability of the virus to replicate in cultured cells was observed, indicating that the 32-kDa protein could play a role in virus attachment to cells (22). The gene for the 32-kDa protein corresponds to open reading frame D8R on *Hin*dIII fragment D of vaccinia virus DNA (22, 25) and encodes a 35, 426-Da protein. This protein contains a large N-terminal domain with sequence homology to carbonic anhydrase and a C-terminal domain with sequence similar to those of the attachment glycoprotein VP7 of rotavirus and of transmembrane proteins (22, 25). Further biochemical analysis showed that the 32-kDa protein is a basic polypeptide, has a predicted pI of 8.67, may form homodimers by disulfide bonds, and is immunogenic (22). To extend our studies on the role of the 32-kDa protein on virus attachment to cells, in this investigation we demonstrated that the recombinant 32-kDa envelope protein of vaccinia virus produced in *Escherichia coli* can bind to cell surfaces and calculated the affinity constant and number of binding sites on cells.

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We have previously shown that the entire coding sequence of the 32-kDa protein can be expressed in *E. coli* by using a bacteriophage T7 bacterial expression system, yielding a protein with the same apparent molecular mass as that polypeptide present in the virus particle (22). To obtain the recombinant protein from lysates of induced *E. coli* cells for binding studies, we took advantage of the low abundance of

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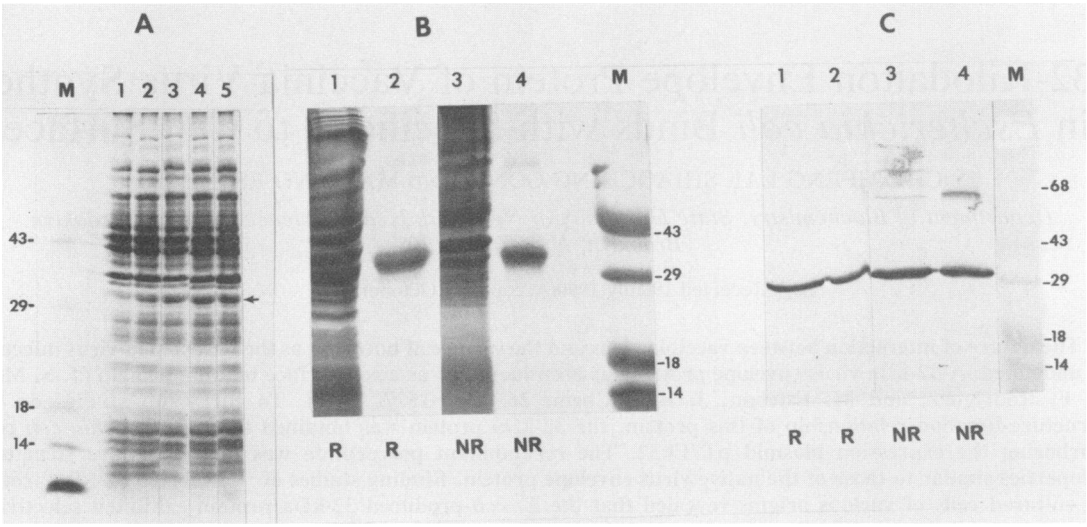


FIG. 1. Preparation of the vaccinia virus 32-kDa protein from lysates of *E. coli*. The 32-kDa encoding gene of vaccinia virus was cloned into the Klenow-treated *Eco*RI site of the procaryotic expression vector pT7-7 as previously described (22). (A) Exponentially growing *E. coli* BL21 (DE3) cells carrying the expression plasmid pT7Ek32 were induced with 400 μm isopropyl-β-D-thiogalactopyranoside. At various times postinduction, portions of samples were analyzed by 12% SDS-PAGE under reducing conditions. Protein profiles were revealed by Coomassie blue R-250 staining. The times of induction were 0 (lane 1), 30 (lane 2), 60 (lane 3), 120 (lane 4), and 240 (lane 5) min. Molecular weight markers are indicated in kilodaltons (lane M), and the migration of the 32-kDa protein is indicated with an arrow. (B and C) Samples of isopropyl-β-D-thiogalactopyranoside-induced *E. coli* cell lysates and of gel-purified 32-kDa protein were run on 12% SDS-polyacrylamide gels and analyzed by Coomassie blue staining (B) or by immunoperoxidase staining after reactivity with rabbit anti-vaccinia virus serum (C). The origins of proteins were as follows: 4-h-induced *E. coli* extract (lanes 1 and 3); gel-purified 32-kDa protein (lanes 2 and 4). The protein samples were analyzed under both reducing (lanes 1 and 2) and nonreducing (lanes 3 and 4) conditions. The molecular weight markers are indicated in kilodaltons (lane M).

a bacterial protein with a similar size present in uninduced cultures. This can be observed after Coomassie Blue R-250 staining of lysates run on sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions (Fig. 1A, arrow). The protein band containing the 32-kDa protein was excised from the gel, electroeluted, concentrated in a speed vacuum, suspended in deonized water, and precipitated with methanol–acetone (50:50, vol/vol), and the pellet obtained after centrifugation was dried and suspended in water. A comparison of the yields obtained from uninduced and induced cultures indicates that the gel-purified protein represents >90% of the vaccinia virus 32-kDa protein relative to *E. coli* proteins.

As shown by Coomassie blue staining (Fig. 1B) or by specific reactivity with rabbit anti-vaccinia virus serum (Fig. 1C), the gel-purified 32-kDa protein (Fig. 1B and C, lanes 2 and 4) forms dimers of about 64 kDa when analyzed under nonreducing conditions (lanes 3 and 4). The amount of dimer was calculated by densitometric analysis. It was estimated that dimers account for about 9% of the 32-kDa protein present in *E. coli* lysates, whereas dimers account for about 27% of the 32-kDa protein after gel purification. Several lines of evidence indicate the *E. coli*-produced 32-kDa protein has structural properties similar to those of the native virus envelope protein. The following similarities were observed: (i) same apparent molecular mass; (ii) same ability to form homodimers, presumably at the single cysteine 262; (iii) same reactivity against anti-32-kDa monospecific antibody and with anti-vaccinia virus serum; and (iv) identity in size of cleavage fragments after protease digestion with trypsin and with V8 protease of *Staphylococcus aureus* (data not shown).

To show that the *E. coli*-expressed 32-kDa protein binds to the cell surface of animal cells, binding experiments were carried out with $^{125}$I-labeled 32-kDa protein. Iodinated 32-kDa protein was added to cells grown in 24-well culture plates ($4 \times 10^5$ cells per well) and incubated on ice for different times. After extensive washings, cell lysates were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The results are shown in Fig. 2. It is clear that binding of $^{125}$I-labeled 32-kDa protein occurs in human HeLa G, mouse L-929, and monkey BSC-40 cells, whereas a twofold lower binding is observed in canine MDCK and in the subclone MDCK-5D11 cells (Fig. 2A). The selective binding of the 32-kDa protein to cells correlated well with the extent of binding of purified vaccinia virions to different cells (Fig. 2B). In this case, binding was performed on ice with 40 PFU of purified vaccinia virions per cell. After incubation for various times on ice, unbound virus was removed by washing, and cell extracts were analyzed by 12% SDS-PAGE and immunoblotted. Protein profiles were revealed after reactivity with rabbit anti-vaccinia virus serum followed by reactivity with $^{125}$I-labeled protein A of *S. aureus*. Binding of virion polypeptides to different animal cells was quantitated by densitometric analysis of the autoradiogram. The results (Fig. 2B) revealed that binding of vaccinia virions to human HeLa G, mouse L-929, and monkey BSC-40 cells is three to fourfold higher than binding to the highly polarized epithelial MDCK-5D11 cells. The binding characteristics of the 32-kDa protein were similar to those of purified vaccinia virus, suggesting a common cell surface receptor.

To demonstrate that binding is not only selective but also specific, we carried out competition experiments with unlabeled, purified vaccinia virus. Vaccinia virus particles compete for binding of $^{125}$I-labeled 32-kDa protein to cells (Fig.

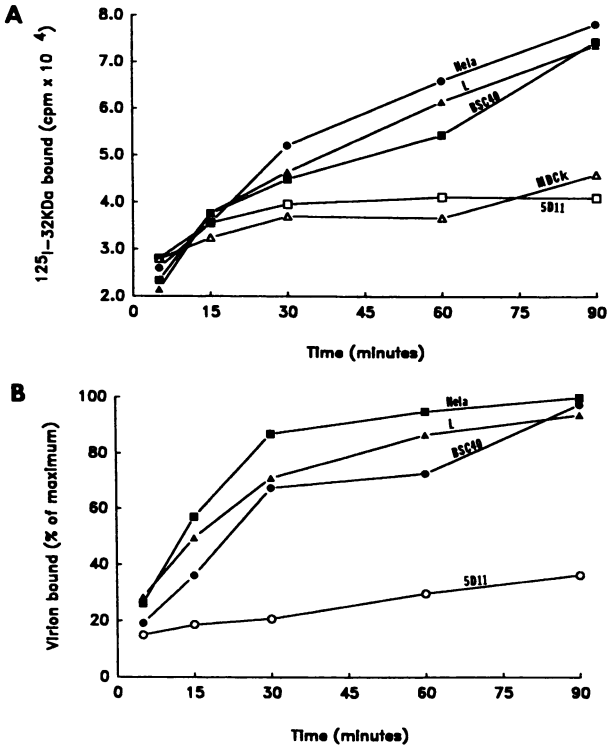


FIG. 2. Selective binding of 32-kDa protein to different animal cells. (A) Binding of labeled 32-kDa protein to cells. Iodinated 32-kDa protein ($5 \times 10^6$ to $10 \times 10^6$ cpm) in 0.20 ml of phosphate-buffered saline (PBS) with 100 μg of bovine serum albumin labeled as previously described (22) was added to cells grown in 24-well plates ($4 \times 10^5$ cells per well) and incubated on ice for different times. After extensive washings with PBS to remove free $^{125}$I-labeled 32-kDa protein, cells were collected in 2× SDS-sample buffer. Samples were counted in a Biogamma counter, and the radioactivity bound was plotted as a function of time. (B) Binding of vaccinia virus particles to cells. Binding of purified wild-type vaccinia virus (4,000 particles per cell) to different animal cells (human HeLa G, mouse L-929, monkey BSC-40, and canine MDCK-5D11 cells) was carried out on ice for different times. After extensive washings to remove unbound virions, cells were collected in 2× SDS-sample buffer, analyzed by 12% SDS-PAGE, and blotted, and protein profiles were revealed after reactivity with rabbit anti-vaccinia virus serum and $^{125}$I-labeled protein A of *S. aureus*. The extent of binding of virion polypeptides to cells was quantitated by using densitometric analysis of the protein profiles in each lane of the autoradiogram. The results in panels A and B are expressed as total cell-associated radioactivity per $4 \times 10^5$ cells in duplicate samples (standard error, <5%). The subclone of the Madin-Darby canine kidney cells, MDCK-5D11, has a transepithelial electrical resistance of 1,000 to 2,000 ohms/cm$^2$ (15).

3). About 50% competition was observed with 1,000 particles per cell, and maximum competition, i.e., about 75%, was observed when a fivefold increase in the amount of virus particles was added. When competition was carried out with heterologous viruses (adenovirus and vesicular stomatitis virus), only vaccinia virus (but not adenovirus or vesicular stomatitis virus) could compete for binding of $^{125}$I-labeled 32-kDa protein to cells (Fig. 3). The results (Fig. 3) show that the 32-kDa protein from *E. coli* binds with specificity to animal cells.

To determine the binding saturability of $^{125}$I-labeled 32-kDa protein to animal cells, dose-dependent binding of $^{125}$I-labeled 32-kDa protein to HeLa cells in culture was performed. The profile of 32-kDa protein binding indicates that binding was saturable (Fig. 4A). Scatchard analysis (30) of these data revealed that the total binding sites for the 32-kDa envelope protein of vaccinia virus were about $4.5 \times 10^4$ sites per cell (Fig. 4B). The apparent dissociation constant was about $1.8 \times 10^{-9}$ M, which was calculated from the slope of the line in Fig. 4B.

Evidence for a possible role of vaccinia virus 32-kDa envelope protein on virus attachment to cells has been provided in this investigation. Interaction of the *E. coli*-expressed 32-kDa protein with the surface of various animal cells of different origins grown in culture was found to be quite specific. Strong binding was observed in human (HeLa G), monkey (BSC-40), and mouse (L-929) cells. However, lower binding was observed in canine polarized epithelial (MDCK and MDCK-5D11) cells (Fig. 2). This differential binding correlates well with the ability or inability of the various cell lines to be infected by vaccinia virus (22). The highly polarized epithelial MDCK-5D11 cells showed reduced binding for both labeled 32-kDa protein and purified virions of vaccinia virus, suggesting that the apical surface contains few binding sites for both the 32-kDa protein and the virus. This is consistent with our findings that vaccinia virus enters highly polarized epithelial cells preferentially by the basolateral surface (29a).

Examination of binding sites and dissociation constants for other viruses revealed that the number of sites and affinity vary, depending on the origins of viruses and of cells. Some of the available information includes binding of reovirus on endothelial cells ($3.4 \times 10^4$ sites per cell with $K_d$ of $4.3 \times 10^{-9}$ (M) (34), rhinovirus on HeLa cells ($2 \times 10^3$ sites per cell with $K_d$ of $4.35 \times 10^{-11}$ M) (5), human cytomegalovirus on fibroblasts ($5.26 \times 10^3$ sites per cell with $K_d$ of $1.1 \times 10^{-9}$ M) (33), herpes simplex virus on human R970-5 cells ($5 \times 10^5$ sites per cell with $K_d$ of $2.6 \times 10^{-7}$ M) (17), and simian virus 40 on polarized epithelial cells ($9 \times 10^4$ sites per cell with $K_d$ of $3.76 \times 10^{-9}$ M) (3). A dissociation constant of about $10^{-9}$ M was also observed for the interaction of human immunodeficiency virus envelope glycoprotein gp120 and its receptor CD4 (19). The ability of the purified vaccinia virions (but not vesicular stomatitis virus or adenovirus) to compete for binding of $^{125}$I-labeled 32-kDa protein might indicate that the 32-kDa protein binds to the same cellular receptor as the virus. The virion envelope contains many copies of the 32-kDa protein and other envelope proteins, and this multivalency may stabilize the interactions between the virus and the receptor. Although the number of binding sites per cell for vaccinia virus has not been determined, it is likely that the number of sites is similar to that found for the 32-kDa protein. A correlation has been found between the number of binding sites for the herpes simplex virus glycoprotein D and the number of binding sites for the virion (17).

Virus attachment and adsorption to cells are the major determinants in virus tropism and virus pathogenesis. Thus, it would be important to identify the structural features involved in the interaction of vaccinia virus 32-kDa protein with a putative cellular receptor. It is of major interest that, among the animal virus receptors clearly identified, three have properties of the immunoglobulin superfamily (poliovirus, rhinovirus, and human immunodeficiency virus), while sialic acid is involved in influenza virus-receptor interaction (35). Some of the animal viruses use binding sites for known physiological ligands. For example, rhinovirus which is an etiological vector responsible for the common cold enters

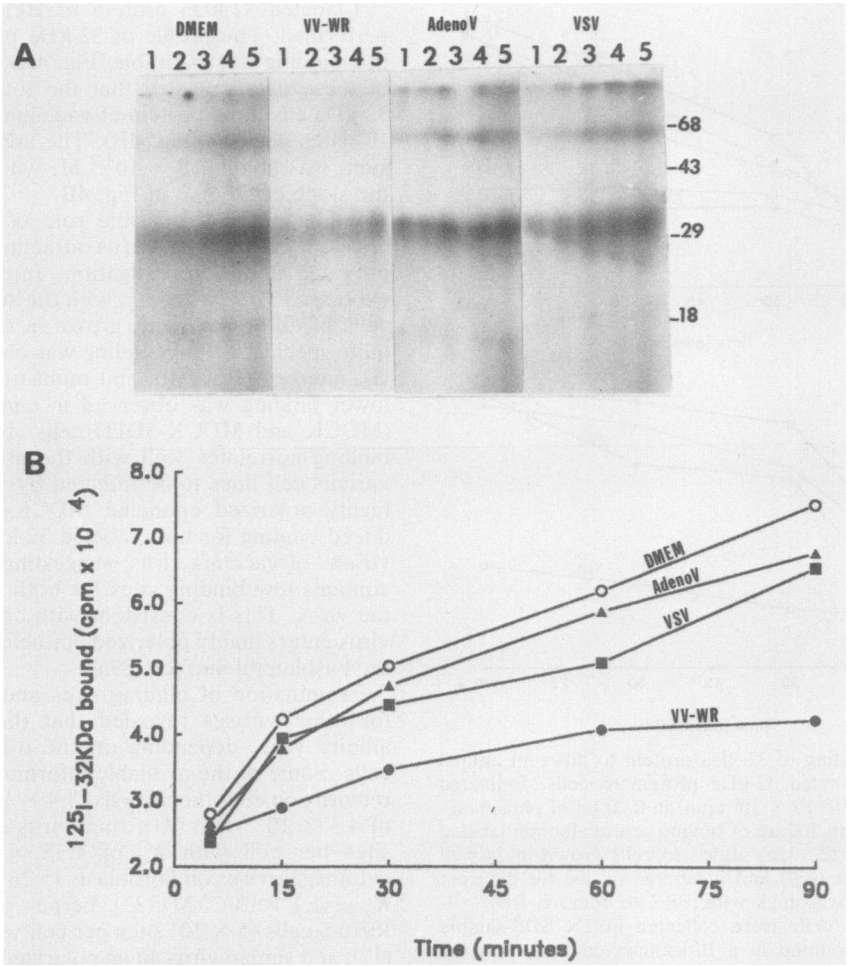


FIG. 3. Competition of 32-kDa protein binding to cells by different viruses. Confluent monolayers of HeLa G cells in 24-well plates ($4 \times 10^5$ cells per well) were incubated on ice for 60 min prior to binding, medium was removed, and cells were washed once with serum-free DME medium. A 0.20-ml volume of inoculum containing DME medium, or DME medium with 40 PFU of vaccinia virus (VV-WR), adenovirus (AdenoV), or vesicular stomatitis virus (VSV) per cell was then added to each well. The mixtures were incubated on ice for 60 min, the inoculum was removed, and the cells were washed with cold PBS. Thereafter, 0.20 ml of $^{125}$I-labeled 32-kDa protein ($5 \times 10^6$ to $10 \times 10^6$ cpm) in PBS containing 100 μg of bovine serum albumin was added to each well, and cells were incubated on ice for different times (5, 15, 30, 60, or 90 min). After unbound $^{125}$I-labeled 32-kDa protein was removed, cells were washed three times with cold PBS, and collected in 2× SDS-sample buffer. Samples were analyzed by 12% SDS-PAGE, and binding was visualized by autoradiography (A). The extent of binding was determined by counting the cell-associated radioactivity in portions of samples in a Biogamma counter (B). Results in duplicate samples are represented (standard error, <5%).

cells by means of the intercellular cell adhesion molecule ICAM-1 (14), human immunodeficiency virus which is responsible for AIDS uses primarily the CD4 glycoprotein receptor (8), Epstein-Barr virus infects T lymphocytes by means of the C3d complement receptor CR2 (12), rabies virus infects cells through the acetylcholine receptor (20), reovirus enters through the β-adrenergic receptor (4), herpes simplex virus type I enters cells by the fibroblast growth factor receptor (18), and it has been proposed that vaccinia virus enters cells by first interacting with the epidermal growth factor receptor (10). It is unlikely that the epidermal growth factor receptor interacts with the 32-kDa protein of vaccinia virus since binding of $^{125}$I-labeled 32-kDa protein to cells could not be competed by epidermal growth factor (22).

Our findings that vaccinia virus 32-kDa envelope protein can be produced in *E. coli* with apparently similar conformation and cell surface binding specificity to the native virus protein suggests that the recombinant 32-kDa protein folds correctly in *E. coli*. The availability of 32-kDa virus protein from recombinant sources will permit preparation of large quantities of homogeneous protein to facilitate additional structural studies and will allow mutagenic experiments to define functional domains of the protein. Elucidation of the structure of the 32-kDa virus protein by physical studies including crystallography and X-ray diffraction, as well as identification of specific cellular receptor, might then be possible with the *E. coli*-expressed 32-kDa protein.

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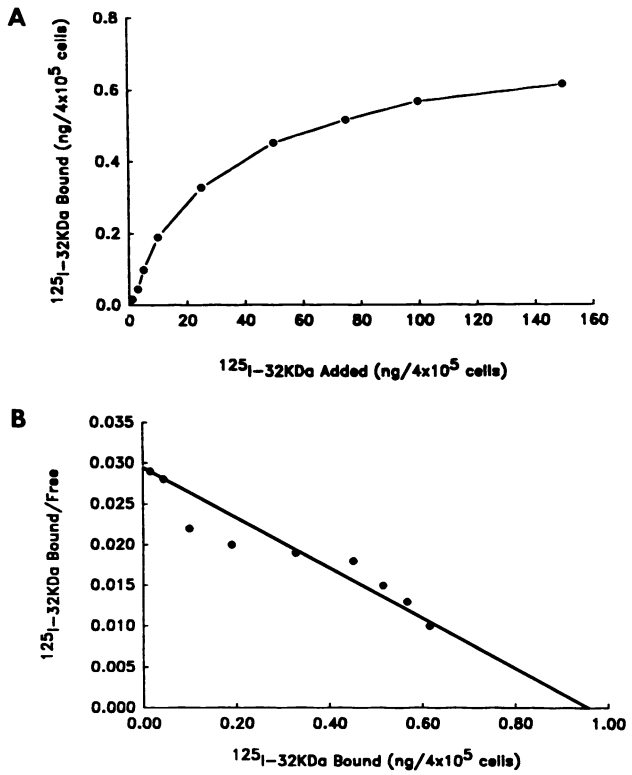


FIG. 4. Saturability of binding of 32-kDa protein to animal cells. Confluent monolayers of HeLa G cells grown in 24-well plates ($4 \times 10^5$ cells per well) were placed on ice for 60 min, medium was removed, and cells were washed with cold PBS. Different amounts of $^{125}$I-labeled 32-kDa protein (0 to 150 ng per well) in 0.20 ml of PBS containing 100 μg of bovine serum albumin per ml were added to each well, and cells were incubated on ice for 90 min. Free $^{125}$I-labeled 32-kDa protein was recovered, and portions of the unbound $^{125}$I-labeled 32-kDa protein were counted, which represented the free ligand. Cells were washed three times with cold PBS and collected in 2× SDS-sample buffer. Portions of collected samples were counted in a Biogamma counter, which represented the $^{125}$I-labeled 32-kDa protein bound to the cultured cells. The amounts of bound $^{125}$I-labeled 32-kDa protein were plotted versus the total amount of $^{125}$I-labeled 32-kDa protein added per well (A). Scatchard analysis of the binding data is shown (B). The specific binding of $^{125}$I-labeled 32-kDa protein to cells was calculated after subtracting the nonspecific binding observed in wells incubated in the presence of an excess of purified vaccinia virus (5,000 particles per cell).

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