JOURNAL OF VIROLOGY, Dec. 1994, p. 8409–8412
0022-538X/94/$04.00+0

Vol. 68, No.12

The Epidermal Growth Factor Receptor Is Not a Receptor for Vaccinia Virus

AMBROS W. HÜGIN* AND CONRAD HAUSER
Clinique de Dermatologie, Hôpital Cantonal Universitaire, 1211 Geneva, Switzerland

Received 10 June 1994/Accepted 15 September 1994

It has been suggested that the epidermal growth factor receptor (EGFR) is a receptor for vaccinia virus. Other reports, although not specifically addressing this question, did not support such a role for the EGFR. We addressed this issue by using wild-type virus and a virus growth factor deletion mutant, as well as sets of cells that do not express EGFR or have been transfected with the human gene for EGFR. The expression of virus growth factor by vaccinia virus or of EGFR by the target cells influenced neither virus adsorption to cells nor penetration. These results indicate that the EGFR is not a receptor for vaccinia virus.

Specific cell surface receptors are required for efficient entry of viruses into cells, and the presence of such receptors is a major factor in virus tropism. Vaccinia virus encodes a protein termed virus growth factor (VGF) with structural and functional homology to epidermal growth factor (EGF) and transforming growth factor alpha (1, 13, 17, 18). On the basis of the hypothesis that such a virally encoded protein could be incorporated into the virus envelope and target the virus to the EGF receptor (EGFR), it was suggested that the EGFR could be a receptor for vaccinia virus (7). Support for such a role was obtained from blocking studies using purified EGF, a panel of synthetic peptides with homology to EGF, and monoclonal anti-EGFR antibodies that competitively inhibit binding of EGF to its receptor (7, 14). These reagents specifically reduced vaccinia virus plaque formation. Because inhibition was maximally 60%, the existence of a second vaccinia virus receptor was postulated (7).

The EGFR seems to be an attractive candidate as a receptor for vaccinia virus, since it could explain (i) the typical clinical manifestations as an expression of the prominent epithelial cell tropism by members of the poxvirus family and (ii) the broad host cell range in tissue cultures, as the EGFR is ubiquitously expressed on cells other than lymphoid cells, which are mostly resistant to vaccinia virus infection (11).

Although not addressing this issue specifically, other reports have questioned the requirement of the EGFR for efficient infection with vaccinia virus (3, 4). The goal of the present investigation was to resolve this dispute by a series of experiments using (i) a control virus and a mutant virus in which the VGF gene has been deleted and (ii) pairs of fibroblast and lymphoid cells that do not express the EGFR or have been transfected with the human EGFR gene, respectively.

Cells. 2.2 is an NIH 3T3 cell clone that does not express the EGFR, and HER 2.2 is a subclone of 2.2 which has been stably transfected with the human EGFR (10). These cells were propagated in Dulbecco modified Eagle medium (DMEM) supplemented with glutamine, antibiotics, and 10% fetal calf serum. EP170.7 cells were obtained after transfection of nontumorigenic hematopoietic cell line 32D with the human EGFR (16). Both cell lines were grown in RPMI supplemented with glutamine, antibiotics, 15% fetal calf serum, and 5% interleukin 3-containing supernatant from WEHI-3 cells (ATCC TIB68).

Virus. For experiments, the following virus strains were used: IHD-J, WR, and its deletion mutant VSC20, which has both copies of the VGF gene replaced with a lacZ-containing cassette (3). To prepare purified virus, HeLa cells (ATCC CCL2) were infected and virus was prepared in accordance with standard procedures (6). Enveloped extracellular virus (EEV) was obtained by harvesting the supernatant of RK13 cells (ATCC CCL37) 24 h after infection with vaccinia virus at a multiplicity of infection of 3. To prepare biotinylated virus, WR virus was purified by being banded twice on a glucose gradient and then biotinylated with NHS-LC-biotin (Pierce, Rockford, Ill.) (12). The specificity of biotinylated vaccinia virus binding to cells was verified by binding studies in the presence of an excess of unlabelled virus. Unlabelled virus reduced specific cell binding of biotinylated virus to less than 8% in a dose-dependent fashion (data not shown).

EGF and vaccinia virus binding studies. To analyze EGFR expression, adherent cells were first detached from culture flasks by incubation with phosphate-buffered saline containing 10 mM EDTA. All cells were resuspended to yield single-cell suspensions, washed once with RPMI, and diluted in RPMI–0.02% (wt/vol) NaN_3 and 1% bovine serum albumin. Cells (5×10^5) were incubated first with EGF-biotin (Molecular Probes, Eugene, Oreg.) and subsequently stained with streptavidin-PE (Southern Associates Inc., Birmingham, Ala.). Similarly, cells were incubated with biotinylated vaccinia virus strain WR to detect virus binding to cells. Analysis was done on a FACscan (Becton Dickinson, San Jose, Calif.).

Plaque assay. For plaque assays, cells were plated in 24-well plates and incubated until they reached confluence. Before addition of virus, cells were incubated at 37°C with serum-free DMEM for 1 to 2 h. Virus was diluted in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered DMEM to give about 100 PFU per well and added to wells in a volume of 0.25 ml. Two hours after infection or the final time point in kinetic experiments, 1 ml of DMEM with 3% fetal calf serum was added per well. Plaques were developed 2 days later by adding 0.5 ml of staining solution (0.3% crystal violet, 10% formalin, 5% ethanol). PFU of four or six wells are given as the mean ± the standard error (SE).

Vaccinia virus purified from infected cells contains distinct phenotypes, described according to morphology and localization as intracellular naked virions (INV) and EEV (2). Although both INV and EEV viruses can infect cells efficiently

* Corresponding author. Mailing address: Clinique de Dermatologie, 4-783, Hôpital Cantonal Universitaire, 1211 Genève, Switzerland. Phone: 41-22-372-9458. Fax: 41-22-372-9460.

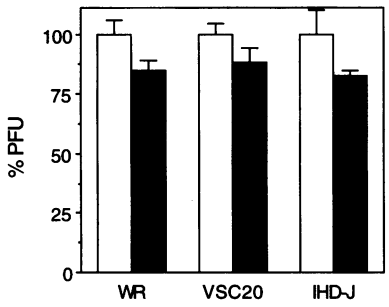

FIG. 1. Plaque formation on 2.2 and HER 2.2 cells by WR, VSC20, and IHD-J. Threefold dilutions of virus were added to 2.2 (open bars) and HER 2.2 (closed bars) cells as indicated, and plaque formation was analyzed 2 days later. PFU of quadruplicates are given as means ± standard errors. The numbers were standardized for individual cells, with the number of plaques obtained for each virus on 2.2 cells corresponding to 100%.

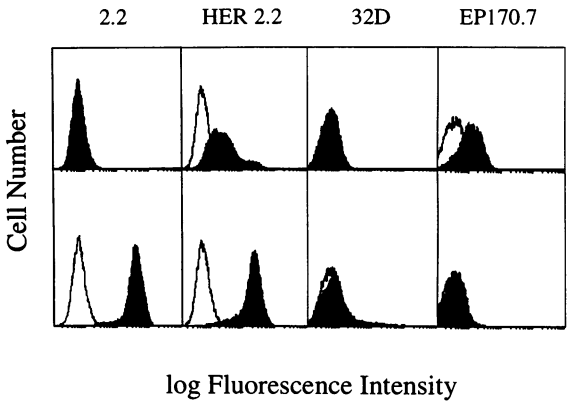

FIG. 2. Vaccinia virus binding to cells and expression of the EGFR. The presence of EGFR on cells was analyzed by incubating cells as indicated with biotinylated EGF and subsequently with streptavidin-PE for analysis with a FACscan (upper row). Vaccinia virus binding to cells was similarly analyzed by using biotinylated vaccinia virus (lower row). Control staining was obtained by incubation with streptavidin-PE alone. Curves for specific staining (closed curves) and control staining (open curves) are overlaid for each panel.

(15), it is not known if they use identical receptors. To exclude the possibility that the previous observations made by blocking the EGFR apply only to one particular form of vaccinia virus, initial experiments were performed with preparations of INV and EEV viruses. These experiments did not reveal any differences in plaque formation on fibroblast cells that do or do not express the EGFR, making it unlikely that the partial inhibition found previously in blocking experiments can be explained by selective blockage of only one phenotype of vaccinia virus (data not shown). EEV (containing <10% INV) was used for subsequent experiments, except for virus-binding studies with biotinylated virus.

Plaque formation, VGF, and EGFR. The gene for VGF encodes hydrophobic amino acids at the amino terminus, which may serve as a signal sequence, and a hydrophobic region at the carboxyl terminus representing a putative transmembrane region (1, 17). While VGF is readily detectable in the supernatant of vaccinia virus-infected cells (17, 18), the pathway of secretion and the possible expression of VGF in a membrane-bound form that might be incorporated into the virus envelope have not been investigated. By using the strain WR virus and its VGF deletion mutant VSC20, we could test if the expression of VGF affects plaque formation. The influence of the EGFR on plaque formation was analyzed by performing assays in parallel on EGFR-negative 2.2 cells and on transfected, EGFR-expressing HER 2.2 cells. Cells were infected with threefold virus dilutions, and plaques developed after 2 days (Fig. 1). Compared with that on 2.2 cells, plaque formation by both viruses, as well as virus strain IHD-J, was always lower on the EGFR-expressing cell line. While the difference varied between experiments (n = 6), apparently depending on the cell density (data not shown), it was always the same for all three viruses. Plaque formation by WR on HER 2.2 cells compared to the other combinations of virus and cells was never increased. Plaque size depended on the virus and the cell line used. VGF deletion mutant VSC20 induced big plaques on 2.2 and HER 2.2 cells, and wild-type WR induced big plaques on 2.2 and small plaques on HER 2.2 cells. This was due to the growth-promoting effect of VGF on HER 2.2 and proves the functionality of the EGFR for the HER 2.2 cell line. Interestingly, titration of progeny virus at various time points after infection of 2.2 or HER 2.2 cells did not reveal a difference in replication rate between the two viruses (data not shown). These experiments revealed that the number of PFU after infection does not correlate with viral VGF or host EGFR expression.

EGFR expression and vaccinia virus binding. Plaque formation is the result of repeated cycles of infection and is only an indirect measure of virus binding to cells. The use of biotinylated virus allows direct analysis of virus binding to cells and, in conjunction with biotinylated EGF, enabled us to correlate expression of the EGFR with binding of vaccinia virus. Cells were stained in parallel with biotinylated EGF and vaccinia virus and analyzed with a FACscan (Fig. 2). Of the first pair of cells, no EGF binding could be detected on 2.2 cells, while the EGFR of the transfected subclone HER 2.2 bound its ligand, confirming expression and proper ligand-binding capacity. Nevertheless, both cells adsorbed vaccinia virus equally well. Thus, the presence of the EGFR is not required for efficient virus binding. Of the second pair of cells analyzed, 32D cells did not bind EGF, while its EGFR-transfected subclone EP170.7 did. Nevertheless, when vaccinia virus adsorption was analyzed, very little virus bound to these cells and especially the presence of EGFR never increased adsorption. Not surprisingly, these cell lines are not permissive for vaccinia virus replication. As adsorption was completely absent in two of five experiments, we consider the weak binding of virus as being unspecific or, alternatively, due to low expression of a vaccinia virus receptor. Since our biotinylated virus preparation contains EEV and INV viruses in nearly equal amounts and we assume that biotinylation is similar for both viruses, we can also exclude the possibility that only one virus phenotype binds to the EGFR. This implies that the EGFR does not bind vaccinia virus or, alternatively, is not sufficient for virus binding to cells. Together, results from these binding studies strongly indicate that the EGFR is not a receptor for vaccinia virus.

Adsorption of virus. Although PFU after infection with IHD-J are similar on 2.2 and HER 2.2 cells, adsorption is a kinetic event, and accelerated adsorption, when measured in PFU, may not be apparent in an endpoint assay, such as a plaque-forming assay done at a single time point. Thus, the rates of adsorption on 2.2 and HER 2.2 cells were compared to investigate the possibility that the presence of the EGFR, acting as a cofactor with a low affinity to vaccinia virus or by affecting the affinity of the vaccinia virus receptor, accelerates irreversible binding. Such a model for adsorption has been

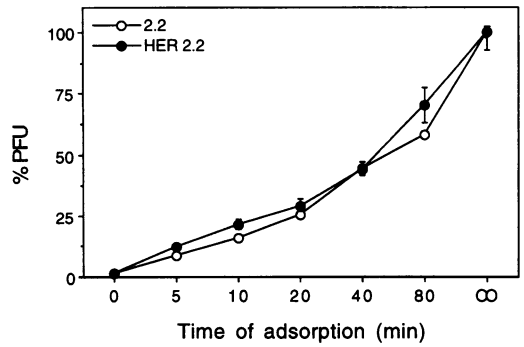

FIG. 3. Adsorption of IHD-J to 2.2 and HER 2.2 cells. Monolayers of cells were infected at 37°C with IHD-J for the periods indicated before being washed twice. Plaques were visualized 2 days later. The symbol ∞ signifies that wells were not washed. Mean numbers of PFU ± standard errors were normalized, with the number of PFU in wells not washed corresponding to 100%.

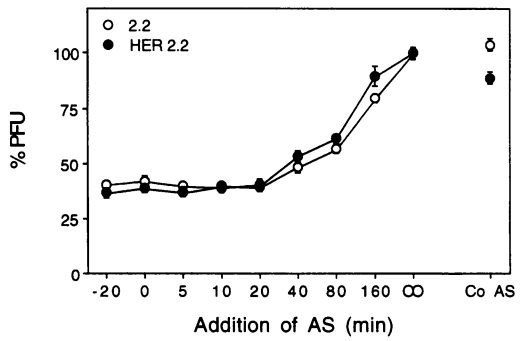

FIG. 4. Penetration of vaccinia virus into 2.2 and HER 2.2 cells. Penetration or resistance to antiserum neutralization was measured by adding vaccinia virus-specific antiserum (AS) at the times indicated to cells previously adsorbed with IHD-J. Plates were shifted from room temperature to 37°C at time zero. The symbol ∞ signifies that no antiserum was added to wells. Mean numbers of PFU ± standard errors were normalized, with 100% corresponding to the PFU for each cell line when no antiserum was added. Co AS represents values obtained when preimmune rabbit serum was added to wells instead of vaccinia virus-specific antiserum at −20 min.

suggested for other viruses (8, 9). Virus in 0.25 ml of DMEM was added to 2.2 and HER 2.2 cell monolayers, and plates were incubated at 37°C. After the periods indicated, wells were washed twice with DMEM and fresh medium was added. For both cell types, the number of plaques increased with time at the same rate (Fig. 3), showing that there is no difference between 2.2 and HER 2.2 cells in the kinetics of irreversible virus adsorption. Similarly, virus adsorption at room temperature was slower but occurred at the same rate for both cell lines (data not shown). Thus, these results exclude an accessory role of the EGFR in vaccinia virus binding to cells.

Penetration of virus. After adsorption, the vaccinia virus envelope fuses with the cell membrane and the virus core is released into the cytoplasm. Cell structures other than the virus receptor can be involved in virus penetration (19). To analyze such a role for the EGFR, penetration studies were performed by using resistance to antiserum after adsorption to cells as an indicator (15). Although not directly measuring virus penetration, such an assay may be biologically more relevant, as virus neutralization can still occur after adsorption to cells but then diminishes with time. Since cell monolayers detached when kept at 4°C, virus adsorption to 2.2 and HER 2.2 cells was done at room temperature. After 5 h, wells were washed twice with DMEM and fresh DMEM was added. At the times indicated before or after transfer of plates to 37°C, a rabbit antiserum to vaccinia virus, diluted in DMEM–1% bovine serum albumin, was added to wells. The final antiserum dilution was 1/150. Two hours after the last time point, 1 ml of DMEM with 3% fetal calf serum was added per well and plaques were visualized 2 days later. These penetration studies (i) show that after 5 h of adsorption at room temperature, the percentage of virus resistant to neutralization is the same for both cell lines (Fig. 4) and (ii) indicate that the presence of the EGFR does not alter the rate by which adsorbed virus becomes resistant to neutralization. Similar results with slower kinetics were obtained when penetration proceeded at room temperature (data not shown). Thus, the presence of the EGFR does not alter virus penetration.

Although the EEV form of vaccinia virus seems to be the biologically relevant form in vivo, additional experiments were performed with a preparation of INV that contains 10% or less EEV. Results from two experiments each measuring adsorption to or penetration of 2.2 and HER 2.2 cells revealed no kinetic differences between the two cell lines (data not shown). Thus, EGFR expression by target cells does not influence adsorption or penetration of either phenotypic form of vaccinia virus.

The conclusions drawn from our results contradict those obtained from blocking studies (7, 14). Our own experiments, including some blocking studies (data not shown), suggest that a plaque assay may not always be a reliable test for analysis of virus binding to its receptor. The readout of such a test depends on several steps of the infectious cycle of a virus, and there can be variation of plaque formation depending on other critical experimental conditions. For example, we found that vaccinia virus plaque formation, especially on L929 cells and A431 cells, previously used for receptor studies (7, 14), was dependent on cell density and probably other factors (data not shown). Consideration should also be given to the fact that manipulation of biologically active proteins, such as receptors for growth hormones, may have secondary effects on cells and plaque formation, respectively. In addition, in plaque assays, virus receptor occupation is far from saturating, even at a high virus particle to infectious unit ratio, which is 1:100 for vaccinia virus but can be up to 1:1,000 for some animal viruses (5). Thus, for effective blocking, probably >99% of receptors have to be occupied and careful kinetic studies of virus adsorption may be required. As a consequence, if a cell expresses different virus receptors, competitive occupation of one receptor will not result in markedly reduced PFU, unless the second receptor is available in much smaller numbers or is less efficient in allowing a productive infection. Thus, for analysis of virus-receptor interactions, one has to consider the inherent limitations of a plaque assay.

In summary, our experiments give clear evidence that the EGFR is not a vaccinia virus receptor and has no role in vaccinia virus adsorption or penetration. This is true for the EEV and INV forms of vaccinia virus. Thus, the identification of a receptor for poxviruses awaits further investigations.

We thank D. Wohlwend for helping with the fluorescence-activated cell sorter analysis; R. Wittek, B. Moss, A. Ullrich, and J. Pierce for providing virus or cells required for these studies; and J.-H. Saurat for support of this project.

REFERENCES

1. **Blomquist, M. C., L. T. Hunt, and W. C. Barker.** 1984. Vaccinia virus 19-kilodalton protein: relationship to several mammalian proteins, including two growth factors. Proc. Natl. Acad. Sci. USA **81:**7363–7367.
2. **Boulter, E. A., and G. Appleyard.** 1973. Differences between extracellular and intracellular forms of poxvirus and their implications. Prog. Med. Virol. **16:**86–108.
3. **Buller, R. M. L., S. Chakrabarti, J. A. Cooper, D. R. Twardzik, and B. Moss.** 1988. Deletion of vaccinia virus growth factor gene reduces virulence. J. Virol. **62:**866–874.
4. **Dales, S.** 1990. Reciprocity in the interactions between the poxvirus and their host cells. Annu. Rev. Microbiol. **44:**173–192.
5. **Dulbecco, R.** 1988. The nature of viruses, p. 1–26. *In* R. Dulbecco and H. S. Ginsberg (ed.), Virology. J. B. Lippincott Co., Philadelphia.
6. **Earl, P. L., N. Cooper, and B. Moss.** 1991. Preparation of cell cultures and vaccinia stocks, p. 16.16.1–16.16.7. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Wiley-Interscience, New York.
7. **Eppstein, D. A., Y. V. Marsh, A. B. Schreiber, S. R. Newman, G. J. Todaro, and J. J. Nestor, Jr.** 1985. Epidermal growth factor occupancy inhibits vaccinia virus infection. Nature (London) **318:**663–665.
8. **Haywood, A.** 1994. Virus receptors: binding, adhesion strengthening, and changes in viral structure. J. Virol. **68:**1–5.
9. **Haywood, A. M.** 1991. Evidence for multiple steps in enveloped virus binding, p. 149–162. *In* S. Ohaki (ed.), Cell and model membrane interaction. Plenum Press, New York.
10. **Honegger, A. M., T. J. Dull, S. Felder, E. Van Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger.** 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell **51:**199–209.
11. **Hügin, A. W.** Unpublished data.
12. **Inghirami, G., M. Nakamura, J. E. Balow, A. L. Notkins, and P. Casali.** 1988. Model for studying virus attachment: identification and quantitation of Epstein-Barr virus-binding cells by using biotinylated virus in flow cytometry. J. Virol. **62:**2453–2463.
13. **King, C. S., J. A. Cooper, B. Moss, and D. R. Twardzik.** 1986. Vaccinia virus growth factor stimulates tyrosine protein kinase activity of A431 cell epidermal growth factor receptors. Mol. Cell. Biol. **6:**332–336.
14. **Marsh, Y. V., and D. A. Eppstein.** 1987. Vaccinia virus and the EGF receptor: a portal for infectivity? J. Cell. Biochem. **34:**239–245.
15. **Payne, L. G., and E. Norrby.** 1978. Adsorption and penetration of enveloped and naked vaccinia virus particles. J. Virol. **27:**19–27.
16. **Pierce, J., M. Ruggiero, T. P. Fleming, P. P. Di Fiore, J. S. Greenberger, L. Varticovski, J. Schlessinger, G. Rovera, and S. A. Aaronson.** 1988. Signal transduction through the EGF receptor transfected in IL-3 dependent hematopoietic cells. Science **239:**628–631.
17. **Stroobant, P., A. P. Rice, W. J. Gullick, D. J. Cheng, I. M. Kerr, and M. D. Waterfield.** 1985. Purification and characterization of vaccinia virus growth factor. Cell **42:**383–393.
18. **Twardzik, D. R., J. P. Brown, J. E. Ranchalis, G. J. Todaro, and B. Moss.** 1985. Vaccinia virus-infected cells release a novel polypeptide functionally related to transforming and epidermal growth factors. Proc. Natl. Acad. Sci. USA **82:**5300–5304.
19. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrins αVβ3 and αVβ5 promote adenovirus internalization but not virus attachment. Cell **73:**309–319.