MOLECULAR AND CELLULAR BIOLOGY, Jan. 1986, p. 332–336
0270-7306/86/010332-05$02.00/0

Vol. 6, No. 1

Vaccinia Virus Growth Factor Stimulates Tyrosine Protein Kinase Activity of A431 Cell Epidermal Growth Factor Receptors

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Received 22 August 1985/Accepted 22 October 1985

Infection of A431 cells with vaccinia virus, or exposure to a mitogenic polypeptide secreted by vaccinia virus-infected cells, induces tyrosine phosphorylation of epidermal growth factor receptors.

An open reading frame that is present twice in the DNA genome of the poxvirus vaccinia virus (VV), is predicted to encode a polypeptide of 15.5 kilodaltons (37). A 50-amino acid portion of the predicted peptide is related in sequence to epidermal growth factor (EGF) and transforming growth factor (TGF) type α (1, 2, 23). Human EGF and rat TGFα show about 40% amino acid homology, and all cysteines, which are known to be important for the secondary structure of EGF (16), are found in homologous positions in TGFα (21). Both EGF and TGFα can bind to EGF receptors, both are mitogenic for appropriate cells, and both can act via EGF receptors to promote the growth of normal cells in soft agarose containing TGFβ (3, 32). Since the VV sequence is as closely related to EGF as EGF is to TGFα, and since in particular the disposition of cysteines is conserved, it has been suggested that the VV sequence codes for a growth factor (1, 2, 23).

The VV gene described above is transcribed early in infection of HeLa cells, producing a 620-nucleotide mRNA that can be translated in vitro to yield a polypeptide of about 19 kilodaltons (7). A similar RNA is found in cells infected by another poxvirus, rabbit poxvirus (7). Recently, a 25-kilodalton glycoprotein (vaccinia growth factor [VGF]) that can bind to EGF receptors has been purified from media of VV-infected BSC-1 cells (34). VGF competes with EGF or TGFα for binding to EGF receptors, and like EGF and TGFα it is mitogenic, yet it is distinguishable from EGF and TGFα by immunological and physical criteria (34). Direct genetic and structural evidence has now been obtained that VGF is the product of the VV gene possessing homology with EGF and TGFα (31; D. R. Twardzik, H. Marquardt, G. J. Todaro, and B. Moss, manuscript in preparation).

The EGF receptor has a tyrosine protein kinase activity that is stimulated by EGF (36). Addition of EGF to human tumor A431 cells, which have a superabundance of EGF receptors (11), stimulates protein phosphorylations at serine, threonine, and tyrosine (17). Phosphate labeling of the EGF receptor at serine and threonine increases by about 100%, but phosphate labeling at tyrosine increases by at least 300% (17). Tyrosine phosphorylation of EGF receptors in EGF-treated fibroblasts has also been detected (9). Most of the phosphotyrosine is found at a single position in the EGF receptor, now identified as tyrosine 1173 (10). This tyrosine is among several that are autophosphorylated in vitro when purified EGF receptors are incubated with EGF and ATP (10). Under some conditions, exposure of A431 cells to EGF can stimulate the de novo phosphorylation of other sites in EGF receptors (19). One such phosphorylation site, now identified as threonine 654 (8, 18, 22), can also be phosphorylated by protein kinase C in vitro (5), and its phosphorylation in EGF-stimulated cells is probably a consequence of activation of protein kinase C when membrane phospholipid turnover is stimulated by EGF (25, 26). Protein kinase C can also be activated in vitro by the tumor promoter tetradecanoyl phorbol acetate (TPA), and TPA promotes phosphorylation of EGF receptors at threonine 654 when added to A431 cells or fibroblasts (4, 5, 8, 9, 13, 19).

In this paper we describe changes in EGF receptor phosphorylation elicited in A431 cells by purified VGF or by infection with VV.

A431 cells were grown in Dulbecco-Vogt modified Eagle medium containing 10% calf serum. Cultures (50% confluent) in 35-mm plastic dishes were labeled for 16 to 18 h with 0.5 or 1.0 mCi of $^{32}P_i$ in 1.0 ml of P_i-free Dulbecco-Vogt modified Eagle medium supplemented with 1% calf serum and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4). ^{32}P-labeled cell cultures were infected with sucrose gradient-purified VV (strain WR) that had been titrated on monolayers of BSC-1 cells. Virus was diluted in P_i-free Dulbecco-Vogt modified Eagle medium–1% calf serum and sonicated, and 0.3-ml volumes were then added to the cell cultures, from which the radioactive medium had been removed. After 30 min at 37°C, the inoculum was removed, and the radioactive medium, supplemented with 40 μg of cytosine arabinoside per ml, was replaced. Fifteen micrograms of anti-EGF receptor immunoglobulin G (IgG) (528 IgG; 20) was added at this time if required. After a further 4 h, cells were washed and lysed in RIPA buffer (28) supplemented with 2 mM EDTA, 50 mM NaF, 14 mM 2-mercaptoethanol, and 0.2 mM Na_3VO_4 (added fresh). A centrifuged lysate (28) from each dish (about 10^5 cells) was incubated at 0°C for 30 min after each of the following additions: 10 μg of 528 IgG (20), 5 μl of rabbit antiserum to mouse IgG (Miles/Yeda), and 4 mg of Pansorbin (Calbiochem). Immunoprecipitates were washed, and the EGF receptor was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by partial acid hydrolysis or tryptic peptide mapping as described (17). Uninfected ^{32}P-labeled A431 cells were treated 1 h before the end of the labeling period with TPA (dissolved in dimethyl sulfoxide; final concentration, 100 ng/ml), an equal volume of dimethyl sulfoxide (final concentration, 0.1%), or 15 μg of 528 IgG per ml. EGF and VGF (purified from VV-infected BSC-1 cell culture medium as described [34]), dissolved in 10 mM acetic acid and 2 mg of serum albumin per ml, were added 10 min before the end of the

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TABLE 1. Phosphotyrosine content of EGF receptors after infection with VV[a]

Expt	Virus multiplicity	% Phosphotyrosine
1	0	0.7
	0.2	0.5
	2.0	1.6
	20.0	6.3
2	0	1.0
	20.0	12.0
	20.0[b]	3.0

[a] $^{32}P_i$-labeled A431 cells were infected with VV at various multiplicities (see the text). Cytosine arabinoside and $^{32}P_i$ were present from 0.5 to 4.5 h after infection. EGF receptors were isolated, and after hydrolysis in 5.7 M HCl at 110°C for 1 h, their phosphoamino acids were separated by two-dimensional thin-layer electrophoresis. Radioactivity in phosphotyrosine, phosphothreonine, and phosphoserine was quantified either by densitometry of autoradiograms (experiment 1) or by liquid scintillation counting (experiment 2). The radioactivity in phosphotyrosine is expressed as a percentage of the total radioactivity in all three phosphorylhydroxyamino acids. Phosphoserine accounted for about two-thirds of the remainder, and phosphothreonine composed one third.

[b] A 15-μg sample of 528 IgG was added from 0.5 to 4.5 h after infection.

labeling period. Control dishes received an equal volume of acetic acid-serum albumin solution.

We tested whether VV infection of A431 cells would induce EGF receptor phosphorylation at tyrosine. VV can grow and form plaques in A431 cell monolayers (S. Chakrabarti and B. Moss, unpublished data). Purified VV was added to $^{32}P_i$-labeled A431 cells for 30 min; the inoculum was then removed and the radioactive medium was replaced together with cytosine arabinoside, which inhibits viral DNA synthesis. Synthesis of early viral RNAs, including the mRNA for the 19-kilodalton polypeptide, is increased in the presence of cytosine arabinoside (38), and titers of VGF secreted into the media are likewise increased (34). Infection with 20 PFU of VV per cell induced tyrosine phosphorylation of the EGF receptor by 4.5 h after infection, and infection with 2 PFU per cell induced a smaller increase (Table 1). EGF receptors from cells infected with 0.2 PFU per cell contained amounts of phosphotyrosine that were too small to quantify but were detectable visually on the autoradiogram. Previous experiments have shown that VGF production increases with multiplicity of infection with VV (34).

To investigate whether the tyrosine phosphorylation stimulated by VV infection involved EGF receptors accessible at the cell surface, we used a monoclonal antibody, 528 IgG, which recognizes an extracellular epitope of the human EGF receptor and inhibits more than 95% of EGF binding to A431 cells (20). Addition of 528 IgG for the time interval between 0.5 and 4.5 h after infection with VV prevented most of the increase in phosphotyrosine in EGF receptors (Table 1).

Next we examined the effects of purified VGF on the phosphorylation state of the EGF receptor. A431 cells were labeled with $^{32}P_i$ for 15 to 18 h, enabling most protein phosphates to reach equilibrium, and then exposed to EGF (0.5 nM) or a similar EGF-competing concentration of high-pressure liquid chromatography-purified VGF. After 10 min, EGF receptors were isolated by immunoprecipitation. Phosphoamino acid analysis showed that VGF, like EGF, stimulated tyrosine phosphorylation of the receptor (Fig. 1A through C, insets). Tryptic peptide mapping revealed that both EGF and VGF induced phosphorylation of the EGF receptor at the peptide containing tyrosine 1173 (peptide 1, Fig. 1A through C). Both factors also stimulated phosphorylation at two related peptides (X and Z, Fig. 1A through C) that were also detected in receptors of TPA-treated cells (Fig. 1D) and contain threonine 654 as the only hydroxyamino acid (18). Accordingly, it appears that VGF stimulates autophosphorylation of the EGF receptor at tyrosine 1173 and activates phosphorylation of the EGF receptor by a protein kinase with identical specificity to protein kinase C.

In most experiments, VGF induced less extensive phosphorylation than EGF, but in some experiments VGF was equally active. The VGF concentration was estimated from competition binding measurements before use in phosphorylation experiments. VGF is relatively hydrophobic, however, and appears to lose potency, perhaps through aggregation or adsorption to surfaces. Since our experiments were performed with amounts of EGF that are not saturating for stimulation of tyrosine phosphorylation (17), variation in the amount of VGF added could be significant. When sufficient purified VGF is available, it will be possible to test whether the maximal phosphorylation with VGF equals the maximal phosphorylation with EGF.

One consequence of exposure of A431 cells to TPA is an inhibition of tyrosine phosphorylation upon subsequent EGF addition (5, 13). Likewise, stimulation of EGF receptor phosphorylation on tyrosine by VGF was prevented by prior exposure of cells to TPA (Fig. 1E).

When cell surface EGF receptors were rendered inaccessible with 528 IgG, subsequent induction of receptor phosphorylation at tyrosine in response to either VGF or EGF (1.7 nM) was inhibited (Table 2) (14).

These results demonstrate that either purified VGF or infection with VV can induce phosphorylation of A431 cell EGF receptors at tyrosine. Thus the functional similarities between VGF and EGF extend beyond the ability of both molecules to bind to EGF receptors and to stimulate DNA synthesis in human fibroblasts (34). Both factors can activate phosphorylation of the receptor at tyrosine 1173, presumably by the receptor's intrinsic kinase, by a mechanism that is sensitive to prior treatment of the cells with TPA or an antagonist antibody. VGF also stimulates receptor phosphorylation at threonine 654; presumably, like EGF, VGF can stimulate phosphatidylinositol turnover (25, 26) and activate protein kinase C.

TGFα, VGF, and EGF can all activate the A431 cell EGF receptor kinase in vitro (24, 31), and we have found that TGFα, like EGF and VGF, stimulates EGF receptor phosphorylation at tyrosine 1173 when added to intact A431 cells (J. A. Cooper, T. Hunter, and G. Todaro, unpublished data). Two monoclonal antibodies can also trigger the EGF receptor kinase (12, 27). It is possible that the three growth factors

TABLE 2. Phosphotyrosine content of EGF receptors after exposure of A431 cells to 528 IgG, VGF, and EGF[a]

Growth factor	% Phosphotyrosine	
	−528 IgG	+528 IgG
None	1.0	0.3
EGF	20.0	3.0
VGF	6.0	1.0

[a] $^{32}P_i$-labeled A431 cells were exposed to 0 or 15 μg of 528 IgG per ml for 60 min. Ten minutes before the end of the incubation, EGF (1.7 nM) or a similar concentration of VGF was added. EGF receptors were isolated by immunoprecipitation, and relative phosphotyrosine content was determined by liquid scintillation counting of separated phosphoamino acids (Table 1). Radioactivity in phosphotyrosine varied from 6 to 176 cpm above background.

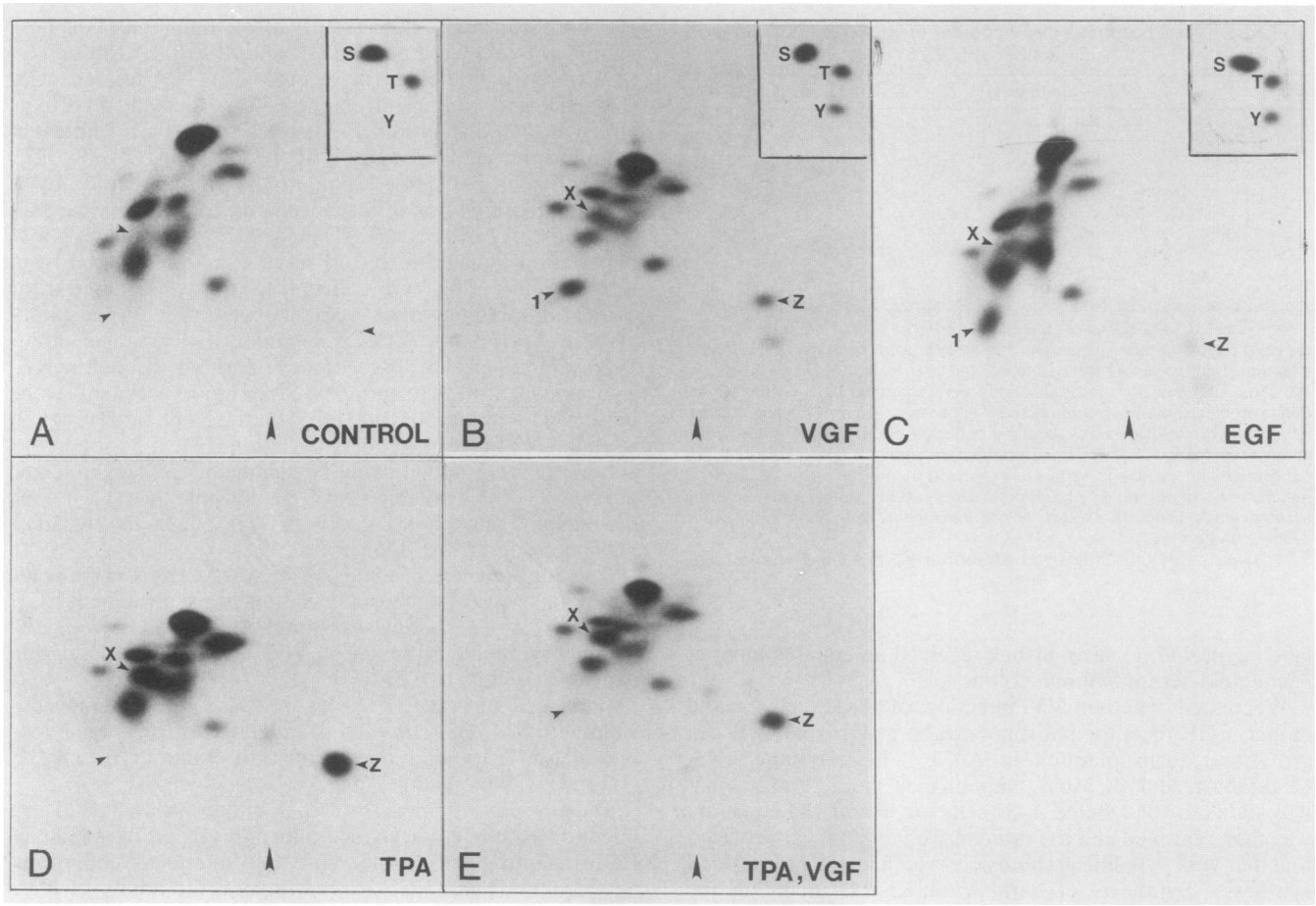

FIG. 1. Effects of VGF, EGF, and TPA on EGF receptor phosphorylation. $^{32}P_i$-labeled A431 cells were exposed to dimethyl sulfoxide (A-C) or TPA (D, E) for 50 min, and then VGF (B, E), EGF (C), or solvent (A) was added for 10 min. EGF receptors were isolated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One-fifth part of each sample was incubated with 5.7 M HCl at 110°C for 1 h, and four-fifths were digested with trypsin. (A through C, inserts) Phosphoamino acids from one-half of each acid hydrolysate were resolved by electrophoresis at pH 1.9 (anode at left) and perpendicularly at pH 3.5 (anode at top). Thin-layer plates were sprayed with ninhydrin to localize nonradioactive phosphoamino acid markers (S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine) and autoradiographed for 4 days at −70°C with a fluorescent screen. (A through E) Phosphopeptides (one-half of each trypsin digest) were resolved by electrophoresis at pH 8.9 (anode at left) and ascending chromatography. Thin-layer plates were exposed for 2 to 2.5 days (radioactivity analyzed ranged from 3,000 to 4,000 cpm Cerenkov). Parts A, C, and D were from one experiment; B and E were from another experiment in which an untreated control appeared similar to A, but in which the effects of EGF and of TPA alone were not tested. Peptide 1 phosphorylation in response to VGF was routinely observed, but the extent of phosphorylation was variable, probably because of variation in the amount of VGF added (see text). Large arrowheads: Points of sample application. Small arrowheads: Positions of peptides 1, X, and Z. Acid digestion of phosphopeptide 1 from VGF-treated cells released phosphotyrosine, and phosphothreonine was found in phosphopeptide Z (data not shown).

and the two antibodies all induce the same conformational change in the EGF binding domain. Alternatively, perhaps different conformational changes in the binding domain, outside the cell, all translate into the same change in the kinase domain, inside the cell, resulting in kinase activity. Indeed, the type of signal that may be transmitted across the plasma membrane from binding domain to kinase domain may be limited to on or off, given the apparently monomeric, simple structure of the EGF receptor (15, 35).

It is useful to compare the production of VGF and TGFα after infection with VV and various acute transforming retroviruses, respectively (33). VGF is encoded by VV, but TGFα is encoded by the cell and secreted in response to transformation. Whereas VGF is released into the culture medium, TGFα is not always detectable in the medium from transformed cells. It may be produced but then become adsorbed quantitatively to the EGF receptors of the cell in which it is synthesized. Since some intracellular EGF receptors are probably functional (29, 39), nascent TGFα could bind to EGF receptors totally within the cell. Such TGF may never leave the cell, but instead be degraded together with the receptor to which it is bound. This may be the case with A431 cells infected with Harvey or Kirsten murine sarcoma virus or Snyder-Theilen feline sarcoma virus, since TGFα was not detected in the culture media (6). Production of TGFα was inferred from the observation that EGF receptor number was decreased more than 70% in the infected cells, and receptors were degraded at a rate approaching that found when uninfected A431 cells were exposed to EGF (6). Tyrosine phosphorylation of EGF receptors was not detected, in accord with the low level of tyrosine protein kinase activity in A431 cells exposed for long periods to EGF (17).

In contrast, VV infection of BSC-1 cells induces considerable quantitites of VGF in a short time interval (34), which

probably accounts for the increased tyrosine phosphorylation of EGF receptors of VV-infected A431 cells. Most of the tyrosine phosphorylation was blocked by an antagonist antibody, suggesting that the bulk of VGF binding to receptors occurs at the cell surface, rather than to nascent receptors inside the cell. Despite these differences, the similarities between the biological and biochemical actions of VGF and TGFα imply that upon infection of normal cells with VV under appropriate conditions, neighboring cells may become mitogenically stimulated by the secreted VGF via an activation of the EGF receptor kinase. This mitogenic effect of VV infection may normally be obscured by the rapid spread of the lethal virus.

We are most grateful to Carol McLeod and John Mendelsohn for the gift of 528 IgG. We thank Jane E. Ranchalis for technical assistance and Beverly Davie for secretarial help.

This work was supported by grant BC-484 from the American Cancer Society.

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