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ARTICLE *in* VIROLOGY · APRIL 1996

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The Protein Kinase Activity of the Large Subunit of Herpes Simplex Virus Type 2 Ribonucleotide Reductase (ICP10) Fused to the Extracellular Domain of the Epidermal Growth Factor Receptor Is Ligand-Inducible

C. C. SMITH,*,†,‡ J. H. LUO,§ and L. AURELIAN*,†,‡,‖,[1]

*Virology/Immunology Laboratories, †Departments of Pharmacology and Experimental Therapeutics and ‖Microbiology, University of Maryland School of Medicine, Baltimore, Maryland 21201; ‡Department of Biochemistry, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205; and §Department of Pathology, Columbia University, New York, New York 10032

Received October 30, 1995; accepted January 9, 1996

The gene coding for the large subunit of herpes simplex virus type 2 ribonucleotide reductase (RR) (ICP10) has a unique 5′ terminal domain the product of which has a serine/threonine (Ser/Thr) protein kinase (PK) catalytic domain preceded by a transmembrane (TM) segment. Because ICP10 localizes on the cell surface and is internalized by the endocytic pathway like an activated growth factor receptor (Hunter *et al.,* 1995, *Virology* 210, 345–360), we asked whether it is ligand-inducible in order to examine whether it has intrinsic transphosphorylating activity. We constructed a chimeric expression vector that contains the extracellular and TM domains of the epidermal growth factor receptor (EGFR) joined to the intracellular PK and RR domains of ICP10 (pCH5) and established constitutively expressing cell lines in NIH3T3 2.2 cells that do not express EGFR. The chimeric protein, designated $p210^{CH5}$, localized to the surface of these cells as determined by immunofluorescent staining with MAb EGFR, and it bound ^{125}I-EGF. $p210^{CH5}$ coprecipitated with protein species p170, p120, p88, p60, p44, p34, and p25. EGF treatment activated the PK activity of $p210^{CH5}$, resulting in its autophosphorylation and the phosphorylation of the p120, p88, and p34 species. Immunoprecipitation/immunoblotting with anti-*ras*-GAP antibody and phosphoamino acid analysis indicated that p120 is *ras*-GAP and it is phosphorylated on Ser/Thr residues. The identities of the phosphorylated p88 and p34 are still unknown. The data indicate that when fused to a ligand-regulated extracellular domain (EGFR), the ICP10 PK auto- and transphosphorylating activities are ligand-inducible. These findings support the interpretation that the ICP10 PK activity is intrinsic and indicate that *ras*-GAP is one of its phosphorylation substrates.

INTRODUCTION

Ribonucleotide reductase (RR) is an essential enzyme for the conversion of ribonucleotides to the corresponding deoxyribonucleotides in eukaryotic and prokaryotic cells, and its activity may represent the rate-limiting step in DNA synthesis and concomitant cell growth (Thelander and Reichard, 1979). Several herpesviruses, including HSV-1, HSV-2, EBV, VZV, pseudorabies virus, and equine herpesvirus types 1 and 3, induce a novel distinct RR activity (Cohen *et al.,* 1977; Henry *et al.,* 1978; Lankinen *et al.,* 1982; Averett *et al.,* 1983; Dutia, 1983; Davison *et al.,* 1986) that may be required for virus growth in nondividing cells (Goldstein and Weller, 1988a,b; Preston *et al.,* 1988; Jacobson *et al.,* 1989). The HSV RR differs from the cellular enzyme in that it is insensitive to dTTP and dATP inhibition and does not have an absolute Mg^{2+} requirement (Langelier and Buttin, 1981). However, like the mammalian and bacterial enzymes, the HSV RR activity is formed by the association of two distinct subunits, the coding regions of which do not overlap. The large subunit (RR1) is a 140-kDa protein, designated ICP6 for HSV-1 and ICP10 for HSV-2. The small subunit (RR2) is a 38-kDa protein encoded by a 1.2-kb mRNA overlapping the 3′ end of the 5.0-kb mRNA that encodes RR1 (Anderson *et al.,* 1981; McLauchlan and Clements, 1983).

The HSV RR1 genes differ from their counterparts in eukaryotic and prokaryotic cells and in other viruses in that they possess a unique one-third 5′-terminal domain (Nikas *et al.,* 1986; Swain and Galloway, 1986). The ICP10 unique domain (ICP10 PK oncogene) causes neoplastic transformation of immortalized cells (Jariwalla *et al.,* 1980; Hayashi *et al.,* 1985; Iwasaka *et al.,* 1985; Smith *et al.,* 1992, 1994). Its protein product has conserved protein kinase (PK) catalytic motifs characteristic of serine/threonine (Ser/Thr)-specific kinases which are preceded by features of a transmembrane (TM) helical segment (Smith *et al.,* 1994; Chung *et al.,* 1989, 1990; Luo and Aurelian, 1992). Two proline-rich regions consistent with core SH3-binding motifs that might be involved in protein–protein interactions (Pawson, 1995) were also identified (Smith *et al.,* 1994).

A wealth of evidence indicates that the PK activity is an intrinsic property of ICP10. Thus: (i) expression of the ICP10 PK oncogene (amino acids 1–411) in eukaryotic or in bacterial expression systems permits the synthesis of an enzymatically active protein (Chung *et al.,* 1989;

[1] To whom correspondence and reprint requests should be addressed. Fax: (410) 706-2513.

0042-6822/96 $18.00

Luo *et al.,* 1991), (ii) ICP10 binds the ^{14}C-labeled ATP analogue *p*-fluorosulfonylbenzoyl 5′-adenosine (FSBA) and binding is specifically competed by another ATP analogue, AMP-PNP (Luo and Aurelian, 1992), (iii) ICP10 kinase activity is retained after electrophoresis on denaturing gels and renaturation on a nitrocellulose membrane (Luo and Aurelian, 1992), (iv) an ICP10 mutant deleted in the conserved PK catalytic motifs is PK negative and does not bind FSBA (Luo and Aurelian, 1992), (v) PK activity is also lost by deletion of the TM segment although the mutant protein retains all known target sites for cellular kinases (Luo and Aurelian, 1992; Smith *et al.,* 1994; Hunter *et al.,* 1995a), and (vi) mutation of Lys^{176} significantly decreases (Luo and Aurelian, 1992), almost abrogates (Peng *et al.,* 1996), PK activity and FSBA binding both in eukaryotic and in prokaryotic cells. It is unlikely that the ATP-binding Lys is located at the exact same site in ICP10 and in putative contaminating eukaryotic and prokaryotic kinases. Recent studies of the HSV-1 RR1 (ICP6) also concluded that the PK activity is intrinsic (Conner *et al.,* 1992; Cooper *et al.,* 1995).

ICP10 is associated with the plasma membrane with a polarity that identifies the TM as a membrane-spanning domain (Strnad and Aurelian, 1978; Smith *et al.,* 1994; Chung *et al.,* 1989; Luo and Aurelian, 1992; Hunter *et al.,* 1995a,b). Immunogold electron microscopy indicates that ICP10 is localized on the cell surface and it is internalized by the endocytic pathway, and deletion of the TM segment eliminates cell surface localization (Smith *et al.,* 1994; Hunter *et al.,* 1995a,b). Consistent with the interpretation that ICP10 kinase activity is intrinsic, the activity is required in order to induce activation of the *ras* signaling pathway and transformation (Smith *et al.,* 1992, 1994; Hunter *et al.,* 1995a).

Although ICP10 fulfills the same stringent criteria for intrinsic kinase activity that were used for established cellular PKs (Gibbs and Zoller, 1991; Taylor *et al.,* 1992), recent conflicting results relative to the RR1 transphosphorylating potential in HSV-infected cells (Ali, 1995) have again raised the question of the intrinsic nature of this activity. Since biochemical criteria for intrinsic activity were already studied, and in order to focus on transphophorylating potential, we constructed a chimeric molecule in which the extracellular domain of ICP10 is replaced by the ligand-binding extracellular region of a growth factor receptor whose ligand is known and used it to examine whether ICP10 PK activity is ligand-inducible. This approach is based on previous studies of chimeric molecules in which the intracellular signals induced upon stimulation with the appropriate ligand were shown to be determined by the cytoplasmic (PK catalytic domain) rather than the extracellular portion of the molecule (Ellis *et al.,* 1987; Lee *et al.,* 1989; Lehvaslaiho *et al.,* 1989; Riedel *et al.,* 1987, 1989, 1994; Di Fiore *et al.,* 1990; Seedorf *et al.,* 1991; Lhotak and Pawson, 1993). Here we describe the results of our studies with a chimeric construct (pCH5) in which both the extracellular domain and the TM segment of ICP10 were replaced by the corresponding domains of the epidermal growth factor receptor (EGFR). The data indicate that the ICP10 PK catalytic domain can function as an inducible kinase, supporting the conclusion that its PK activity is intrinsic.

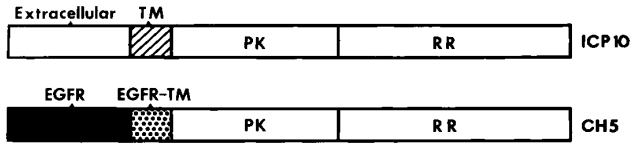

FIG. 1. Schematic representation of ICP10 and the epidermal growth factor (EGFR)/ICP10 hybrid protein p210^{CH5}. ICP10 consists of an extracellular domain (at residues 1–85), transmembrane segment (TM) (residues 86–106), protein kinase (PK) catalytic domain (residues 107–411), and ribonucleotide reductase (RR) domain (residues 411–1144). p210^{CH5}, the product of the pCH5 chimera, contains the extracellular and TM domains from EGFR (amino acids 1–687) and the PK and RR domains from ICP10 (amino acids 107–1144).

MATERIALS AND METHODS

Plasmids

pCH5 consists of the EGFR extracellular and TM domains and the ICP10 intracellular PK domain (Fig. 1). To construct pCH5, the EGFR cDNA (obtained from A. Ullrich) was digested with *Hin*dIII and *Eco*RI. The resulting 2.3-kb fragment that encodes the EGFR extracellular and TM domains was ligated into the TM-deleted ICP10 mutant pJHL15 that contains an *Eco*RI linker (Luo and Aurelian, 1992). Prior to ligation pJHL15 had been digested with *Hin*dIII/*Eco*RI to remove a 261-bp fragment encoding ICP10 residues 1–87. The resulting chimera, designated pCH1, was digested with *Hin*dIII/*Sca*I, and a 3.4-kb fragment that encompasses the EGFR extracellular and TM domains and ICP10 PK amino acids 106–351 was ligated into the *Hin*dIII/*Sca*I-collapsed pJW17N that contains the constitutive simian cytomegalovirus IE94 promoter and a SV_2neo cassette (Luo and Aurelian, 1992). pCH5 contains DNA sequences encoding EGFR amino acids 1–687 and ICP10 amino acids 107–1144.

Cells

NIH3T3 mouse clone 2.2 fibroblasts which do not express detectable levels of endogenous EGFR, erbB2, erbB3, or erbB4 (Levaslaiho *et al.,* 1989; Lee *et al.,* 1989; Margolis *et al.,* 1990; Osherov *et al.,* 1993; Kraus *et al.,* 1993) were obtained from I. Lax (Rorer Biotech, Inc.) and maintained in Dulbecco's modified minimal essential medium (DMEM) with 10% fetal bovine serum (FBS).

Antibodies

Polyclonal antibody anti-LA-1 to a peptide located upstream of the ICP10 TM (amino acids 13–26) and monoclonal antibodies (MAbs) H3 and G8 that respectively

recognize determinants located at ICP10 amino acids 414–1144 and 1–283 were described (Aurelian *et al.,* 1989; Chung *et al.,* 1991). MAb EGFR 528, which recognizes the EGFR extracellular domain, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-*ras*-GAP antibody (06-153) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Metabolic labeling

Labeling and preparation of whole cell extracts was as described (Smith *et al.,* 1994; Luo and Aurelian, 1992; Hunter *et al.,* 1995a). Cells were labeled with [^{35}S]-methionine (100 μCi/ml; sp act 1120 Ci/mmol; Dupont NEN Research Products, Boston, MA) in methionine-free DMEM with 10% FBS (18 hr, 37°) and resuspended in ice-cold RIPA buffer [150 m*M* NaCl, 20 m*M* Tris–HCl (pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO), 100 kallikrein units/ml aprotinin (Sigma)]. In some experiments, cells were serum starved by growth (24 hr) in DMEM with 0.5% FBS and labeled with [^{32}P]-orthophosphate (500 μCi/ml; NEN) in phosphate-free EMEM with 0.5% FBS (2 hr, 37°). They were stimulated (15 min, 37°) with 1 μg/ml epidermal growth factor (EGF) or mock-treated with phosphate-buffered saline, pH 7.3 (PBS). Labeled cells were resuspended in a buffer consisting of 50 m*M* HEPES (pH 7.5), 0.15 *M* NaCl, 1% NP-40, 5 m*M* EDTA, 10 m*M* sodium pyrophosphate, 1 m*M* sodium vanadate, 100 m*M* sodium fluoride, 1 m*M* PMSF, and 100 kallikrein units/ml aprotinin, incubated on ice for 15 min, and disrupted by sonication for 1 min at maximum setting in an Ultrasonics Model 220F sonicator. The extracts were clarified by centrifugation (30 min) at 16,000 *g* and the supernatants were used in immunoprecipitation.

Immunofluorescent staining

Immunofluorescent staining of unfixed (membrane) cells was done as described (Wymer *et al.,* 1989; Smith *et al.,* 1994). Flow cytometric analysis was performed on a Beckton–Dickinson FACScan cytometer equipped with a 150-mW laser. The cells were excited with 488 nm and fluorescence was collected through a 530/30 filter. Ten thousand events were stored in list mode and later analyzed using the Consort 40 analysis software from Beckton–Dickinson.

Immunoprecipitation and immunoblotting

For immunoprecipitation, cell extracts were incubated (1 hr, 4°) with 10–15 μl of antibody and (30 min, 4°) with 100 μl of protein A–Sepharose CL4B beads (50% v/v, Sigma). Beads were washed three times with ice-cold RIPA buffer and bound proteins were eluted by boiling (5 min) in 50 μl of denaturing solution [150 m*M* Tris–HCl (pH 7.0), 5.7% SDS, 14% β-mercaptoethanol, 17% sucrose, 0.04% bromthymol blue]. Proteins were resolved by SDS–PAGE on polyacrylamide gels and visualized by autoradiography. In some experiments, the resolved proteins were transferred to nitrocellulose membranes and immunoblotted with specific antibodies. Detection was with the ECL detection system. For immunoprecipitation/immunoblotting experiments with anti-*ras*-GAP antibody, cells were resuspended in a buffer consisting of 50 m*M* HEPES, pH 7.5, 0.15 m*M* NaCl, 1% Triton X-100, 10% glycerol, 100 m*M* sodium fluoride, 1 m*M* sodium vanadate, 10 m*M* sodium pyrophosphate, 1 m*M* PMSF, and 100 kallikrein units/ml aprotinin. After 30 min of incubation on ice, the extracts were clarified by centrifugation at 16,000 *g* for 30 min. Quantitative analyses were done by densitometric scanning of samples equilibrated for protein content using a Molecular Dynamics Image Quant computing densitometer Model 300B (Smith *et al.,* 1994).

EGF binding

EGF binding was measured as described by Lhotak and Pawson (1993). Cells were plated in 12-well plates at a density of 1×10^5 cells/well. The next day the plates were cooled on ice, washed twice with ice-cold washing buffer (PBS with 1 mg/ml BSA), and incubated (2 hr) on ice in washing buffer containing 1 ng/ml ^{125}I-EGF (Amersham) in the presence or absence of 100-fold excess cold EGF (Upstate Biotechnology). Cells were washed five times with ice-cold washing buffer, lysed in 0.2% SDS–0.2 *N* NaOH and counted in a scintillation gamma counter (Beckman). Cell protein was measured from duplicate wells treated as above but without the addition of radioactive isotope. Protein was assayed using the BCA protein assay kit (Pierce Chemicals, Rockford, IL) according to the manufacturer's instructions.

Phosphoamino acid analysis

Extracts of CH5 cells were assayed for *in vitro* PK activity by immunocomplex assay with MAb H3, and the ^{32}P-labeled p210^{CH5} and p120 were identified. Gel sections corresponding to p210^{CH5} and p120 were excised, homogenized in 2 ml of 50 m*M* ammonium bicarbonate with 0.1% SDS–1 m*M* EDTA–10 μg of bovine serum albumin per milliliter (16 hr, 37°). The eluted proteins were cleared of gel debris by filtration through a 0.22-μm cellulose acetate filter (Spin-X, Co-Star) and were trichloroacetic acid (20% final concentration) precipitated. Following centrifugation (15 min, 16,000 *g,* 4°), the precipitates were washed with cold absolute ethanol and cold ethanol–diethyl ether (50% v/v), suspended in 100 μl of boiling 6 *N* HCl, and incubated 1 hr at 110°. The hydrolysates were lyophilized and resolubilized in 10 μl of a marker mixture containing 1 mg each of phosphoserine, phosphothreonine, and 04-phosphotyrosine per milliliter (Sigma). Phosphoamino acids were separated by two-

dimensional paper electrophoresis at pH 2.3 for 2.4 hr at 400 V in 1 *M* acetic acid followed by pH 5.0 for 2.5 hr at 400 V in 0.1 *M* pyridine acetic acid. The positions of the markers were ascertained by staining with ninhydrin spray reagent (Sigma) and the ^{32}P-labeled amino acids were visualized by autoradiography (Chung *et al.,* 1989).

RESULTS

Stable transfection and expression of EGFR/ICP10 hybrid protein

We have previously shown that: (i) the ICP10 TM segment is a membrane-spanning domain and (ii) ICP10 PK activity is required to cause activation of the *ras* signaling pathway (Smith *et al.,* 1994; Hunter *et al.,* 1995a). Based on these observations, and in order to further examine whether ICP10 has intrinsic kinase activity, we constructed an expression vector for EGFR/ICP10 chimera (pCH5) which contains DNA sequences that encode a protein (p210^{CH5}) in which the EGFR extracellular and TM domains (EGFR amino acids 1–687) are joined to the ICP10 intracellular domains (ICP10 amino acids 107–1144). The vector also contains a SV_2neo cassette to facilitate establishment of stably transfected cell lines. pCH5 retains ICP10 intracellular sequences that contain all the known targets for cellular kinases. Therefore, if the ICP10 PK activity is due to a contaminating, albeit invisible, cellular PK, ICP10 should be constitutively phosphorylated in cells that express pCH5. Alternatively, if the ICP10 PK activity is intrinsic, phosphorylation should be ligand-stimulated.

NIH3T3 2.2 cells that do not express detectable levels of EGFR, erbB2, erbB3, or erbB4 (Levaslaiho *et al.,* 1989; Lee *et al.,* 1989; Margolis *et al.,* 1990; Osherov *et al.,* 1993; Kraus *et al.,* 1993) were transfected with pCH5 followed by G418 selection (400 μg) as described (Luo and Aurelian, 1992; Smith *et al.,* 1994). Individual colonies were independently isolated and stable cell lines were established. Three cell lines (CH5a, CH5b, and CH5f) were chosen for further passage and analysis. To determine whether the hybrid proteins are expressed in these cell lines, total extracts of cells labeled with [^{35}S]-methionine (5×10^6 cells; 1200 μg protein) were precipitated with MAb H3 (recognizes a determinant in the ICP10 RR domain) or with MAb EGFR (recognizes a determinant in the EGFR extracellular domain) and separated by SDS–PAGE as previously described (Luo and Aurelian, 1992; Smith *et al.,* 1992, 1994).

As shown in Fig. 2 for one representative cell line (CH5a), a protein (p210^{CH5}) with the expected molecular weight for the chimera protein was precipitated by MAb H3 from [^{35}S]methionine-labeled CH5 cells (Fig. 2A, lane 4). Its expression levels were similar in all three independently established cell lines, as determined by densitometric scanning of the immunoprecipitates (densitometric integration units = 3348 ± 150). Proteins with respective molecular weights of 170, 120, 88, 60, 44, 34, and 25 kDa coprecipitated with p210^{CH5} (Fig. 2A, lane 4). The coprecipitating proteins are not proteolytic breakdown products of the 210-kDa chimera protein nor are they unrelated proteins that share an epitope with p210^{CH5}, since similar results were obtained with MAb G8 (Fig. 2A, lanes 7 and 8) that recognizes a different ICP10 epitope (Chung *et al.,* 1991) or MAb EGFR (Fig. 2B, lane 2) that is unrelated to ICP10. The levels of the coprecipitating p60 and p34 were significantly lower in the MAb EGFR than the MAb H3 immunoprecipitates. This may reflect a different stoichiometric interaction than that seen for the other coprecipitating proteins which appear to be equally distributed in the MAb H3 and MAb EGFR immunoprecipitates. Proteins were not precipitated from NIH3T3 2.2 cells (Fig. 2A, lane 2; Fig. 2B, lane 1) nor by normal rabbit serum (NRS) (Fig. 2A, lane 3). Proteins p170 and p25 are consistent with the complex of the guanine exchange factor hsos and the adaptor protein Grb_2 (Chardin *et al.,* 1993; Li *et al.,* 1993), and immunoblotting of the MAb H3 precipitates with antibody to hsos or Grb_2 confirmed their identity (C. C. Smith *et al.,* in preparation). These proteins bind ICP10 in stably transfected human cells (Smith *et al.,* 1994) and or NIH3T3 cells (C. C. Smith *et al.,* in preparation).

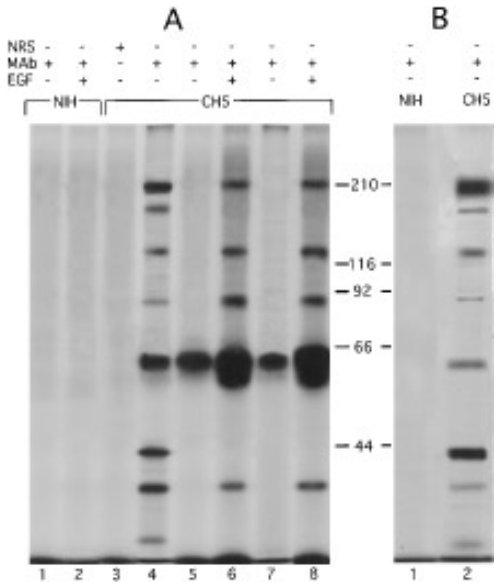

FIG. 2. Expression and phosphorylation of p210^{CH5}. (A) Extracts from [^{35}S]methionine-labeled CH5a cells (lanes 3 and 4) were immunoprecipitated with Mab H3 (lane 4) or normal rabbit serum (NRS) (lane 3). Extracts from serum-starved CH5a (lanes 5–8) or NIH3T3 2.2 cells (lanes 1 and 2) labeled with [^{32}P]orthophosphate and treated with PBS (lanes 1, 5, and 7) or with 1 μg/ml EGF (lanes 2, 6, and 8) for 15 min were immunoprecipitated with MAb H3 (lanes 1, 2, 5, and 6) or MAb G8 (lanes 7 and 8). Precipitated proteins were resolved by 9.5% SDS–PAGE. (B) Extracts from [^{35}S]methionine-labeled NIH3T3 2.2 (lane 1) or CH5a (lane 2) cells were immunoprecipitated with MAb to EGFR. Precipitated proteins were resolved by 9.5% SDS–PAGE. Similar results were obtained for all independently established CH5 cell lines. Molecular weight markers are listed in the margins.

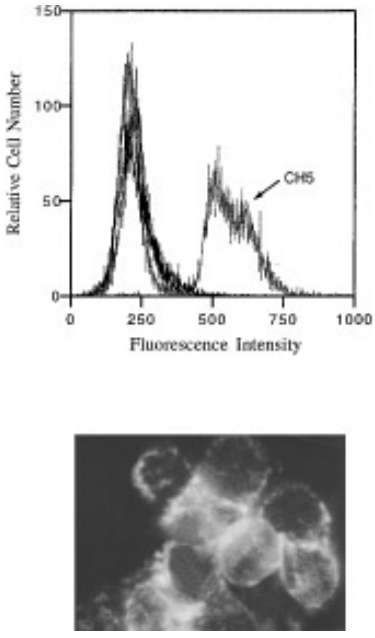

FIG. 3. FACS analysis of CH5b (arrow) and NIH3T3 2.2 cells stained in membrane immunofluorescence with Mab EGFR. Scans of CH5b and NIH3T3 2.2 cells stained with NRS or anti-LA-1 antibody overlap the NIH3T3 2.2 cells stained with MAb EGFR. Similar results were obtained for all independently established CH5 cell lines.

p210^{CH5} localizes on the cell surface

We used membrane immunofluorescent staining with the EGFR MAb in order to determine whether p210^{CH5} localizes to the cell surface. As shown in Fig. 3, 97–99% of the CH5 cells stained with the EGFR antibody. NIH3T3 2.2 cells did not stain with MAb EGFR and preimmune serum was negative.

p210^{CH5} binds EGF

To determine whether the hybrid protein retains EGF binding potential, we examined the ability of CH5 cells to bind ^{125}I-labeled EGF. The protocol, similar to that previously described for EGFR chimeric proteins (Lee *et al.,* 1989), involved cell exposure to ^{125}I-EGF in the presence or absence of 10- to 100-fold excess cold EGF. CH5 cells bound the ^{125}I-labeled EGF (275 cpm/μg protein) and the radioactivity was competed by cold EGF. This is shown in Fig. 4 for a 100-fold excess of EGF. ^{125}I-EGF did not bind to NIH3T3 2.2 cells, consistent with previous reports (Margolis *et al.,* 1990). We estimate that CH5 cells express $\sim 1 \times 10^5$ specific EGF-binding sites/cell. This compares to 2×10^6 receptors/cell in A431 cells studied in parallel (data not shown). These findings indicate that (i) CH5 cells express specific EGF-binding sites on the cell surface which are not present on NIH3T3 2.2 cells and (ii) the levels of EGF-binding receptors on CH5 cells are 20-fold lower than those in A431 cells (Haigler *et al.,* 1978; Lehvaslaiho *et al.,* 1989) but within the range previously reported for EGFR hybrid proteins (Lehvaslaiho *et al.,* 1989; Lhotak and Pawson, 1993; Riedel *et al.,* 1987; Lax *et al.,* 1989).

ras-GAP binds p210^{CH5}

ras-GAP is a 120-kDa protein that functions as a negative regulator of *ras* (Satoh *et al.,* 1990a,b; Molloy *et al.,* 1992). It stimulates the otherwise weak intrinsic GTPase activity of *ras,* thereby promoting its return to an inactive, GDP-bound state (Trahey and McCormick, 1987; McCormick, 1989). Since *ras*-GAP binds to ICP10 in stably transfected (constitutively expressing) human cells (Smith *et al.,* 1994), and a p120 species coprecipitates with p210^{CH5}, these studies sought to determine whether: (i) *ras*-GAP binds p210^{CH5} and (ii) binding is dependent on EGF treatment. To address these questions, CH5 cells were serum starved and exposed to EGF (or PBS control) for 15 min as described above. Extracts obtained at this time were immunoprecipitated with MAb H3 or NRS control and the proteins, resolved by SDS–PAGE and transferred to nitrocellulose membranes, were immunoblotted with anti-*ras*-GAP antibody. As shown in Fig. 5 for CH5f cells, *ras*-GAP was observed in both the EGF-treated (Fig. 5, lane 4) and untreated (Fig. 5, lane 3) cells. It was not seen in the NRS immunoprecipitates (Fig. 5, lanes 1 and 2). Similar results were obtained for all three CH5 lines and for precipitates obtained with MAbs G8 or EGFR. We interpret these findings to indicate that the coprecipitating p120 is *ras*-GAP and its binding to p210^{CH5} is ligand-independent.

EGF activates the PK activity of p210^{CH5}

Ligand binding to the extracellular domain of EGFR triggers autophosphorylation and transphosphorylation of several intracellular substrates on tyrosine residues

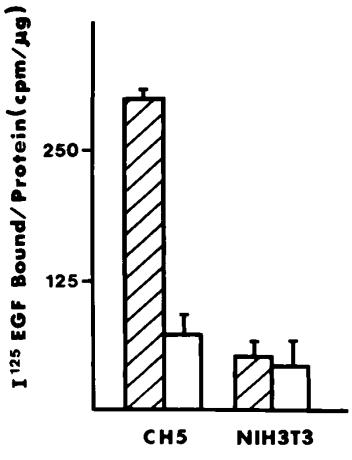

FIG. 4. Binding of ^{125}I-EGF to the p210^{CH5} chimera. CH5f and NIH3T3 2.2 cells (1×10^5 cells/well) were incubated on ice with 1 ng/ml ^{125}I-EGF in the absence (▨) or presence (□) of 100-fold excess nonradioactive EGF. The results represent mean values from three independent experiments. Similar results were obtained for all independently established CH5 cell lines.

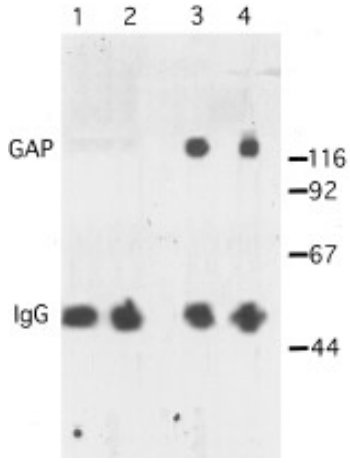

FIG. 5. *ras*-GAP binds the p210^{CH5} chimera protein. Extracts from serum-starved (24 hr, 0.5% FBS) CH5f cells treated with PBS (lanes 1 and 3) or with 1 μg/ml EGF (lanes 2 and 4) were immunoprecipitated with MAb G8 (lanes 3 and 4) or NRS (lanes 1 and 2) and electrotransferred to nitrocellulose membranes. They were immunoblotted with anti-*ras*-GAP antibody. Similar results were seen for all three CH5 lines and for immunoprecipitates obtained with MAb H3 or MAb EGFR. Molecular weight markers are shown in the right margin.

(van der Geer *et al.,* 1994). Since the hybrid p210^{CH5} protein contains the ICP10 PK catalytic domain which is Ser/Thr specific, the question arises whether its PK activity is triggered by EGF binding. To address this question, CH5 cells were serum starved by 24 hr of incubation in medium containing 0.5% FBS and labeled (2 hr, 37°) with [^{32}P]orthophosphate before stimulation (15 min) with 1 μg/ml EGF or PBS. NIH3T3 2.2 cells served as control. Cell extracts obtained at this time were precipitated with MAb H3 and analyzed by SDS–PAGE. Autophosphorylated p210^{CH5} was precipitated from EGF-treated (Fig. 2A, lane 6) but not untreated (Fig. 2A, lane 5) CH5 cells. Other phosphorylated proteins precipitated from EGF-treated, but not untreated, CH5 cells were p120 (*ras*-GAP), p88, p60, and p34 (Fig. 2A, lane 6). A phosphorylated 60-kDa protein was also precipitated from PBS-treated CH5 cells (Fig. 2A, lane 5) but its levels were significantly (three- to fivefold) higher in the precipitates from the EGF-treated cells (Fig. 2A, lane 6). Although p170, p25, and p44 proteins coprecipitated with p210^{CH5} from [^{35}S]methionine-labeled cells (Fig. 2A, lane 4) they were not phosphorylated (Fig. 2A, lane 6), suggesting that ICP10-mediated phosphorylation is specific and only some of the proteins which bind p210^{CH5} are phosphorylated by its ligand-induced activation. Similar results were obtained with MAb G8 (Fig. 2A, lanes 7 and 8), indicating that this was not an artifact due to the use of a specific MAb in the immunocomplex PK assay. Phosphorylated proteins were not seen in the precipitates from the EGF-treated NIH3T3 2.2 cells (Fig. 2A, lane 2) and NRS was negative (data not shown).

Immunoblotting of the MAb H3 precipitates from EGF-treated (Fig. 2A, lane 6) CH5 cells with anti-*ras*-GAP antibody confirmed that the phosphorylated p120 is *ras*-GAP (Fig. 6A, lane 1). *ras*-GAP was also seen in immunoblotting (with anti-*ras*-GAP antibody) of the precipitates from untreated CH5 cells (Fig. 6A, lane 2), but it was not seen in precipitates from NIH3T3 cells (Fig. 6A, lane 3). Phosphoamino acid analysis of the phosphorylated p210^{CH5} and *ras*-GAP coprecipitated with p210^{CH5} from EGF-treated CH5 cells indicated that both are phosphorylated on serine/threonine (Figs. 6B and 6C), consistent with the specificity of ICP10 PK. These findings indicate that EGF stimulation induces the kinase activity of p210^{CH5}, and *ras*-GAP is a phosphorylation substrate of the EGF-activated p210^{CH5}. The identity of the other proteins phosphorylated by EGF treatment of CH5 cells is still unclear.

DISCUSSION

The HSV-2 RR1 gene differs from its counterparts in eukaryotic and prokaryotic cells and in other viruses in that it possesses a unique 5′-terminal domain that has transforming activity (ICP10 PK oncogene) and encodes a protein that has conserved PK catalytic motifs preceded by a TM segment and an extracellular domain (Smith *et al.,* 1994; Chung *et al.,* 1989, 1990; Hunter *et al.,* 1995a).

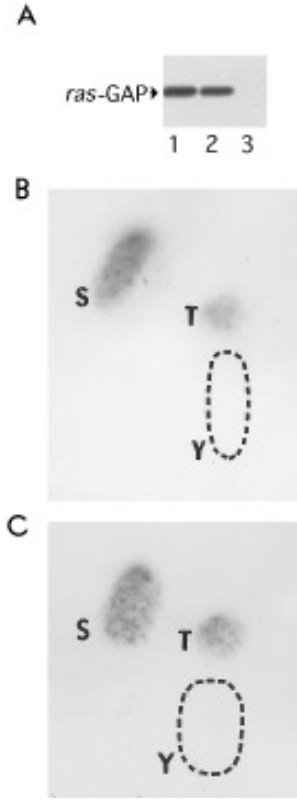

FIG. 6. (A) Immunoblotting of the MAb H3 PK immunocomplex assays of EGF-treated (lane 1) or untreated (lane 2) CH5a cells or of NIH3T3 2.2 cells (lane 3) with anti-*ras*-GAP antibody. (B) Phosphoamino acid analysis of the phosphorylated *ras*-GAP from EGF-treated CH5f cells. (C) Phosphoamino acid analysis of the phosphorylated p210^{CH5} from EGF-treated CH5f cells.

The oncoprotein is located on the cell surface and is internalized by the endocytic pathway. It induces activation of the *ras* signaling pathway and causes increased cell proliferation and anchorage-independent growth. Membrane anchorage and PK activity are required for signaling and anchorage-independent growth (Smith *et al.,* 1994; Hunter *et al.,* 1995a). Comparison of the ICP10 PK oncoprotein to the superfamily of Ser/Thr GFRs in the current versions of SwissProt (v27.0) and Genbank (v80.0) databases and analysis by the GCG/Wisconsin (v8.0) DISTANCES program indicate that the oncoprotein is a member of a novel subfamily of Ser/Thr GFR kinases (Hunter *et al.,* 1995b). One outstanding difference between the ICP10 PK oncoprotein and known members of the superfamily is the presence of two proline-rich regions that closely resemble core SH3-binding motifs, at positions 150 and 396 within the PK catalytic domain (Smith *et al.,* 1994). The exact function of these motifs is still unknown. However, a bacterially expressed oncoprotein mutant that retains only the proline-rich SH3-binding motif at position 150 binds Grb_2 *in vitro* after it is purified to homogeneity (M. Santana-Verano, personal communication), suggesting that these motifs may be involved in the binding of signaling proteins.

A wealth of evidence indicates that the ICP10 PK activity is intrinsic. Thus: (i) expression of the ICP10 PK oncogene (amino acids 1–411) in eukaryotic or in bacterial expression systems permits the synthesis of an enzymatically active protein (Luo *et al.,* 1991), (ii) ICP10 binds the ^{14}C-labeled ATP analogue FSBA and binding is inhibited by another ATP analogue, AMP-PNP (Luo and Aurelian, 1992), (iii) kinase activity is retained after ICP10 electrophoresis on denaturing gels and renaturation on a nitrocellulose membrane (Luo and Aurelian, 1992), (iv) an ICP10 mutant deleted in the PK catalytic domain is PK negative and does not bind FSBA (Luo and Aurelian, 1992), (v) kinase activity is not due to contaminating cellular kinases since an ICP10 mutant deleted in the TM segment retains all known target sites for cellular kinases but is PK negative (Luo and Aurelian, 1992; Smith *et al.,* 1994; Hunter *et al.,* 1995a)—the deleted TM is not the binding site for cellular kinases since it is a transmembrane-spanning segment (Hunter *et al.,* 1995a,b), and (vi) mutation of Lys^{176} significantly decreases (Luo and Aurelian, 1992), almost abrogates (Peng *et al.,* 1996), the PK activity and FSBA binding of ICP10 expressed in eukaryotic cells and bacteria and it is unlikely that the putative eukaryotic and bacterial contaminating kinases have the same ATP binding site as that mutated in ICP10.

It is noteworthy that similar types of evidence have been used to establish that cellular kinases have intrinsic enzymatic activity (Gibbs and Zoller, 1991; Taylor *et al.,* 1992). Notwithstanding, recent conflicting results relative to the transphosphorylating potential of RR1 PK (Ali, 1995) have raised the possibility that the ICP10 PK activity is due to a contaminating, albeit invisible, cellular kinase, most notably CKII (Langelier, personal communication). In addition to the above-summarized evidence which argues against this interpretation, ICP10 PK is Mn^{2+}-dependent for optimal activity (Peng *et al.,* 1996) while CKII is Mg^{2+}-dependent for optimal PK activity (Gatica *et al.,* 1993) and ICP10 PK activity does not require monovalent cations and is not inhibited by zinc sulfate (Peng *et al.,* 1996), unlike CKII (Gatica *et al.,* 1993). However, since most of these studies did not specifically address the question of transphosphorylation, we took advantage of the observation that ICP10 has properties of a GFR to construct a chimera protein in which the function/specificity of ICP10 kinase can be examined under conditions of controlled ligand-induced activation.

Hybrid receptors were previously used to elucidate the biological and biochemical activities of GFR kinases, the ligand of which is unknown. Chimeras were constructed between different tyrosine kinases including EGFR/fms, EGFR/v-src (Riedel, 1994), EGFR/neu (Lehvaslaiho *et al.,* 1989), and EGFR-ret (Santoro *et al.,* 1994). Hybrids constructed from receptor molecules were functional with respect to protein expression, cell surface localization, and ligand binding (Lehvaslaiho *et al.,* 1989; Lhotak and Pawson, 1993; Riedel, 1994; Santoro *et al.,* 1994). They also displayed ligand-responsive kinase activity and, at least in some cases, mitogenic responses (Santoro *et al.,* 1994). The contribution of the TM origin was addressed in a study of EGFR-elk receptors and found to be insignificant. However, unlike the wild type EGFR expressed in the same cells, the EGFR-elk chimeras did not induce neoplastic changes or enhance cell growth (Lhotak and Pawson, 1993). A hybrid consisting of the extracellular and TM domains of EGFR and the intracellular domain of v-src (which is not a plasma membrane-spanning protein) displayed a ligand-unresponsive v-src tyrosine kinase and did not evidence cell-transforming activity or increased DNA synthesis (Riedel, 1994). Our construction strategy was based on these findings. The EGFR ligand-binding extracellular domain was selected for these studies because it had been previously used to construct chimeric proteins in which intracellular signals induced by EGF stimulation were shown to be mediated by the PK catalytic domains rather than the extracellular EGFR domains or the formation of heterodimers with erbB2, erbB3, or erbB4 (Ellis *et al.,* 1987; Lee *et al.,* 1989; Lehvaslaiho *et al.,* 1989; Riedel *et al.,* 1989; Di Fiore *et al.,* 1990; Seedorf *et al.,* 1991; Lhotak and Pawson, 1993; Osherov *et al.,* 1993; Kraus *et al.,* 1993). To control for the potential contribution of conformational changes resulting from its deletion, the ICP10 RR domain was retained. Stably transfected lines were established in NIH3T3 2.2 cells because they do not contain endogenous EGFR, erbB2, erbB3, or erbB4 (Levaslaiho *et al.,* 1989; Lee *et al.,* 1989; Margolis *et al.,* 1990; Kraus *et al.,* 1993; Osherov *et al.,* 1993) and therefore the EGF

signal must be mediated by the EGFR/ICP10 hybrid protein ($p210^{CH5}$).

We found that $p210^{CH5}$, which retains the ligand-regulated extracellular domain and TM segment of EGFR, is expressed in stably transfected cell lines, localizes to the cell surface, and binds EGF. The proportion of cells expressing high levels of $p210^{CH5}$ on the cell surface, the estimated number of EGF-binding sites, and the levels of bound ^{125}I-EGF were within the range previously described for EGFR chimera proteins (Lehvaslaiho *et al.,* 1989; Lhotak and Pawson, 1993; Riedel *et al.,* 1987; Lax *et al.,* 1989). Similar results were obtained for all three independently established CH5 lines, suggesting that this is not an artifact, unique to one cell line. Immunoprecipitation/immunoblotting assays similar to those previously used to determine GFR binding to signaling proteins (Lowenstein *et al.,* 1992; Li *et al.,* 1993) indicated that $p210^{CH5}$ binds proteins p88, p60, p44, and p34, the identities of which are still unknown, as well as proteins p170 and p25, which are consistent with the hsos–Grb_2 complex (Chardin *et al.,* 1993; Li *et al.,* 1993) and were identified as such by immunoblotting of the precipitates with the respective antibodies (C. C. Smith *et al.,* in preparation), and a p120 species that is recognized by anti-*ras*-GAP antibody. This is consistent with previous findings that hsos-Grb_2 and *ras*-GAP bind to ICP10 in the stably transfected transformed human cells JHLa1 (Smith *et al.,* 1994). *ras*-GAP binding to $p210^{CH5}$ was independent of EGF stimulation, suggesting that it is similar to that evidenced by ICP10 (in JHLa1 cells), but differs from that evidenced by EGFR which complexes with signaling proteins only after it is ligand-activated (Lowenstein *et al.,* 1992; van der Geer *et al.,* 1994). Presumably, ligand-triggered activation is required for EGFR binding of signaling proteins in order to phosphorylate the Tyr residues recognized by SH2 modules on these proteins (van der Geer *et al.,* 1994). Binding by ICP10 and the EGFR/ICP10 hybrid, on the other hand, is likely to involve SH3 modules the ligands of which are unphosphorylated proline-rich regions (Pawson, 1995).

Significantly, $p210^{CH5}$ evidenced ligand-inducible kinase activity. Thus, $p210^{CH5}$ was phosphorylated in EGF-treated, but not untreated cells, and EGF treatment induced the phosphorylation of the coprecipitating p120 (*ras*-GAP) protein. Phosphoamino acid analysis of $p210^{CH5}$ and the coprecipitated *ras*-GAP indicated that they are phosphorylated on Ser/Thr residues, supporting the conclusion that *ras*-GAP is a substrate for the ICP10 kinase activity (Smith *et al.,* 1994). This is consistent with previous findings that *ras*-GAP proteins are activated by growth factor stimulation (Trahey and McCormick, 1987; McCormick, 1989). Other phosphorylated proteins coprecipitating with $p210^{CH5}$ are p88, p60, and p34. The p60 species was also phosphorylated in EGF-untreated serum-starved CH5 cells, but its levels were increased three- to fivefold after EGF treatment, suggesting that it is also phosphorylated by $p210^{CH5}$. The identities of these phosphorylated proteins are still unclear. Other proteins which coprecipitate with $p210^{CH5}$ from [^{35}S]methionine-labeled cells (p170, p25, and p44) were not phosphorylated after EGF treatment, suggesting that the transphosphorylating activity of $p210^{CH5}$ is specific. The levels of EGF that induced phosphorylation (100–1000 ng/ml) are within the range previously described for EGFR chimera proteins (Lhotak and Pawson, 1993; Santoro *et al.,* 1994).

The hybrid receptor $p210^{CH5}$ replicates the behavior of the wild type ICP10 in that it binds *ras*-GAP as well as other cellular proteins potentially involved in signaling, and when its PK activity is stimulated by EGF treatment, it phosphorylates a number of proteins including *ras*-GAP. However, phosphorylation is not indiscriminate, as some of the proteins that bind $p210^{CH5}$ are not phosphorylated by EGF treatment. By demonstrating that the ICP10 PK catalytic domain can function as an inducible kinase, our findings support previous conclusions that the ICP10 auto- and transphosphorylating activities are intrinsic (Smith *et al.,* 1994; Chung *et al.,* 1989; Luo and Aurelian, 1992). The alternative interpretation is that PK activity is mediated by a cellular kinase which is intimately associated with ICP10 but, miraculously, it remains invisible under all the experimental conditions studied so far and does not contribute to the specific properties [viz. cation requirements (Peng *et al.,* 1996)] of the ICP10 kinase activity. This interpretation implies that EGF activates the invisible contaminant without transmitting its signal through $p210^{CH5}$, which is its only receptor in CH5 cells. In addition to contradicting all available data on ligand-induced signaling, this fanciful interpretation is illogical and cannot be taken seriously.

Further studies are required to determine whether the EGF-initiated signal in CH5 cells is routed through the same pathway as that described for ICP10 (Smith *et al.,* 1994; Hunter *et al.,* 1995a) to cause increased cell proliferation. Ongoing studies are designed to address this question and identify the other proteins that bind $p21^{CH5}$ and are phosphorylated by EGF treatment. The effect of ligand stimulation on activation of the *ras* signaling pathway, DNA synthesis, and anchorage-independent growth and the role of the ICP10 proline-rich sequences in *ras*-GAP binding are also under investigation.

ACKNOWLEDGMENTS

This study was supported by Public Health Service Grant CA39691 from the National Cancer Institute. We thank Deniece Garnett for help with the figures and Dr. J. V. Ordonez for help with the FACS analysis.

REFERENCES

Ali, M., McWheeney, D., Milosavljevic., A., Jurka, J., and Jariwalla, R. J. (1991). Enhanced malignant transformation induced by expression of a distinct protein domain of ribonucleotide reductase large subunit from herpes simplex virus type 2. *Proc. Natl. Acad. Sci. USA* 88, 8257–8261.

Ali, M. A. (1995). The 140-kDa RR1 protein from both HSV-1 and HSV-2 contains an intrinsic protein kinase activity capable of autophosphorylation but it is transphosphorylation defective. *Virology* 207, 409–416.

Anderson, K. P., Frink, R. J., Devi, G. B., Gaylord, B. H., Costa, R. H., and Wagner, E. K. (1981). Detailed characterization of the mRNA mapping in the *Hin*dIII fragment K region of the herpes simplex virus type 1 genome. *J. Virol.* 37, 1011–1027.

Averett, D. R., Lubbers, C., Elion, G. B., and Spector, T. (1983). Ribonucleotide reductase induced by herpes simplex virus type 1 virus. *J. Biol. Chem.* 258, 9831–9838.

Aurelian, L., Terzano, P., Smith, C. C., Chung, T. C., Shamsuddin, A., Costa, S., and Orlandi, C. (1989). Amino terminal epitope of herpes simplex virus type 2 ICP10 protein as a molecular diagnostic marker for cervical intraepithelial neoplasia. *In* "Cancer Cells," Vol. 7, "Molecular Diagnostics of Human Cancer," pp. 187–191. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Chardin, P., Carnonis, J. H., Gale, N. W., Van Aelst, L., Schlesinger, J., Willer, M. H., Bar-Sagi, D. (1993). Human Sos1 — A guanine nucleotide exchange factor for *ras* that binds to Grb2. *Science* 260, 1338–1343.

Chung, T. C., Luo, J. H., Wymer, J. P., Smith, C. C., and Aurelian, L. (1991). Leucine repeats in the large subunit of herpes simplex virus type 2 (HSV-2) ribonucleotide reductase (ICP10) are involved in RR activity and subunit complex formation. *J. Gen. Virol.* 72, 1139–1144.

Chung, T. D., Wymer, J. P., Kulka, M., Smith, C. C., and Aurelian, L. (1989). Protein kinase activity associated with the large subunit of the herpes simplex virus type 2 ribonucleotide reductase (ICP1). *J. Virol.* 63, 3389–3398.

Chung, T. D., Wymer, J. P., Kulka, M., Smith, C. C., and Aurelian, L. (1990). Myristylation and polylysine-mediated activation of the protein kinase domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *Virology* 179, 168–178.

Cohen, J. C., Henry, B. E., Randall, C. C., and O'Callaghan, D. (1977). Ribonucleotide reductase activity in hydroxyurea-resistant herpesvirus replication. *Proc. Soc. Exp. Biol. Med.* 155, 395–399.

Conner, J., Cooper, J., Furlong, J., and Clements, J. B. (1992). An autophosphorylating but not transphosphorylating activity is associated with the unique N-terminus of the herpes simplex virus type 1 ribonucleotide reductase large subunit. *J. Virol.* 66, 7511–7516.

Cooper, J., Conner, J., and Clements, J. B. (1995). Characterization of the novel protein kinase activity present in the R1 subunit of herpes simplex virus ribonucleotide reductase. *J. Virol.* 69, 4979–4985.

Davison, A. J., and Scott, J. E. (1986). The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* 67, 1759–1816.

Di Fiore, P. P., Segato, O., Taylor, W. G., Aaronson, S. A., and Pierce, J. H. (1990). EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. *Science* 248, 79–83.

Dutia, B. M. (1983). Ribonucleotide reductase induced by herpes simplex virus has a virus-specified constituent. *J. Gen. Virol.* 64, 513–521.

Ellis, L., Morgan, D. O., Jong, S. M., Wang, L. H., Roth, R. A., and Rutter, W. J. (1987). Heterologous transmembrane signaling by a human insulin receptor v-ros hybrid in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* 84, 5101–5105.

Gatica, M., Hinrichs, M. V., Jedlicki, A., Allende, C. C., and Allende, J. E. (1993). Effect of metal ions on the activity of casein kinase II from *Xenopus laevis*. *Fed. Eur. Biochem. Soc.* 315, 173–177.

Gibbs, C. S., and Zoller, M. J. (1991). Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. *J. Biol. Chem.* 266, 8923–8931.

Goldstein, D. J., and Weller, S. K. (1988a). Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: Characterization of an ICP6 deletion mutant. *Virology* 166, 41–51.

Goldstein, D. J., and Weller, S. K. (1988b). Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: Isolation and characterization of an ICP6 lacZ insertion mutant. *J. Virol.* 62, 196–205.

Haigler, H., Ash, J. F., Singer, S. J., and Cohen, S. (1978). Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc. Natl. Acad. Sci. USA* 75, 3317–3321.

Hayashi, Y., Iwasaka, T., Smith, C. C., Aurelian, L., Lewis, G. K., and Ts'o, P. O. P. (1985). Multistep transformation by defined fragments of herpes simplex virus type 2 DNA: Oncogenic region and its gene product. *Proc. Natl. Acad. Sci. USA* 82, 8493–8497.

Henry, B. E., Glaser, R., Hewetson, J., and O'Callaghan, D. J. (1978). Expression of altered ribonucleotide reductase activity associated with the replication of Epstein–Barr virus. *Virology* 89, 262–271.

Hunter, J. C. R., Smith, C. C., Bose, D., Kulka, M., Broderick, R., and Aurelian, L. (1995a). Intracellular internalization and signaling pathways triggered by the large subunit of HSV-2 ribonucleotide reductase (ICP10). *Virology* 210, 345–360.

Hunter, J. C. R., Smith, C. C., and Aurelian, L. (1995b). The HSV-2 LA-1 oncogene is a member of a novel family of serine/threonine receptor kinases. *Int. J. Oncol.* 7, 515–522.

Iwasaka, T., Smith, C., Aurelian, L., and Ts'o, P. O. P. (1985). The cervical tumor-associated antigen (ICP10/AG-4) is encoded by the transforming region of the genome of herpes simplex virus type 2. *Jpn. J. Cancer Res.* 76, 946–958.

Jariwalla, R. J., Aurelian, L., and Ts'o, P. O. P. (1980). Tumorigenic transformation induced by a specific fragment of DNA from herpes simplex virus type 2. *Proc. Natl. Acad. Sci. USA* 77, 2279–2283.

Jacobson, J. G., Leib, D. A., Goldstein, D. J., Bogard, C. L., Schaffer, P. A., Weller, S. K., and Coen, D. M. (1989). A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells. *Virology* 173, 276–283.

Kraus, M. H., Fedi, P., Starks, V., Muraro, R., and Aaronson, S. A. (1993). Demonstration of ligand-dependent signaling by the erbB3 tyrosine kinase and its constitutive activation in human breast tumor cells. *Proc. Natl. Acad. Sci. USA* 90, 2900–2904.

Langelier, Y., and Buttin, G. (1981). Characterization of ribonucleotide reductase induction in BHK-21/C13 Syrian hamster cell line upon infection by herpes simplex virus (HSV). *J. Gen. Virol.* 57, 21–31.

Lankinen, H., Graslund, A., and Thelander, L. (1982). Induction of a new ribonucleotide reductase after infection of mouse L cells with pseudorabies virus. *J. Virol.* 41, 893–900.

Lax, I., Bellor, F., Howk, R., Ullrich, A., Givol, D., and Schlessinger, J. (1989). Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules. *EMBO J.* 8, 421–427.

Lee, J., Dull, T. J., Lax, I., Schlessinger, J., and Ullrich, A. (1989). HER cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor. *EMBO J.* 8, 167–173.

Lehvaslaiho, H., Lehtola, L., Sistonen, L., and Alitalo, K. (1989). A chimeric EGF-R–neu proto-oncogene allows EGF to regulate neu tyrosine kinase and cell transformation. *EMBO J.* 8, 159–166.

Lhotak, V., and Pawson, T. (1993). Biological and biochemical activities of a chimeric epidermal growth factor–ELK receptor tyrosine kinase. *Mol. Cell. Biol.* 13, 7071–7079.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993). Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* 363, 85–88.

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Scholnick, E., Bar-Sagi, D., and Schlessinger, J. (1992). The SH2 and SH3 domain containing protein Grb2 links receptor tyrosine kinases to ras signaling. *Cell* 70, 431–442.

Luo, J. H., and Aurelian, L. (1992). The transmembrane helical segment but not the invariant lysine is required for the kinase activity of the large subunit of herpes simplex virus type 2 ribonucleotide reductase. *J. Biol. Chem.* 267, 9645–9653.

Luo, J. H., Smith, C. C., Kulka, M., and Aurelian, L. (1991). A truncated protein kinase domain of the large subunit of the herpes simplex type 2 ribonucleotide reductase (ICP10) expressed in *Escherichia coli. J. Biol. Chem.* 266, 20976–20983.

Margolis, B., Bellot, F., Honegger, A. M., Ullrich, A., Schlessinger, J., and Zilberstein, A. (1990). Tyrosine kinase activity is essential for the association of phospholipase C γ with the epidermal growth factor receptor. *Mol. Cell. Biol.* 10, 435–441.

McCormick, F. (1989). ras GTPase activating protein: Signal transmitter and signal terminator. *Cell* 56, 5–8.

McLauchlan, J., and Clements, J. B. (1983). DNA sequence homology between two co-linear loci on the HSV genome which have different transforming abilities. *EMBO J.* 2, 1953–1961.

Molloy, C. J., Fleming, T. P., Bottaro, D. P., Cuadrado, A., and Aaronson, S. A. (1992). Platelet-derived growth factor stimulation of GTPase-activating protein tyrosine phosphorylation in control and c-H-ras-expressing NIH 3T3 cells correlates with p21ras activation. *Mol. Cell. Biol.* 12, 3903–3909.

Nikas, I., McLauchlan, J., Davison, A. J., Taylor, W. R., and Clements, J. B. (1986). Structural features of ribonucleotide reductase. *Proteins: Struct. Funct. Genet.* 1, 376–384.

Osherov, N., Gazit, A., Gilon, C., and Levitzki, A. (1993). Selective inhibition of the epidermal growth factor and HER/neu receptors by tyrphostins. *J. Biol. Chem.* 268, 11134–11142.

Paradis, H., Gaudreau, P., Massie, B., Lamarche, N., Guilbault, C., Gravel, S., and Langelier, Y. (1991). Affinity purification of active subunit 1 of herpes simplex virus type 1 ribonucleotide reductase exhibiting a protein kinase function. *J. Biol. Chem.* 266, 9647–9651.

Pawson, T. (1995). Protein modules and signaling networks. *Nature* 373, 573–580.

Peng, T., Colin-Hunter, J. R., and Nelson, J. (1996). The novel protein kinase of the RR1 subunit of herpes simplex virus has autophosphorylation and transphosphorylation activity that differs in its ATP requirements for HSV-1 and HSV-2. *Virology* 216, 184–196.

Preston, V. G., Darling, A. A., and McDougall, I. M. (1988). The herpes simplex virus type 1 temperature-sensitive mutant ts 1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. *Virology* 167, 458–467.

Riedel, H. (1994). Epidermal growth factor (EGF) modulation of feline sarcoma virus *fms* tyrosine kinase activity, internalization, degradation, and transforming potential in an EGF receptor/vfms chimera. *J. Virol.* 68, 411–424.

Riedel, H., Dull, T. M., Honegger, A. M., Schlessinger, J., and Ullrich, A. (1989). Cytoplasmic domains determine signal specificity, cellular routing characteristics and influence ligand binding of epidermal growth factor and insulin receptors. *EMBO J.* 8, 2943–2954.

Riedel, H., Schlessinger, J., and Ullrich, A. (1987). A chimeric, ligand-binding v-erbB/EGF receptor retains transforming potential. *Science* 236, 197–200.

Santoro, M., Wong, W. T., Aroca, P., Santos, E., Matoskova, B., Grieco, M., Fusco, A., and Di Fiore, P. P. (1994). An epidermal growth factor receptor/*ret* chimera generates mitogenic and transforming signals: Evidence for a *ret*-specific signaling pathway. *Mol. Cell. Biol.* 14, 663–675.

Satoh, T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T., and Kaziro, Y. (1990a). Accumulation of p21ras GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA* 87, 7926–7929.

Satoh, T., Endo, M., Nakafuku, M., Nakamura, S., and Kaziro, Y. (1990b). Platelet-derived growth factor stimulates formation of active p21ras GTP complex in Swiss mouse 3T3 cells. *Proc. Natl. Acad. Sci. USA* 87, 5993–5997.

Seedorf, K., Felder, S., Millauer, B., Schlessinger, J., and Ullrich, A. (1991). Analysis of platelet-derived growth factor receptor domain function using a novel chimeric receptor approach. *J. Biol. Chem.* 266, 12424–12431.

Smith, C. C., Kulka, M., Wymer, J. P., Chung, T. D., and Aurelian, L. (1992). Expression of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) is required for virus growth and neoplastic transformation. *J. Gen. Virol.* 73, 1417–1428.

Smith, C. C., Luo, J. H., Hunter, J. C. R., Ordonez, J. V., and Aurelian, L. (1994). The transmembrane domain of the large subunit of HSV-2 ribonucleotide reductase (ICP10) is required for protein kinase activity and transformation-related signaling pathways that result in *ras* activation. *Virology* 200, 598–612.

Strnad, B., and Aurelian, L. (1978). Proteins of herpesvirus type 2. III. Isolation and immunologic characterization of a large molecular weight viral protein. *Virology* 87, 401–415.

Swain, M., and Galloway, D. A. (1986). Herpes simplex virus specifies two subunits of ribonucleotide reductase encoded by 3′-coterminal transcripts. *J. Virol.* 57, 802–808.

Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., and Sowadski, J. M. (1992). Structural framework for the protein kinase family. *Annu. Rev. Cell Biol.* 8, 429–462.

Thelander, L., and Reichard, P. (1979). Reduction of ribonucleotides. *Annu. Rev. Biochem.* 48, 133–158.

Trahey, M., and McCormick, G. (1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* 238, 542–545.

van der Geer, P., Hunter, T., and Lindberg, R. A. (1994). Receptor protein–tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.* 10, 251–337.

Wymer, J. P., Chung, T. C., Chang, Y. N., Hayward, G. S., and Aurelian, L. (1989). Identification of immediate-early-type cis-response elements in the promotor for the ribonucleotide reductase large subunit form herpes simplex virus type 2. *J. Virol.* 63, 2773–2784.