Foot-and-mouth disease virus (FMDV) binds to cellular integrins through an RGD motif in its capsid protein, VP1. It is unclear, however, what kind of cellular event(s) are triggered after the binding of VP1 to the cells. In this study, we show that aqueous soluble recombinant DNA-derived VP1 (rVP1) of FMDV induced apoptosis of BHK-21 cells after binding to integrins. In addition, treatment of BHK-21 cells with rVP1 resulted in deactivation of Akt and enhancement of several pro-apoptotic responses such as dephosphorylation of glycogen synthase kinase-3β and cleavage of procaspase-3, -7, and -9. Additional studies revealed that the rVP1 treatment caused apoptosis of cancer cells, including MCF-7 (a breast carcinoma cell line with a functional deletion of the caspase-3 gene) and PC-3 (a sphingosine 1-phosphate receptor subtype 3-deficient androgen-independent prostate cancer cell line). These results suggest that rVP1 of FMDV may be used selectively as a potent apoptotic agent for human cancer by modulating the Akt signaling pathway and that its effect is not primarily dependent on either activation of procaspase-3 or deactivation of sphingosine 1-phosphate receptor subtype 3.

Foot-and-mouth disease virus (FMDV) is the etiological agent of foot-and-mouth disease, a deadly epidemic that affects many economically important domestic livestock such as cattle, pigs, goats, and sheep (1). There are seven serotypes of FMDV that all belong to the Aphthovirus genus of the Picornaviridae (2).

The capsid of FMDV is made up of 60 copies each of four proteins, VP1, VP2, VP3, and VP4 (2). Although, in some cases, FMDV may use heparin sulfate as an alternative receptor for internalization (3), it usually infects cells by attaching to integrin receptors through a long conformationally flexible loop (G-H loop) of VP1 (4, 5). The sequence of this loop contains a conserved RGD tripeptide motif, which is characteristic of the ligands that bind to integrin receptors (3, 6–9).

Integrins belong to a family of cell-surface α-β heterodimeric glycoproteins that are responsible for a variety of processes, including the induction of signal transduction pathways that modulate cell proliferation, morphology, migration, and apoptosis (10). To date, four species of integrins (αβ1, αβ6, αβ9, and αβ3) have been shown to mediate FMDV infection (6, 11, 12).

Regulation of apoptosis, or programmed cell death, allows the organism to replace aged cells, to control the cell number and tissue size, and to protect itself from rogue cells that may lead to lethality (13). Apoptosis requires integration and fine-tuning of multiple proteins that are either regulators or executors of the survival and death processes. Cancer, autoimmune diseases, immunodeficiency disease, reperfusion injury, and neurodegenerative disorders are characterized by dysregulation of apoptosis. Integrin signaling can activate the Akt pathway (14), an anti-apoptotic mechanism utilized by many types of cells (15). It has been shown that Akt mediates cell survival in response to stimulation by growth factors (16, 17). Akt is a serine/threonine kinase that binds phosphorylated lipids at the membrane in response to activation of phosphatidylinositol 3-kinase (PI3K) (18). Activation of PI3K through G protein-coupled receptors such as sphingosine 1-phosphate (S1P) receptors or use of PI3K inhibitors (e.g. LY294002) may thus modulate the activity of Akt (19). Phosphorylated Akt can activate anti-apoptotic or inhibit pro-apoptotic processes in the cell by phosphorylating Bad, Forkhead transcription factors, glycogen synthase kinase-3β (GSK-3β), and caspase-9 (20–25). On the other hand, deactivation of Akt has been shown to activate pro-apoptotic responses (26). Both GSK-3β and caspase-9 are pro-apoptotic factors (27, 28). Cleavage of caspase-9 results in induction of a caspase cascade, including the processing of procaspase-3 and -7 (29). The initiation of the caspase cascade eventually leads to apoptosis (28).

Although integrins have been shown to mediate FMDV infection (6, 11, 12), whether the binding of FMDV and VP1 to integrins results in activation, deactivation, or any cellular response is unclear. Full-length and truncated forms of recombinant DNA-derived VP1 (rVP1) of FMDV have been generated previously by several investigators (30). To solve the problem of poor water solubility, however, rVP1 has been routinely used together with denaturing agents such as urea (31). The presence of denaturing agents has made it quite difficult to evaluate the biological effects of rVP1. Recently, we have not only expressed and isolated two aqueous soluble forms of rVP1, but also showed that, like native VP1, they can elicit protective immunity against FMDV (31). In this study, we further investigated the binding of purified rVP1.
VP1 of FMDV Induces Apoptosis
52169

FIG. 1. rVP1 treatment causes apoptosis, and this effect is reversed by anti-VP1 and anti-integrin αβ2 antibodies as well as by fibronectin. A, BHK-21 cells were treated with or without rVP1. After incubation, cells were harvested, and the genomic DNA was resolved by 1.2% agarose gel electrophoresis. Lane 1, DNA marker; lane 2, normal control; lane 3, after treatment with 0.5 μM rVP1. B, BHK-21 cells transfected with plasmid pEGFP-C3 were incubated with or without 1 μM rVP1 for the time periods indicated. The cells were observed under a fluorescence microscope, and their nuclei were stained with DAPI (magnification in all panels, ×200; in inset, ×800). C, the cells were treated for 16 h with serial concentrations of rVP1 and wild-type rVP1 (rVP1w) as indicated. D, some cells were treated with PBS (control), 25 μg/ml lipopolysaccharides (LPS; also as negative control), 10 μM rVP1(pEGFP-C3) were incubated with or without 1 μM rVP1 for 8 h; the other cells were pretreated with fibronectin (Fn), anti-integrin αβ2 antibodies, or anti-FMDV neutralizing antibodies (RN; 1:5000 dilution) for 30 min before rVP1 treatment. The apoptotic index was determined by measuring the percentage of cells with morphologically changed nuclei. Data represent means ± S.D. (n = 5).

to integrins and examined their cellular effects, mechanism of action, and potential applications.

EXPERIMENTAL PROCEDURES

Materials—The PI3K inhibitor LY294002 and antibodies against Akt; phospho-Ser473 Akt; phospho-Ser9 GSK-3β; caspase-3, -7, and -9; and procaspase-3, -7, and -9 were obtained from Cell Signaling Technology, Inc. (Temecula, CA). Mouse anti-actin antibody, anti-integrin VLA-5 (α5β1) monoclonal antibody, and horse radish peroxidase-coupled anti-mouse IgG secondary antibodies were purchased from Chemicon International, Inc. (Temecula, CA). Effecte transfection reagent was obtained from QIAGEN Inc. (Valencia, CA). Lipofectamine Plus™ was obtained from Invitrogen. Recombinant human platelet-derived growth factor (PDGF)-BB was from PeproTech EC Ltd. (London, United Kingdom). GSK-3β inhibitor 1 (GSK-3βi) came from Calbiochem. 4,6-Diamidino-2-phenylindole dilactate (DAPI) was from Sigma. Polypeptide P29 (NGSSVKDDESTIVNRVIDQVLAQKAERTL), representing residues 131–159 of VP1 of the FMDV O/Taiwan/97 strain, was synthesized using an ABI peptide synthesizer (32).

Site-directed Mutagenesis—Site-directed mutagenesis was performed according to the protocol of Du et al. (33) on the VP1 gene using primers that mutate Cys237 to serine and Arg45 to glycine. The primers that we used were as follows: VP1R145G-F, 5'-CGGTGACACCAGCACTAACAGCAGTTCTCAAGC-3', VP1R145G-R, 5'-CTTG-AAGGTCACTCCACAGCGTGTGCTGGTCACCG-3'; VP1C-187S-F, 5'-GAATTAAGAGAGCAGGCACTACATGCTCCAGGCCCCTTC-3'; and VP1C187S-R, 5'-GAATTCGAGAAAGGGGCTGTGGACGTGATGTGCTGGTC-3'. The nucleotide sequences of truncated and mutant VP1 constructs were confirmed by automated DNA sequencing.

Expression and Purification of VP1—The mutant VP1 gene in the expression vector pET24a(+) was expressed in Escherichia coli and purified according to our procedure described previously (31). Preliminary experiments showed that the apoptotic effect of the monomeric rVP1 mutant (rVP1C187S) was of the same magnitude as that of rVP1 without mutation at position 187. This monomeric rVP1 mutant was thus used in all experiments and is hereafter referred to as rVP1 in this study. After being processed according to our purification method, wild-type and mutant rVP1 become water-soluble (up to 5 mg/ml). Moreover, the lyophilized powder can be easily reconstituted in water without any precipitation. Because our purification process is quite simple (31), it can be easily used for large-scale production of rVP1.

Cell Lines and Treatments—BHK-21 cells are known to be permissive for FMDV to bind and replicate through the RGD motif (34). In this study, BHK-21 cells and several human cancer cell lines such as MCF-7, 22Rv1, and PC-3 were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at 2 × 10⁴ cells/well in a 12-well plate (400 μl/well) 1 day before the experiments. The cells were washed twice with phosphate-buffered saline (PBS) and treated with rVP1 in Dulbecco’s modified Eagle’s medium without fetal calf serum. Some of the cells were then lysed with 0.2 ml of boiling protein loading buffer (Invitrogen) at the indicated time points, and 20-μl samples were analyzed for Akt and GSK-3β phosphorylation by Western blotting. The remaining cells were incubated overnight at 37 °C for apoptosis experiments.

DNA Fragmentation Assay—In brief, BHK-21 cells were cultured in monolayer cultures overnight and treated with various amounts of rVP1. The treated cells were washed with ice-cold PBS and transferred to a centrifuge tube. The cells were recovered by centrifugation at 1500 × g for 10 min at 4 °C and resuspended in 100 μl of solution containing 10 mM Tris-Cl and 1 mM EDTA (pH 8.0). One ml of extraction buffer consisting of 0.1 M EDTA, 0.5% (w/v) SDS, 20 μg/ml pancre...
for 5 min, washed with 70% ethanol, and separated by electrophoresis. After 3 days of culture, the cells were harvested, and the cell lysate was subjected to Western blot analysis. The viability of the transfected cells with or without 0.5 µM rVP1 treatment was determined. The viability of the transfected cells was examined by counting the percentage of morphologically normal cells. Data represent the means of two experiments.

Assay for Cell Survival and Apoptosis—BHK-21 cells (5 × 10^4 cells/ml) were transfected with plasmid pEFGP-C3 (0.5 µg/well), which encodes green fluorescent protein (GFP), in Effectene for 24–48 h. After incubation with rVP1 at the indicated time points, the surviving and dead cells were distinguished by examination under a fluorescence microscope. To visualize DNA condensation in nuclei, the control and treated cells were stained with DAPI (0.5 µg/ml) and observed using an Olympus IX70 fluorescence microscope. The number of GFP-transfected cells was severely reduced when the treatment lasted for 24 h (Fig. 1B, left panels). Staining of cell nuclei with DAPI confirmed that rVP1-treated cells had nuclear condensation indicative of apoptosis (Fig. 1B, right panels). The apoptotic effect of rVP1 was not due to site-directed mutagenesis at position 187, as wild-type rVP1 also showed a similar effect (Fig. 1C, open bars). However, both FMDV and FMDV inactivated by BEI, an agent known to inactivate FMDV without damaging viral proteins (37), did not show any apoptotic effect (Fig. 1D).

RESULTS

rVP1 Induces Apoptosis—VP1 of FMDV contains an RGD motif, which is characteristic of the ligands that bind to cellular integrins (4, 8, 36). Although we previously produced aqueous soluble rVP1, it was found to form monomers or dimers depending upon the redox conditions of the experiments (31). To avoid any uncertainty in this study, we thus generated a monomeric rVP1 mutant by changing Cys187 to Ser by site-directed mutagenesis and investigated the cellular effect of this rVP1. Treating BHK-21 cells with 1 µM rVP1 for 24 h resulted in DNA fragmentation (Fig. 1A), one of characteristic features of apoptosis (28). To evaluate whether DNA fragmentation was accompanied by cell death and DNA condensation, we transfected BHK-21 cells with plasmid pEFGP-C3 and observed them under a fluorescence microscope. The number of GFP-transfected cells (green color under fluorescence) was reduced when the cells were treated with 1 µM rVP1 for 2 h. The number of GFP-transfected cells was severely reduced when the treatment lasted for 24 h (Fig. 1B, left panels). Staining of cell nuclei with DAPI confirmed that rVP1-treated cells had nuclear condensation indicative of apoptosis (Fig. 1B, right panels). The apoptotic effect of rVP1 was not due to site-directed mutagenesis at position 187, as wild-type rVP1 also showed a similar effect (Fig. 1C, open bars). However, both FMDV and FMDV inactivated by BEI, an agent known to inactivate FMDV without damaging viral proteins (37), did not show any apoptotic effect (Fig. 1D).

Indirect Immunofluorescence—The binding of rVP1 and binary ethyleneimine (BEI)-inactivated FMDV to the cells was detected following an established procedure (35), with minor modifications. Briefly, BHK-21 cells were grown on a glass coverslip overnight and then treated with rVP1 (1 µM) or BEI-inactivated FMDV (125 µg/ml) at 4 °C for 2 h. Cells on the coverslip were washed with PBS once and immersed in 100% methanol at −20 °C for 10 min. Fixed cells were rinsed three times with PBS, blocked in 1% bovine serum albumin in PBS at room temperature for 40 min, washed with PBS, and then incubated with primary antibodies (rabbit anti-FMDV neutralizing antibodies) at 1:500 in 1% bovine serum albumin in PBS for 1 h at 4 °C. After the primary antibody reaction, cells were washed twice with PBS, incubated with secondary antibodies (fluorescein isothiocyanate-labeled pig anti-rabbit IgG) at 1:1000 in 1% bovine serum albumin in PBS for 60 min at 30 °C under low light conditions, and washed twice with PBS. Coverslips were mounted on slides using Gel/Mount (Biomedica, Foster City, CA). The slides were air-dried for 1 h and stored at room temperature in the dark. Cells were observed using an Olympus IX70 fluorescence microscope.
mM), respectively. Pretreatment of the cells with antibodies before addition of rVP1 revealed that the apoptotic effect of rVP1 was blocked specifically not only by rabbit anti-FMDV neutralizing antibodies, but also by anti-integrin $\alpha_5\beta_1$ antibodies (Fig. 1D).

Because fibronectin is a natural ligand for the integrin $\alpha_5\beta_1$ receptor (9, 36), we investigated whether addition of fibronectin could reverse the apoptotic effect caused by rVP1. Our results show that fibronectin at 0.8 mM abolished the apoptotic effect of 1 mM rVP1 on BHK-21 cells (Fig. 1D). To further determine whether the apoptotic effect of rVP1 could be obtained only after its interaction with integrin receptors, we constructed a mutant VP1 protein containing GGD instead of RGD and found that this mutant (rVP1(R145G)) caused little, if any, apoptosis (Fig. 1D). In addition, we were able to show that rVP1, like BEI-inactivated FMDV, bound to BHK-21 cells and that the binding was blocked by anti-integrin $\alpha_5\beta_1$ antibody (Fig. 2A).

Moreover, we transfected BHK-21 cells with plasmid pIBSY1-VP1, an expression vector containing the VP1 gene, to express rVP1 intracellularly. Although rVP1 was significantly expressed in the transfected cells, little apoptosis was observed even after 3 days of culture (Fig. 2, B and C). On the other hand, addition of rVP1 to the culture medium resulted in the death of the transfected cells (Fig. 2C). These results suggest that induction of apoptosis by rVP1 is most likely due to its binding to integrin receptors.

rVP1 Treatment Deactivates Akt—Binding of growth factors such as PDGF to cells activates PI3K/Akt pathways and causes cell proliferation (17). On the other hand, deactivation of Akt causes apoptosis (28). To determine whether rVP1 has any effect on the Akt pathway after binding to integrin, we treated BHK-21 cells with PDGF and rVP1. As expected, PDGF activated Akt phosphorylation in a concentration-dependent manner, and this effect was inhibited by the PI3K inhibitor LY294002 (Fig. 3A and B). When BHK-21 cells were treated with rVP1 for 30 min, Akt phosphorylation was inhibited, in contrast to the activation induced by PDGF (Fig. 3C). The effect was more pronounced when the incubation time was increased to 60 min (Fig. 3C). This inhibitory effect of rVP1 on Akt was reversed not only by increasing concentrations of PDGF (0.1–10 $\mu$g/ml) (Fig. 3D), but also by pretreatment of cells with anti-integrin $\alpha_5\beta_1$ antibodies for 30 min (Fig. 3C). Because BEI-inactivated FMDV, unlike rVP1, did not cause apoptosis (Fig. 1D), we also investigated its effect on Akt. BEI-inacti-
rVP1 induces cancer cell apoptosis irrespective of the absence of caspase-3 and S1P receptor subtype 3 (S1P3).—The Akt pathway has been a major target for treatment of all four major human cancers, i.e., breast, prostate, lung, and colorectal (39–42). To evaluate further the apoptotic effect and mechanism of action of rVP1, we concentrated on cells that might not be responsive to it. One of these cancer cells is MCF-7, a breast carcinoma cell line with a functional deletion of the caspase-3 gene (43). Buckley et al. (43) found that seven peptides containing an RGD motif were unable to induce apoptosis of MCF-7 cells and concluded that the apoptotic effect of these RGD motif-containing peptides is caspase-3-dependent. To evaluate whether the apoptotic effect of rVP1 would also be caspase-3-dependent, we treated MCF-7 cells with rVP1 to evaluate their apoptosis. Our results show that rVP1 caused apoptosis of MCF-7 cells in a concentration-dependent manner and that this effect was reversed by addition of increasing concentrations of GSK-3β (Fig. 6, A and B), suggesting that the apoptotic effect of rVP1 is not necessarily dependent on caspase-3.

Other cancer cell lines that we selected for study were PC-3 and 22Rv1, both of which are prostate cancer cells. 22Rv1 cells express S1P3 and responds to androgen stimulation, whereas PC-3 cells do not (44). Androgen-independent prostate cancer cells such as PC-3 are usually much more lethal and difficult to treat. As S1P binds to S1P3 to mediate Akt activation and cross-talk with PDGF receptors (44), rVP1 could cause deactivation of Akt via S1P3. In this case, treating PC-3 with rVP1 would not result in apoptosis. Our results show that rVP1 not only caused apoptosis of both cell types, but also showed greater apoptotic effect on PC-3 cells, suggesting that the apoptotic effect of rVP1 is not via deactivation of S1P3 (Fig. 6C).

**DISCUSSION**

The capsid protein(s) of FMDV (45) and a variety of pathogens, including adenovirus 2 (46), coxsackievirus A9 (47), and echovirus 22 (48), contain an RGD motif that can bind to cellular integrin receptors such as αvβ3, αvβ5, αvβ6, and αvβ8 (6, 11, 12). Whether the binding of the RGD motif of these pathogens to the receptors elicits any cellular events has not been well documented. Our results in this study show clearly that rVP1 of FMDV bound to the integrin receptors and resulted in cellular apoptosis. Because integrin-mediated cell attachment has been shown to regulate both PI3K and Akt (49, 50), our observation that rVP1 treatment resulted in apoptosis and inhibition of Akt within the same effective dose range (Figs. 1C and 3G) is consistent with the proposal that the effect of rVP1 is most likely via its deactivation of Akt after its binding to integrin. It must be noted, however, that FMDV and BEI-inactivated FMDV caused activation of Akt and resulted in less cellular apoptosis (Figs. 1D and 3E). These results suggest that, although both FMDV and VP1 bind to integrin, the former acts as an agonist and the latter as an antagonist of integrin receptors. Which portion(s) of FMDV enables it to act
as an agonist to activate Akt remains to be elucidated.

The Akt signaling pathway has been shown to regulate cell survival via both BAD and caspase-3 in Chang liver cells (51) and via only caspase-3 in cardiomyocytes (52). Recently, Zhang et al. (53) found that the Akt pathway in HeLa cells affect cell survival via regulation of not only caspase-3, but also caspase-9 and GSK-3β. It thus appears that, depending on cell types and stimuli, the Akt pathway may regulate cell survival and apoptosis via modification of different downstream molecular targets. Although we have not tested all the potential downstream targets, our results so far reveal clearly that rVP1 treatment of BHK-21 fibroblast cells down-regulates the Akt pathway, re-targets, which reveals the biological and pharmacological importance of RGD motif-containing molecules. In this study, we found that aqueous soluble rVP1, an RGD motif-containing protein, was extremely potent in causing apoptosis of MCF-7, PC-3, and 22Rv1 cells (ED50 = < 1 μM). Its effect was not completely dependent on either caspase-3 or S1P3 (Fig. 6). Moreover, as more integrin α5β1 receptors on the cells have been linked to a higher metastatic potential of cancer cells (56), rVP1 may selectively cause the apoptosis of these cancer cells and prevent the occurrence of metastasis.

FIG. 6. Effect of rVP1 treatment on MCF-7, 22Rv1, and PC-3 cells. A, MCF-7 cells were treated with PBS (control), 0.8 μM rVP1, 0.8 μM rVP1 + 10 μM GSK-3βi, and 0.8 μM rVP1 + 30 μM GSK-3βi. The cells were observed under a microscope, and their nuclei were stained with DAPI (magnification ×400). B, MCF-7 and BHK-21 cells were treated with increasing concentrations of rVP1 as indicated. C, 22Rv1 and PC-3 cells were treated with increasing concentrations of rVP1 as indicated. Data represent means ± S.D. (n = 4). *p < 0.05.

FMDV affects many domestic livestock and results in economic disaster. However, our finding that a recombinant form of VP1 of FMDV can kill cancer cells shows that the capsid protein of this pathogen may be beneficial for human beings.

Acknowledgments—We thank Dr. Shui-Tein Chen for providing cyclic RGD and Dr. Ming-Hwa Jong for providing BEI-inactivated FMDV.

REFERENCES

1. Woolhouse, M., Chase-Topping, M., Haydon, D., Friar, J., Matthews, L., Hughes, G., Shaw, D., Wilesmith, J., Donaldson, A., Cornell, S., Keseling, M., and Grenfell, B. (2001) Nature 411, 258–259
2. Belscham, G. J. (1993) Prog. Biophys. Mol. Biol. 60, 241–260
3. Sa-Carvalho, D., Rieder, E., Baxt, B., Rodarte, R., Tanuri, A., and Mason, P. W. (1997) J. Virol. 71, 5115–5123
4. Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., King, A., Lea, S., Lewis, R., Newman, J., Parry, N., Rowlands, D., Stuart, D., and Fry, E. (1993) Nature 362, 566–568
5. Achariya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D., and Brown, F. (1989) Nature 337, 709–716
6. Jackson, T., Blakemore, W., Newman, J. W., Knowles, N. J., Mould, A. P., Humphries, M. J., and King, A. M. (2000) J. Gen. Virol. 81, 1383–1391
7. Mateu, M. G., Valero, M. L., Andreu, D., and Domingo, E. (1996) J. Biol. Chem. 271, 12814–12819
8. Ruslaha, E. (2005) Matrix Biol. 24, 459–465
9. Pytel, R., Pierschbacher, M. D., and Ruslaha, E. (1985) Cell 40, 191–198
10. Hynes, R. O. (1992) Cell 99, 11–25
11. Jackson, T., Sheppard, D., Denyer, M., Blakemore, W., and King, A. M. (2000) J. Virol. 74, 4949–4956
12. Jackson, T., Mould, A. P., Sheppard, D., and King, A. M. (2002) J. Virol. 76, 935–941
13. Li, M. O., Sarkissian, M. R., Mehal, W. Z., Rakic, P., and Flavell, R. A. (2003) Science 302, 1560–1563
14. King, W. G., Mattaliano, M. D., Chan, T. O., Tsichlis, P. N., and Brugge, J. S. (1997) Mol. Cell. Biol. 17, 4406–4414
15. Toker, A. (2000) Mol. Pharmacol. 57, 652–658
16. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) Cell 88, 435–437
17. Schneller, M., Vuori, K., and Ruslaha, E. (1997) EMBO J. 16, 5600–5607
VP1 of FMDV Induces Apoptosis

18. Hemmings, B. A. (1997) Science 275, 628–630
19. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
20. Belham, C. L., Wu, S., and Avruch, J. (1999) Curr. Biol. 9, R93–R96
21. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Science 282, 1314–1321
22. Brunet, A., Bonni, A., Zigmund, M. J., Lin, M. Z., Joo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868
23. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2905–2927
24. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 378–387
25. Benetti, L., Munger, J., and Roizman, B. (2003) J. Virol. 77, 6567–6573
26. Luo, H. R., Hattori, H., Hossain, M. A., Huang, Y., Lee-Kwon, W., Donowitz, M., Nagata, E., and Snyder, S. H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11712–11717
27. Loberg, R. D., Vesely, E., and Brosius, F. C., III (2002) J. Virol. 76, 41667–41673
28. Srinivasula, S. M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R. C., Wang, L., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) J. Biol. Chem. 271, 27099–27106
29. Bayrýy, J., Prabhudas, K., Gopalakrishna, S., Patil, P. K., Ramakrishna, C., Misra, L. D., and Suryanarayana, V. V. (1999) Microb. Immunol. 43, 765–771
30. Wang, J. H., Liang, C.-M., Peng, J.-M., Shieh, J. J., Jong, M.-H., Lin, Y. L., Steber, M., and Liang, S.-M. (2003) Vaccine 21, 3721–3729
31. Shieh, J. J., Liang, C.-M., Chen, C. Y., Lee, F., Jong, M.-H., Lai, S. S., and Liang, S.-M. (2001) Vaccine 19, 4002–4010
32. Du, Z., Regier, D. A., and Desrosiers, R. C. (1995) BioTechnology 13, 376–378
33. Baix, B., Morgan, D. O., Robertson, B. H., and Timpone, C. A. (1984) J. Virol. 51, 298–305
34. Nishida, K., Misumi, O., Yagisawa, K., Kurihara, H., Nagata, T., and Kuroiwa, T. (2004) J. Histochem. Cytochem. 52, 843–849
35. Pierschbacher, M. D., and Ruoslahti, E. (1984) Nature 309, 30–33
36. Patil, P. K., Suryanarayana, V., Bist, P., Bayrýy, J., and Naturajan, C. (2002) Vaccine 20, 1163–1168
37. Martinez, A., Alonso, M., Castro, A., Perez, C., and Moreno, F. J. (2002) J. Med. Chem. 45, 1292–1299
38. Brognard, J., Clark, A. S., Ni, Y., and Dennis, P. A. (2001) Cancer Res. 61, 3986–3997
39. Roy, H. K., Olusola, B. F., Clemens, D. L., Karolski, W. J., Ratashak, A., Lynch, H. T., and Smyrk, T. C. (2002) Carcinogenesis 23, 201–205
40. Hutchinson, J., Jin, J., Cardiff, R. D., Woodgett, J. R., and Muller, W. J. (2001) Mol. Cell. Biol. 21, 2203–2212
41. Roy, H. K., Olusola, B. F., Clemens, D. L., Karolski, W. J., Ratashak, A., Lynch, H. T., and Smyrk, T. C. (2002) Carcinogenesis 23, 201–205
42. Hutchinson, J., Jin, J., Cardiff, R. D., Woodgett, J. R., and Muller, W. J. (2001) Mol. Cell. Biol. 21, 2203–2212
43. Buckle, C. D., Pilling, D., Henriquez, N. V., Parsonage, G., Threlfall, R., Scheel-Toellner, D., Simmons, D. L., Akbar, A. N., Lord, J. M., and Salmon, M. (1999) Nature 397, 534–539
44. Bauduin, L. M., Jiang, Y., Zaslavsky, A., Ishii, I., Chun, J., and Xu, Y. (2004) FEBS J. 18, 341–343
45. Surovoi, A., Ivanov, V. T., Chepurkin, A. V., Ivanishchenkov, V. N., and Driagalin, N. N. (1988) Bioorg. Khim. 14, 965–968
46. Crow, T. J., Mathias, P., Cheshes, D. A., and Nemere, G. R. (1993) Cell 73, 309–319
47. Roivainen, M., Hyytiä, T., Piirainen, L., Kalkkinen, N., Stanway, G., and Hovi, T. (1991) J. Virol. 65, 4735–4740
48. Stanway, G., Kalkkinen, N., Roivainen, M., Ghazi, F., Khan, M., Smyth, M., Meurman, O., and Hyytiä, T. (1994) J. Virol. 68, 8232–8238
49. Vauri, K. (1996) J. Membr. Biol. 165, 191–199
50. Marte, B. M., and Downward, J. (1997) Trends Biochem. Sci. 22, 355–358
51. Lee, Y. I., Kang-Park, S., and Do, S. I. (2001) J. Biol. Chem. 276, 16969–16977
52. Wu, W., Lee, W. L., Wu, Y. Y., Chen, D., Liu, T.-J., Jiang, A., Sharma, P. M., and Wang, P. H. (2000) J. Biol. Chem. 275, 40113–40119
53. Zhang, H. M., Yuan, J., Cheung, P., Lu, H., Yanagawa, B., Chau, D., Stephan-Toyz, N., Wong, B. W., Zhang, J., Wilson, J. E., McManus, B. M., and Yang, D. (2003) J. Biol. Chem. 278, 33011–33019
54. Kiaris, H., and Schally, A. V. (1999) Proc. Soc. Exp. Biol. Med. 221, 87–88
55. Tsuura, T., Naito, M., Tomida, A., Fujita, N., Mashima, T., Sakamoto, H., and Haga, N. (2003) Cancer Sci. 94, 15–21
56. Stoeber, O., Liu, W., Reimuth, N., Fan, F., Parry, G. C., Parikh, A. A., McCarty, M. F., Bucana, C. D., Mazar, A. P., and Ellis, L. M. (2003) Int. J. Cancer 104, 496–503
