

# Activation of Mitogen-activated Protein Kinase Pathway by the Antiandrogen Hydroxyflutamide in Androgen Receptor-negative Prostate Cancer Cells<sup>1</sup>

Yi-Fen Lee,<sup>2</sup> Wen-Jye Lin,<sup>2</sup> Jiaoti Huang, Edward M. Messing, Franky L. Chan, George Wilding, and Chawnsang Chang<sup>3</sup>

George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, and The Cancer Center, University of Rochester Medical Center, Rochester, New York 14642 [Y-F. L., W.-J. L., J. H., E. M. M., C. C.]; The Chinese University of Hong Kong, Shatin, Hong Kong, China [F.L.C.]; and University of Wisconsin Comprehensive Cancer Center, Madison, Wisconsin 53792 [G. W.]

## ABSTRACT

Whereas hydroxyflutamide (HF) has been used as an antiandrogen to block androgen-stimulated prostate tumor growth, the antiandrogen withdrawal syndrome that allows antiandrogens to stimulate prostate tumor growth still occurs in many patients treated with androgen ablation therapy. This was previously explained by mutations in the androgen receptor (AR) and/or modulation from AR coregulators, so that HF becomes an AR agonist. Using immunohistochemical analysis, we analyzed four prostate cancer patients undergoing androgen ablation therapy with flutamide and compared their phospho-extracellular signal-regulated kinase 1/2 levels in prostate cancer biopsies before receiving HF and after experiencing disease progression while taking HF. We found a significant increase of activated mitogen-activated protein (MAP) kinase in prostate tumors from patients receiving HF during androgen ablation therapy. *In vitro* studies showed that HF induced a rapid activation of the Ras/MAP kinase pathway in human prostate cancer DU145 cells which lack the AR, as well as in PC-3AR2 and CWR22 cells which express the AR. Cycloheximide failed to inhibit this activation, but both AG1478, an inhibitor of the epidermal growth factor receptor (EGF-R), and an EGF-R-neutralizing antibody blocked this HF-mediated activation of MAP kinase, which suggests that the activation of Ras/MAP kinase by HF is a membrane-initiated, non-AR-mediated, and nongenomic action. The consequence of this activation may result in increasing cell proliferation and cyclin D1 expression. This raises a concern for using HF in the complete-androgen-ablation therapy in prostate cancer treatment and provides a possible pathway that might contribute to the HF withdrawal syndrome.

## INTRODUCTION

Prostate cancer is the most common noncutaneous cancer in men and the second leading cause of cancer-related death (1). The lack of effective therapies for advanced prostate cancer reflects, in part, the lack of knowledge about the molecular mechanism involved in the development and progression of this disease (2, 3). In particular, little is known about the mechanisms that trigger the conversion of an initially androgen-dependent cancer to androgen independence. When prostate cancers first occur, they are dependent on androgens for growth and can be treated successfully with androgen ablation therapy. However, after prolonged antiandrogen therapy, eventually the cancer acquires the ability to proliferate (4, 5). Defining the mechanisms that might lead to improved outcomes from this disease is a critical step.

The failure of antiandrogen therapy may be associated with the elevation of multiple polypeptide growth factors (6, 7). For example,

EGF,<sup>4</sup> transforming growth factor  $\alpha$ , insulin-like growth factor-1, interleukin 6, keratinocyte growth factor, and fibroblast growth factor family members are suggested to play important roles in fueling androgen-independent growth. Many of these growth factors and their receptors activate Ras family members to mediate a signal transduction cascade of successive phosphorylation steps leading to the activation of MAP kinases (8). Several studies have linked the increased activation of MAP kinases to the progression of carcinomas of the kidney, liver, and prostate (9, 10). For example, Gioeli *et al.* (11) found that the level of activated MAP kinase increased with an increasing Gleason score and prostate tumor stage. Additionally, tumor samples from two patients showed no activation of MAP kinase before androgen ablation therapy, but high levels of activated MAP kinase developed when tumors recurred after androgen ablation (11).

The MAP kinase family includes the ERKs (or p42/p44), the JNKs/stress-activated protein kinases, and p38<sup>HOG</sup>. Reports show that the ERK and JNK pathways are stimulated by receptor protein tyrosine kinases in various cell types; however, p38<sup>HOG</sup> is not commonly activated by growth factors (12–15). Activation of MAP kinase and its upstream regulator Ras, is linked to cell proliferation and tumor progression and Voller *et al.* (16) demonstrated that the functional activation of Ras-dependent signaling could convert androgen-dependent cells to androgen independence.

Because of its competitive inhibition of androgen binding to the AR, HF is used as an antiandrogen to treat prostate cancer. However, *in vitro* studies also suggested that HF could activate the mutated AR that is, at times, found in prostate tumors (16–19). This could explain the “flutamide withdrawal syndrome,” in which patients who experience an increase in PSA while taking flutamide have a PSA decrease after cessation of flutamide treatment (20–22). However, the transient and incomplete nature of the response to antiandrogen withdrawal, as well as its failure to occur in many patients, implies that there are mechanisms other than AR mutations that contribute to tumor progression.

We demonstrated that, at a clinically relevant concentration, 1  $\mu$ M HF rapidly activates the Ras-MAP kinase signal pathway that consequently leads to cell proliferation in an AR-independent manner. This finding not only helps explain the flutamide withdrawal syndrome but may also guide new strategies to prevent the emergence of androgen independence.

## MATERIALS AND METHODS

**Immunocytochemistry.** Immunocytochemical stainings were performed on formalin-fixed, paraffin-embedded tissue sections using antibodies to ERK1 and ERK2 (SC-94 and SC-154, respectively; 1/400 dilution), and phospho-ERK1/2 (SC-7383; 1/50 dilution) all from Santa Cruz Biotechnology (Santa Cruz, CA). Sections were cut at 4–5  $\mu$ m and deparaffinized according to

Received 4/5/02; accepted 9/5/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by NIH Grants DK60905 and DK60948 and by a George Whipple Professorship Endorsement.

<sup>2</sup> Y-F. L., and W.-J. L. contributed equally to this article.

<sup>3</sup> To whom requests for reprints should be addressed, at George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, and The Cancer Center, University of Rochester Medical Center, Rochester, NY 14642. Phone: (716) 273-4501; Fax: (716) 756-4133; E-mail: chang@urmc.rochester.edu.

<sup>4</sup> The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; MAP, mitogen-activated protein; JNK, c-jun NH<sub>2</sub>-terminal kinase; HF, hydroxyflutamide; PSA, prostate-specific antigen; FBS, fetal bovine serum; RIPA, radioimmunoprecipitation assay (buffer); MEK, MAP kinase kinase; DHT, dihydrotestosterone; LUC, luciferase.

established procedures and quenched with 3% hydrogen peroxide for 6 min. Antigen unmasking with heat retrieval in citrate buffer (pH 6.0) was accomplished by placing slides in a microwave (1500 W) pressure cooker for 30 min. Slides were rinsed and stained for 45 min with primary antibody, and then incubated for 20 min with secondary antibody and streptavidin-horseradish peroxidase. Slides were developed with 3-amino-9-ethylcarbazole+ and rinsed and counterstained with Mayer Hematoxylin Blue in 0.3% ammonia water.

**Transient Transfection Assay.** DU145 cells were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells ( $1 \times 10^5$ ) were seeded in 35-mm plates and transfected with the SuperFect transfection reagent (Qiagen, Chatworth, CA). After 4 h, the medium was changed to serum-free DMEM for 24 h. Thereafter, the cells were treated with HF, EGF, and vehicle for 18 h and then lysed for luciferase assay. Luciferase activity was normalized for transfection efficiency using *pRL-TK* as an internal control. Luciferase assays were performed using dual-luciferase reporter system (Promega, Madison, WI).

**Immunoblot of Phosphorylated p44/42 MAP Kinase.** This assay uses a polyclonal antibody specific against the activated p44/42 MAP kinases (Cell Signaling Technology, Beverly, MA), which detects p44 and p42 MAP kinase (ERK1 and 2) only when catalytically activated by dual phosphorylation at Thr202 and Tyr204. DU145 cells were seeded at  $1 \times 10^5$  cells/100-mm plate and were allowed to attach overnight; then the medium was replaced with serum-free DMEM for 24 h. The cells were pretreated with MAP kinase inhibitors, EGFR antibodies, or cycloheximide for 1 h, followed by HF, EGF, or vehicle treatment. Cells were washed twice with PBS, harvested at indicated times (Fig. 2), and lysed with RIPA buffer for 30 min on ice. The protein concentrations were determined by Bradford assay, and equal amounts of protein were resolved by SDS-PAGE. Gels were transferred, immunoblotted with p44/42 MAP kinase antibody (1/1000), and then incubated with secondary antibody. Proteins were visualized by the enhanced chemiluminescence system (NEN Life Science Products, Boston, MA). p44/42 MAP kinase (total ERK1/2) was blotted as a control.

**Cell Proliferation Assay.** DU145 cells were seeded at  $2 \times 10^4$ /ml on 35-mm plates and allowed to attach overnight. Cells were kept in serum-free DMEM for 24 h, and then medium was replaced with either DMEM-10% FBS or DMEM-0.5% FBS and treated as indicated in Fig. 5. Cells were trypsinized and counted with a hemacytometer at different times after treatments. For the antisense oligonucleotide experiments, cells were incubated with a fixed ratio

of oligonucleotide *versus* SuperFect (2.5  $\mu$ l of SuperFect:100 nm oligo) for 4 h, and then the oligo-containing medium was replaced with DMEM-0.5% FBS. Cells were trypsinized and counted by hemacytometer at indicated times (Fig. 5).

**Immunoprecipitation.** Serum-starved and treated DU145 cells were lysed with RIPA buffer for 30 min on ice. Lysates were then centrifuged at  $12,000 \times g$  for 10 min at 4°C, and protein concentrations were determined by the Bradford assay. Cell lysates (500  $\mu$ g) were incubated for 4 h with either anti-Ras or anti-EGFR antibody (1/100; Santa Cruz Biotechnology) to the protein-antibody mixture and then added in 20  $\mu$ l of protein A/G plus-agarose (Santa Cruz Biotechnology) for another 4-h incubation with constant rotation. The immunoprecipitates were washed four times with cold PBS, resolved by SDS-PAGE, and immunoblotted by anti-Raf-1 antibody, or anti-phosphotyrosine monoclonal antibody (Oncogene Research; Calbiochem, La Jolla, CA). The results were visualized by chemiluminescence.

## RESULTS

**Increase of Phospho-ERK1/2 Level in the Patients' Tumors after Patients Developed Flutamide Withdrawal Syndrome.** To evaluate the role that the activation of MAP kinase plays in HF withdrawal in prostate cancer, four prostate cancer patients undergoing androgen ablation therapy with flutamide were examined. We compared their phospho-ERK1/2 levels in prostate cancer biopsies before and after the development of the flutamide withdrawal syndrome. We have examined each slide and scored the number of the cells stained positive for phospho-ERK1/2 under the  $\times 10$  high-power field. We found that phosphorylated or activated MAP kinase was undetectable before androgen ablation therapy with flutamide treatment (Fig. 1C). In contrast, 30, 41, 52, and 45% of the cells were stained positive for phospho-ERK1/2 in the recurrent tumors of all four patients whose disease was progressing while receiving flutamide and who developed flutamide withdrawal syndrome when the medication was discontinued immediately after the second biopsy (Fig. 1D). Mayer hematoxylin blue staining for pathological morphological examination is shown in Fig. 1, A and B. It is possible that

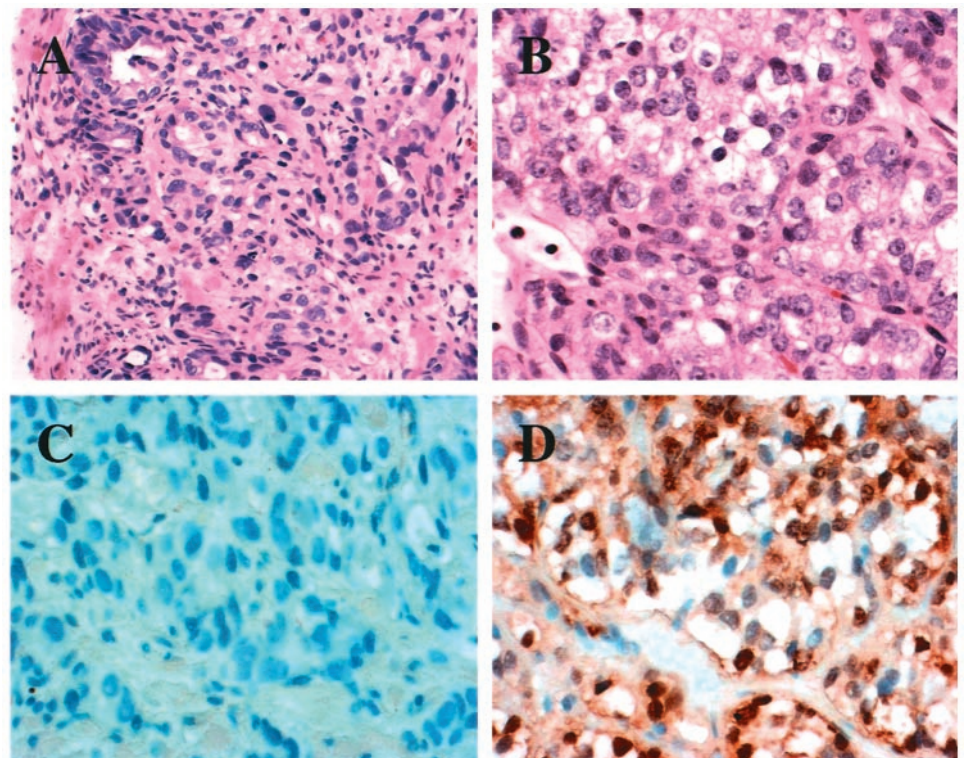


Fig. 1. Elevated levels of active MAP kinase in the prostate cancer specimens from a patient whose tumors progressed on androgen ablation therapy. Prostate tumor tissue sections from the same patient with either the pre-androgen ablation (A and C) or post-androgen ablation plus flutamide therapy (B and D) were immunohistochemically stained for phospho-ERK1/2. Sections were counterstained with Mayer hematoxylin blue (A and B).  $\times 400$ .

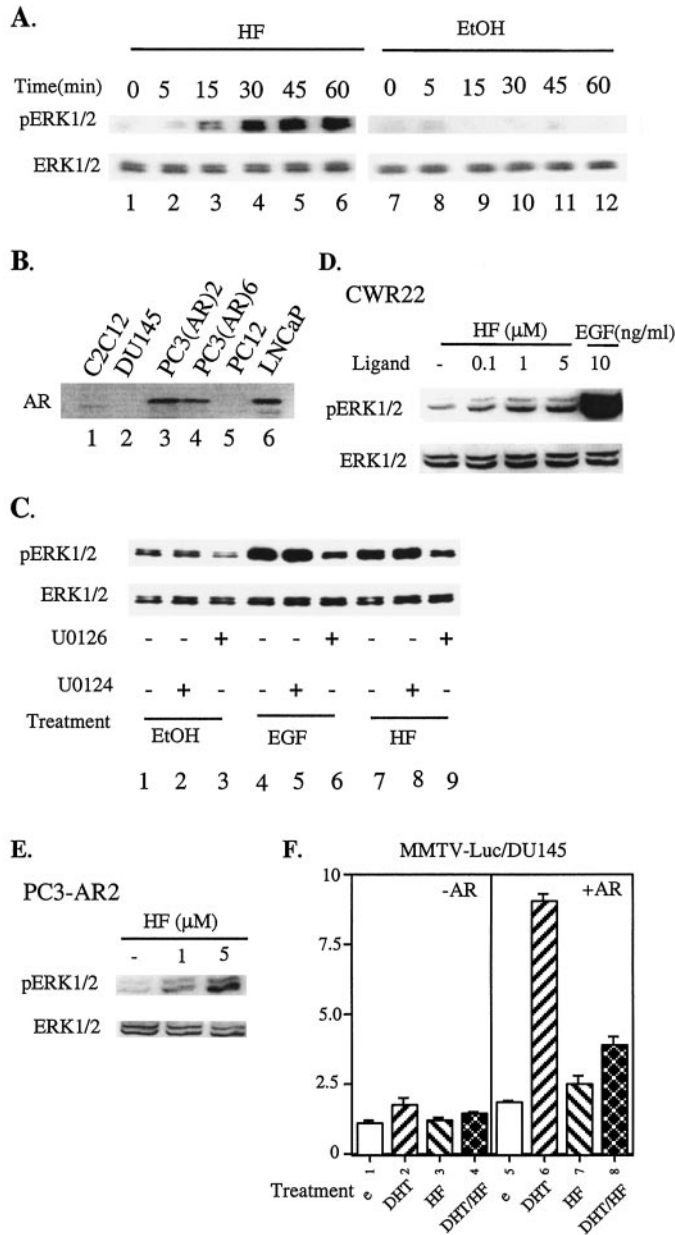


Fig. 2. Activation of the MAP kinase pathway by HF in prostate cancer cells. In A, DU145 cells were grown in 100-mm dishes and serum-starved for 24 h. The cells were treated with 1  $\mu$ M HF (Lanes 1–6) or ethanol (EtOH) vehicle (Lanes 7–12) for different times as indicated in the figure. The cells were lysed on ice. Equal amounts of cell lysate were analyzed by 12% SDS-PAGE and, subsequently, immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. In B, different cell lines, at 70–80% confluence, were lysed and immunoblotted with anti-AR polyclonal antibody, NH27. In C, DU145 cells were preincubated with MEK1/2 inhibitor U0126, or U0124 before HF, EGF, and ethanol (EtOH) treatment. Cells were lysed and immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. CWR22 (D) and PC3-AR (E) cells were grown in 100-mm dishes and serum starved for 24 h. The cells were treated with HF, EGF, or ethanol (EtOH) vehicle for 15 min and immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. In F, the antiandrogen effect of HF. MMTV-ARE-Luc reporter plasmid was cotransfected with or without pSG5AR into DU145 cells. After 18 h, cells were treated with 1 nM DHT, 1  $\mu$ M HF, or both, for another 18 h, and then harvested. Cell lysates were collected and assayed for the luciferase activity.

factors other than HF during complete androgen ablation therapy may contribute to the activation of MAP kinase. The effects of HF-mediated activation of MAP kinase in the prostate cancer cells was examined as follows.

**Activation of MAP Kinase Pathway by HF in DU145 Cells.** To investigate whether HF has any effect on the MAP kinase signal transduction pathway, we performed Western blotting using an anti-

body that specifically recognizes dually phosphorylated MAP kinase (phospho-ERK1/2) in AR-negative DU145 human prostate cancer cells. Our results showed that HF has no influence on total MAP kinase (ERK1/2) protein expression. HF at 1  $\mu$ M can activate MAP kinase within 15 min and reach maximum activation in 30 min (Fig. 2A, Lanes 2 and 4). DU145 cells (passage number 61–65; ATCC HTB-81) are documented as an AR-negative cell line. This is also demonstrated in our Fig. 2B, which shows no visible AR band using AR antibody NH27. Our data in Fig. 1, A and B, demonstrate that HF can activate MAP kinase via a non-AR-mediated mechanism.

Next, we found that U0126, a specific MEK inhibitor, but not U0124, a structurally similar compound without inhibiting effects, can block HF-mediated MAP kinase activation. (Fig. 2C, Lanes 7 versus Lanes 9 and 8).

For comparison, we also found that HF-mediated MAP kinase activation could be detected in other selected prostate cancer cells with endogenous AR expression. For example, HF activates MAP kinase in the CWR22 and in the PC-3 stably transfected with AR (PC3-AR2; Fig. 2, D and E), but not in LNCaP (data not shown). It is unclear whether endogenous AR in these cells plays any role in the activation of the MAP kinase pathway. Because our data demonstrate that HF can activate the MAP kinase pathway in AR-negative cells, such as DU145 cells, we concluded that AR may not be a determining factor to mediate the activation of MAP kinase by HF. We noted that the activation of MAP kinase by HF is, however, relative weak as compared with EGF activation of MAP kinase. Nevertheless, because the concentration of HF needed to activate the MAP kinase is much lower than the concentration commonly available in the treatment of prostate cancer, our data implies that HF at pharmacological concentrations ( $10^{-6}$  to  $10^{-5}$  M) may become a potent activator to stimulate MAP kinase pathway. To ensure that the HF that we used indeed exerts an antiandrogenic effect, the AR/dihydrotestosterone (DHT)-mediated PSA transcriptional activity was examined by adding HF. As shown in Fig. 2F, 1  $\mu$ M of HF represses DHT-induced PSA-Luciferase (Luc) activity. Together, data from Fig. 2 suggest that HF can activate the MAP kinase pathway within 15 min without the involvement of the AR-mediated mechanism.

**HF-mediated MAP Kinase Activation Is via Ras and Raf Pathway.** To assess whether Ras and Raf are upstream regulators of HF-mediated MAP kinase activation, we used a Ras-Raf immunoprecipitation assay. As shown in Fig. 3A, Lane 2 versus Lane 3 and Lane 4 versus Lane 3, Raf was detected in the Ras immunocomplex

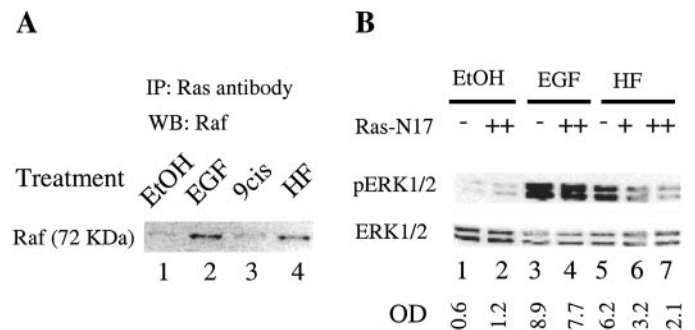


Fig. 3. HF activates Ras→Raf→MAP kinase pathway. In A, DU145 cells were treated with ethanol (Lane 1), 10 ng/ml EGF (Lane 2), 1  $\mu$ M 9-cis retinoic acid (Lane 3), and 1  $\mu$ M HF (Lane 4) after 24-h serum starvation. Cells were lysed, and 300  $\mu$ g of total protein were immunoprecipitated with anti-Ras antibody, pulled down by protein A/G agarose beads, and detected with an anti-Raf antibody. In B, DU145 cells were transfected with pCDNA3.1 or pCDNA3.1-Ras N17 as indicated. After 24-h serum starvation, the cells were treated with different ligands for 20 min, and then harvested. Western blots were performed with antibodies against phospho-ERK1/2 and -ERK1/2. The density of phospho-ERK1/2 shown as absorbance (OD) was determined by the Versa Doc Imaging System (Bio-Rad) and quantified by Quantity One software. *kDa*, *M<sub>t</sub>* in thousands.

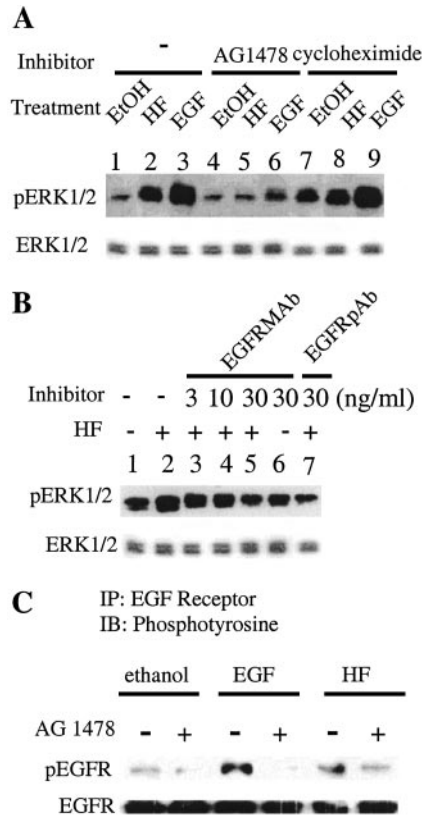


Fig. 4. Inhibition of HF-mediated MAP kinase activation by EGFR inhibitors. DU145 cells were seeded and preincubated with (A) EGFR inhibitor tyrphostin AG1478 (100 nM) or cycloheximide (100  $\mu$ g/ml) and (B) EGFRmAb-528 or EGFRpAb-1005 for 1 h before the EGF or HF treatment, respectively. After a 15-min treatment, cells were lysed and immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. C, effect of the EGFR inhibitor, tyrphostin AG1478, on HF-mediated tyrosine phosphorylation of the EGFR. Serum-starved DU145 cells were preincubated with 100 nM AG1478 before stimulation with 10 ng/ml EGF, 1  $\mu$ M HF, or 10 nM of DHT for 20 min. EGFR was immunoprecipitated from the cell lysates, and the phosphotyrosine level was determined by anti-phosphotyrosine antibody. All of the results were visualized using enhanced chemiluminescence.

in lysates from cells treated with HF or EGF, but not in lysates from cells treated with 9 *cis*-retinoic acid. These data demonstrate that HF promotes the association between Ras and Raf that may result in the activation of their downstream MAP kinase. Transfection with a dominant negative H-Ras mutant, N17 (Ras-N17), further proved this observation. The phospho-ERK1/2 levels in HF-treated or EGF-treated cells were decreased with cotransfection of Ras-N17 into DU145 cells, in a dose-dependent manner (Fig. 3B, Lanes 5, 6, 7, 3, and 4), but not in ethanol-treated cells (Lane 1 and 2). The absorbance of phospho-ERK1/2 was scanned and quantified by Versa Doc Imaging System and Quantity One software.

**An EGFR Inhibitor and Its Neutralized Antibody Can Inhibit HF-mediated Activation.** We hypothesized that the EGFR might be involved in the upstream events for the HF-mediated Ras-Raf-MAP kinase pathway. Preincubation of DU145 cells with 100 nM tyrphostin AG1478, a selective inhibitor of the EGFR tyrosine kinase, for 1 h resulted in decreased levels of phospho-ERK1/2 induced by either HF or EGF (Fig. 4A, Lane 5 versus Lane 3 and Lane 6 versus Lane 4).

Ten ng/ml cycloheximide, a protein synthesis inhibitor, failed to block MAP kinase activation mediated by HF or EGF (Lanes 8 and 9). However, a slight increment was observed when ethanol was added to cycloheximide treatment (Lane 7). These data strongly suggest that the activation of MAP kinase could be a nongenomic effect and did not involve new protein synthesis.

The treatment of DU145 cells with monoclonal antibody

EGFRmAb-528 or polyclonal antibody EGFRpAb-1005 for 1 h also abrogated activation of phospho-ERK1/2 induced by either EGF or HF (Fig. 4B) in a dose-dependent manner (Lanes 3–5). Because these antibodies have been demonstrated to bind to a cell surface epitope of the EGFR and to antagonize ligand-stimulated EGFR tyrosine kinase activity, our data suggest that the activation by HF of the MAP kinase pathway could be exerted through a membrane receptor tyrosine kinase-mediated pathway without the involvement of new protein synthesis in an AR-deficient environment. Whether the EGFR itself, served as the membrane mediator for the activation by HF of the Ras-Raf-MAPK pathway remains unclear.

The  $M_r$  170,000 EGFR exercises its biological effects in response to the binding of specific polypeptide ligands, including EGF and transforming growth factor  $\alpha$ . This leads to the activation of EGFR catalytic tyrosine kinase domain, autophosphorylation of specific residues in its COOH terminus, and recruitment and phosphorylation of signaling proteins. As shown in Fig. 4C, HF causes the autophosphorylation of EGFR as detected by the immunoprecipitation with an antibody to the EGFR and immunoblotting with anti-phosphotyrosine. However, AG1478 can inhibit both EGF- and HF-mediated EGFR autophosphorylation, without affecting total EGFR concentration.

**HF Promoted the Cell Proliferation and Cyclin D1 Expression.** Because the activation of MAP kinase might result in cell proliferation, we determined whether HF treatment affected cell proliferation. We found that HF, like EGF, can promote cell proliferation in a low-serum (0.5% FBS) environment after serum starvation (Fig. 5A). In contrast, if we maintain the cells in 10% FBS medium, HF or EGF causes no significant stimulation of cell proliferation (data not shown). To further link cell proliferation with the HF-mediated activation of Ras/MAP kinase pathway, we transfected the DU145 cells with antisense of Ras, Raf, and HIV as a control antisense. As expected, cells transfected with antisense of Ras and Raf oligomers that block the activation of MAP kinase did not show any significant increase of cell number after 24-h HF or EGF treatment. However, the cells doubled in number when we transfected control antisense oligomers and treated with HF or EGF (Fig. 5B). In conclusion, we demonstrated that HF activated the MAP kinase pathway and promoted cell proliferation. The blocking of Ras and Raf can reverse the proliferation mediated by HF and EGF. These data provided evidence that HF, like EGF, promotes cell proliferation through the Ras-Raf-MAPK pathway.

To further dissect the mechanism of how HF, like EGF, promotes cell proliferation, we tested several potential G<sub>1</sub> or S-phase targets that might be influenced by HF or EGF. We found that cyclin D1 is induced after HF or EGF treatment (Fig. 6A, Lane 1 versus Lane 2; Lane 1 versus Lane 3). In contrast, there is no significant change in other cell cycle-related gene products including p27, p21, Ki67, and proliferating cell nuclear antigen (data not shown). The HF- or EGF-mediated *cyclin D1* gene expression was confirmed by a cyclin D1 promoter study (23). As shown in Fig. 5B, 10<sup>-6</sup> M HF or 30 ng/ml EGF induced cyclin D1 promoter (-1745D1-Luc) activity about 2-fold (Lane 1 versus Lane 2). Importantly, cotransfection of the dominant negative of Ras and Raf attenuated this HF- or EGF-mediated induction. An increase in cyclin D1 concentration may trigger transition from G<sub>1</sub> to S phase and may eventually result in increased cell proliferation. That the expression of cyclin D1 could be induced by HF further clarifies how HF promotes DU145 cell growth.

## DISCUSSION

Flutamide was the first androgen-receptor blocker to achieve widespread use. It is metabolized into HF, the biologically active form of

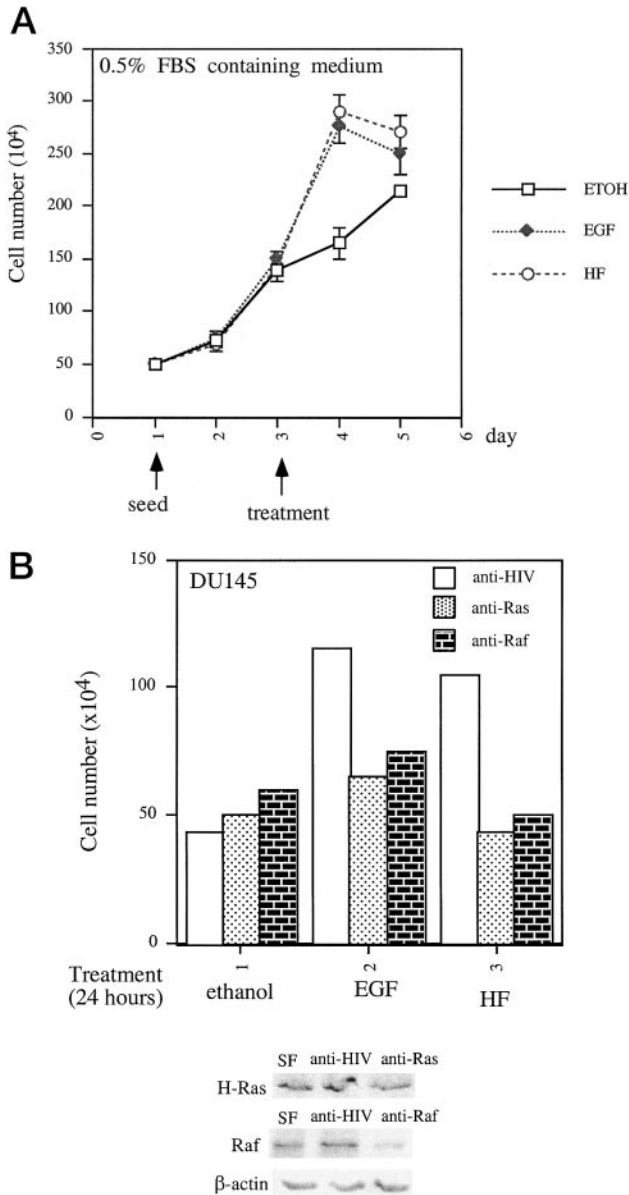


Fig. 5. HF promoted the cell proliferation. In A, DU145 cells were seeded in 10% FBS DMEM, and 24 h later, cells were changed to serum-free medium. Forty-eight h later, the medium was changed to 0.5% FBS medium, and the cells were treated with ethanol, HF, or EGF. Cells were counted by hemacytometer every 24 h. The results were the average from three independent experiments, and statistical analysis (*t* test) was performed and showed that 24 h of HF treatment, as well as of EGF treatment, stimulated the DU145 cell growth significantly, whereas ethanol treatment did not ( $P < 0.05$ ). In B, DU145 cells were seeded and transfected with antisense oligonucleotide of Ras (IRIS 2503: 5'-TCCGTCATCGCTCCTCAGGG-3'), Raf (IRIS: 5132: 5'-TCCGCTGTGACATGCATT-3'), and HIV (5'-TCAGTAATAGCCCCACATGG-3') (32, 33) by SuperFect (Qiagen). Twenty-four h after serum starvation, we changed the cells to a medium containing 0.5% FBS, and treated the cells as described in A. Cells were counted by hemacytometer every 24 h. The cell lysates were blotted with anti-H-Ras and anti-Raf antibodies, and β-actin was blotted for loading control. SF is superfect liposome transfected control.

the drug. In 1993, Kelly and Scher described four patients with progressive metastatic prostate cancer combined-androgen-blockade (simultaneous administration of castration and anti-androgen therapy) therapy. After selective discontinuation of flutamide treatments, the patients showed a biochemical and objective improvement (20). This phenomenon has also been reported for cyproterone acetate (24), nilutamide (25), and bicalutamide (26), as well as for progestational agents (27). It is apparent that prolonged therapy with flutamide may select for tumor cells that are stimulated by HF and, thus, contribute

to prostate cancer progression. Discontinuation of treatment with stimulatory antiandrogens results in a related withdrawal syndrome. The pathophysiology of anti-androgen-withdrawal syndrome is not completely understood, although AR gene mutations seem to be a part of the explanation. However, the transient and incomplete response of the tumor to anti-androgen withdrawal, as well as the failure of the withdrawal syndrome to occur in many patients, implies that there are

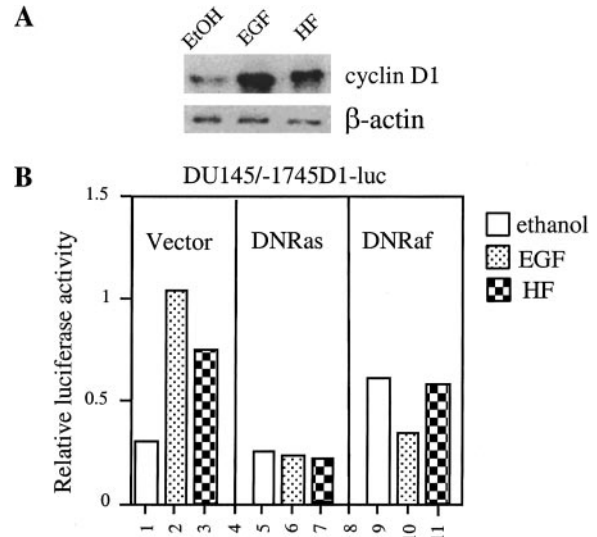


Fig. 6. HF-enhanced cyclin D1 expression. In A, DU145 cells were treated with HF (1 μM), or EGF (10 ng/ml), or ethanol (*EtOH*) vehicle for 12 h after the serum starvation. Cells were lysed and blotted with anti-cyclin D1 antibody. In B, DU145 cells were serum starved for 24 h and then cotransfected with dominant negative (DN)Ras or DNRaf together with -1754D1 Luc reporter with SuperFect (Qiagen). After 4 h, the medium was changed to normal medium (10% serum) for 18 h, and then changed to serum starvation condition for another 24 h, and then treated with HF, EGF, or ethanol vehicle control. Cells were lysed for luciferase activity analysis.

### Dual Roles of HF in Prostate Cancer Progression

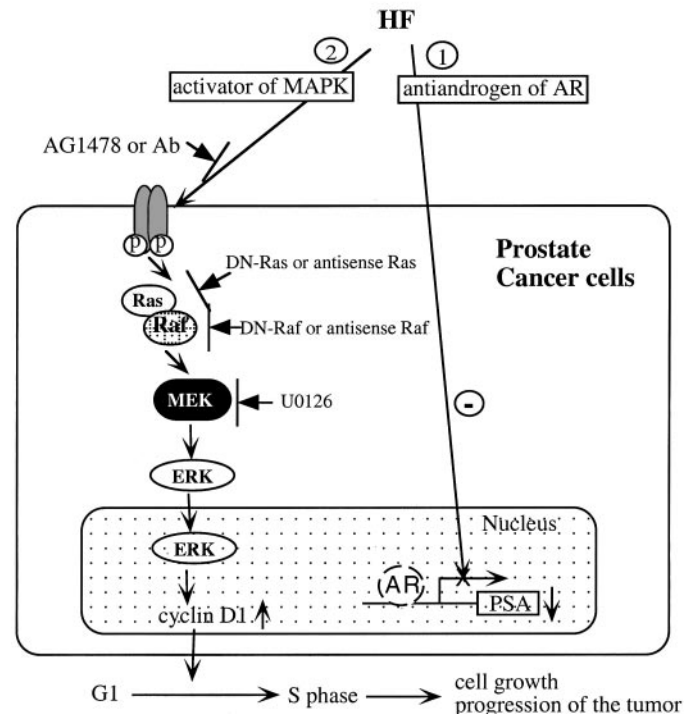


Fig. 7. The model for the HF action in prostate cancer cells. Ab, antibody.

mechanisms other than AR mutations that contribute to tumor progression. The progression of prostate cancer to androgen-independent disease is also associated with the elevation and autocrine production of multiple polypeptide growth factors (6, 7). It is widely suspected that the paracrine and autocrine loops that exist play an important role in the loss of hormone dependence, as well as in the dependence of metastatic potential. The growth factors and receptors associated with prostate cancer progression regulate cell growth, at least partly, through regulation of the activity of Ras family members. *Ras*, a proto-oncogene, is dependent on protein-protein interactions to cause its ultimate dissociation from GDP. The dissociation renders Ras free to bind to GTP and initiate a complex signaling cascade that leads to the activation of the MAP kinases ERK1 and ERK2. Several small molecular inhibitors such as growth factors or their receptors that target specific steps of the MAP kinase cascade have recently entered the clinical arena (28–31).

In this report, we provide another possible mechanism to explain HF withdrawal syndrome. We demonstrated that HF may have dual roles in the modulation of prostate tumor growth; these data are summarized in Fig. 7. First, when prostate cancer patients have relatively high concentrations of androgens, HF may function as an effective antiandrogen, competing with androgens for binding to the AR. The effect is the inhibition of androgen-mediated prostate cancer growth. At later stages, when prostate tumors become androgen independent, the continuation of HF treatment triggers MAP kinase pathway activation, with subsequent stimulation of prostate tumor growth. This phenomenon may contribute to the flutamide withdrawal syndrome and may explain why, in the case metastatic carcinoma, little prolongation of survival can be demonstrated for combined-androgen blockade (simultaneous administration of castration and anti-androgen therapy) compared with androgen-deprivation monotherapy. Moreover, this observation offers potential therapeutic targets that may prolong the antitumor effect of flutamide. Finally, our studies demonstrate that advanced prostate cancer cells have variable responses to therapeutic strategies; hence, the characterization and treatment of hormone-refractory prostate cancer remains a difficult, yet intriguing, challenge for continuing research in this field.

## ACKNOWLEDGMENTS

We thank Drs. M.-C. Hung (University of Texas M.D. Anderson Cancer Center, Houston, TX) and K.-L. Guan (The University of Michigan Medical School, Ann Arbor, MI) for providing plasmids.

## REFERENCES

- Amanatullah, D. F., Reutens, A. T., Zafonte, B. T., Fu, M., Mani, S., and Pestell, R. G. Cell-cycle dysregulation and the molecular mechanisms of prostate cancer. *Front. Biosci.*, *5*: 372–390, 2000.
- Small, E. J. Advances in prostate cancer. *Curr. Opin. Oncol.*, *11*: 226–235, 1999.
- Nupponen, N., and Visakorpi, T. Molecular biology of progression of prostate cancer. *Eur. Urol.*, *35*: 351–354, 1999.
- Brandstrom, A., Westin, P., Bergh, A., Cajander, S., and Damber, J. E. Castration induces apoptosis in the ventral prostate but not in an androgen-sensitive prostatic adenocarcinoma in the rat. *Cancer Res.*, *54*: 3594–3601, 1994.
- McConkey, D. J., Greene, G., and Pettaway, C. A. Apoptosis resistance increases with metastatic potential in cells of the human LNCaP prostate carcinoma line. *Cancer Res.*, *56*: 5594–5599, 1996.
- Culig, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Hittmair, A., Zhang, J., Thurnher, M., Bartsch, G., and Klocker, H. Regulation of prostatic growth and function by peptide growth factors. *Prostate*, *28*: 392–405, 1996.
- Culig, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res.*, *54*: 5474–5478, 1994.
- Schlessinger, J., and Ullrich, A. Growth factor signaling by receptor tyrosine kinases. *Neuron*, *9*: 383–391, 1992.
- Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara, A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., and Hori, M. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. *Hepatology*, *27*: 951–958, 1998.
- Magi-Galluzzi, C., Mishra, R., Fiorentino, M., Montironi, R., Yao, H., Capodiceci, P., Wishnow, K., Kaplan, L., Stork, P. J., and Loda, M. Mitogen-activated protein kinase phosphatase 1 is overexpressed in prostate cancers and is inversely related to apoptosis. *Lab. Invest.*, *76*: 37–51, 1997.
- Gioeli, D., Mandell, J. W., Petroni, G. R., Frierson, H. F. Jr., and Weber, M. J. Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res.*, *59*: 279–284, 1999.
- Elion E. A. Signal transduction: routing MAP kinase cascades. *Science (Wash. DC)*, *281*: 1625–1626, 1998.
- Lewis, T. S., Shapiro, P. S., and Ahn, N. G. Signal transduction through MAP kinase cascades. *Adv. Cancer Res.*, *74*: 49–139, 1998.
- Keyse, S. M. Protein phosphatases and the regulation of MAP kinase activity. *Semin. Cell Dev. Biol.*, *9*: 143–152, 1998.
- Thomson, S., Mahadevan, L. C., and Clayton, A. L. MAP kinase-mediated signalling to nucleosomes and immediate-early gene induction. *Semin. Cell Dev. Biol.*, *10*: 205–214, 1999.
- Voeller, H. J., Wilding, G., and Gelmann, E. P. v-rasH expression confers hormone-independent *in vitro* growth to LNCaP prostate carcinoma cells. *Mol. Endocrinol.*, *5*: 209–216, 1991.
- Culig, Z., Hobisch, A., Cronauer, M. V., Cato, A. C., Hittmair, A., Radmayr, C., Eberle, J., Bartsch, G., and Klocker, H. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. *Mol. Endocrinol.*, *17*: 1541–1550, 1993.
- Taplin, M. E., Buble, G. J., Shuster, T. D., Frantz, M. E., Spooner, A. E., Ogata, G. K., Keer, H. N., and Balk, S. P. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.*, *332*: 1393–1398, 1995.
- Taplin, M. E., Buble, G. J., Ko, Y. J., Smal, E. J., Upton, M., Rajeshkumar, B., and Balk, S. P. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Res.*, *59*: 2511–2515, 1999.
- Kelly, W. K., and Scher, H. I. Prostate specific antigen decline after antiandrogen withdrawal: the flutamide withdrawal syndrome. *J. Urol.*, *149*: 607–609, 1993.
- Kelly, W. K., Slovin, S., and Scher, H. I. Steroid hormone withdrawal syndromes. Pathophysiology and clinical significance. *Urol. Clin. North Am.*, *24*: 421–431, 1997.
- Scher, H. I., and Kelly, W. K. J. Flutamide withdrawal syndrome: its impact on clinical trials in hormone-refractory prostate cancer. *Clin. Oncol.*, *11*: 1566–1572, 1993.
- Lin, S.-Y., Xia, W., Wang, J. C., Kwong, K. Y., Spohn, Y., Wen, Y., Pestell, R. G., and Hung, M.-C. b-Catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. USA*, *97*: 4262–4266, 2000.
- Sella, A., Flex, D., Sulkes, A., and Baniel, J. Antiandrogen withdrawal syndrome with cyproterone acetate. *Urology*, *52*: 1091–1093, 1998.
- Huan, S. D., Gerridzen, R. G., Yau, J. C., and Stewart, D. J. Antiandrogen withdrawal syndrome with nilutamide. *Urology*, *49*: 632–634, 1997.
- Small, E. J., and Carroll, P. R. Prostate specific antigen decline after bicalutamide withdrawal: evidence for an antiandrogen withdrawal syndrome. *Urology*, *43*: 408–410, 1994.
- Dawson, N. A., and McLeod, D. G. Dramatic prostate specific antigen decrease in response to discontinuation of megestrol acetate in advanced prostate cancer: expansion of the anti-androgen withdrawal syndrome. *J. Urol.*, *153*: 1946–1947, 1995.
- Peng, D., Fan, Z., Lu, Y., DeBlasio, T., Scher, H., and Mendelsohn, J. Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27KIP1 and induces G<sub>1</sub> arrest in prostatic cancer cell line DU145. *Cancer Res.*, *56*: 3666–3669, 1996.
- Pietrzkowski, Z., Mulholland, G., Gomella, L., Jameson, B. A., Wernike, D., and Baserga, R. Inhibition of growth of prostatic cancer cell lines by peptide analogues of insulin-like growth factor 1. *Cancer Res.*, *53*: 1102–1106, 1993.
- Putz, T., Culig, Z., Eder, I. E., Nessler-Menardi, C., Bartsch, G., Grunick, H., Uberall, F., and Klocker, H. Epidermal growth factor (EGF) receptor blockade inhibits the action of EGF, insulin-like growth factor I, and a protein kinase A activator on the mitogen-activated protein kinase pathway in prostate cancer cell lines. *Res.*, *59*: 227–233, 1999.
- Fong, C.-J., Sherwood E. R., Mendelsohn J., Lee C., and Kozlowski, M. Epidermal growth factor receptor monoclonal antibody inhibits constitutive receptor phosphorylation, reduces autonomous growth, and sensitizes androgen-independent prostatic carcinoma cells to tumor necrosis factor  $\alpha$ . *Cancer Res.*, *52*: 5887–5892, 1992.
- Chen, G., Oh, S., Monia, B. P., and Stacey, D. W. Antisense oligonucleotides demonstrate a dominant role of c-Ki-RAS proteins in regulating the proliferation of diploid human fibroblasts. *J. Biol. Chem.*, *271*: 28259–28265, 1996.
- Monia, B. P., Sasmor, H., Johnston, J. F., Freier, Su M., Lesnik, E., Muller, M., Geiger, T., Altmann, K.-H., Moser, H., and Fabbro, D. Sequence-specific antitumor activity of a phosphorothioate oligodeoxyribonucleotide targeted to human C-raf kinase supports an antisense mechanism of action *in vivo*. *Proc. Natl. Acad. Sci. USA*, *93*: 15481–15484, 1996.