dine dimers must be replicated so that they may generate the ss DNA to activate recA (*12*).

We suggest that other eukaryotic checkpoint control proteins may also participate in DNA damage processing. For example, S-phase checkpoint control may require different proteins to process the type of DNA damage that is induced after DNA replication is inhibited. *POLε* is required for S but not for G_2 checkpoint control (*7*); perhaps this is because *POLε* is specifically required for processing after DNA replication is disturbed. Differences in processing may explain the perplexing patterns of checkpoint gene requirements among species. For example, *rad1*$^+$ and *RAD17* in fission and budding yeasts encode putative 3′-5′ exonucleases and both are required for the G_2 checkpoint in their respective cell types (Fig. 4). However, *rad1*$^+$, yet not *RAD17*, is also required at the S-phase checkpoint (*13*). Similarly, *cdc2*$^+$ and *CDC28* in fission and budding yeasts encode conserved protein kinases. *cdc2*$^+$, yet not *CDC28*, is needed for S-phase checkpoint control (*14*). Perhaps *rad1*$^+$ and *cdc2*$^+$ in fission yeast are required for DNA damage processing after S-phase inhibition, but their homologs in budding yeast are not.

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9. Two breaks that cause checkpoint-mediated arrest—an HO endonuclease–induced break during mitosis (*15*) (S. Kim and T. Weinert, unpublished data) and double strand breaks produced during meiosis (D. Lydall and T. Weinert, unpublished data)—both become processed to give a 3′ overhang (implying degradation by a 5′-3′ exonuclease). How a putative 3′-5′ exonuclease (Rad17p) mediates arrest, and perhaps processing, at these two breaks is unclear.
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18. Yeast strains used were *MAT***a** *bar1::hisG ade2-1 can1-100 cdc15-2 ura3 leu2-3,112 his3-11 trp1-1* and contained various combinations of *cdc13-1*, *rad9::HIS3*, and *rad24::TRP1*. The *bar1* mutation was present to ensure that G_1 arrest was efficient. The *cdc15-2* mutation was present to ensure that checkpoint control mutants replicated their DNA only once. At 36°C, *cdc15* mutants initiate anaphase but are unable to complete nuclear division. We modified the method of Garvik *et al.* and cut purified yeast DNA with Eco RI before applying the DNA to a slot blot apparatus (*8*). Different filters were probed with GT, AC (from pHR85-31), and *URA3* ss RNA probes. Eco RI was necessary because in some experiments ss DNA was present in a non–strand- and non–locus-specific fashion. This ss DNA did not depend on the presence or absence of *CDC13*. Our interpretation is that it represented replication forks moving through the chromosome. This inconsistent *cdc13*-independent signal disappeared after cutting of the DNA with Eco RI, presumably because the chance that a replication fork was attached to the locus we were probing was reduced. After cutting with Eco RI, most (>95%) of the DNA did not bind to the filter, even after it was denatured. We assume this was because smaller fragments of DNA bind the filter less efficiently. The ss DNA index increased for two reasons. First, ss DNA is created in vivo. Second, as ss DNA is created, Eco RI sites are destroyed, so that the size of the ss DNA increased and it bound the nylon membrane more efficiently. Therefore we cannot make quantitative deductions about the amount of ss DNA produced.
19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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27. We thank B. Garvik and L. Hartwell for invaluable discussions and advice about the *cdc13* defect; N. Seaver for help with flow cytometry; A. Adams, L. Hatfield, and M. Ramaswami for criticizing the manuscript; and all the yeasty boys and girls in Tucson for input. D.L. thanks E. Ayres for encouragement. Supported by grants to T.W. from NIH (GM 45276-01), the American Cancer Society, and the March of Dimes foundation.

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Activation of the Estrogen Receptor Through Phosphorylation by Mitogen-Activated Protein Kinase

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The phosphorylation of the human estrogen receptor (ER) serine residue at position 118 is required for full activity of the ER activation function 1 (AF-1). This Ser^{118} is phosphorylated by mitogen-activated protein kinase (MAPK) in vitro and in cells treated with epidermal growth factor (EGF) and insulin-like growth factor (IGF) in vivo. Overexpression of MAPK kinase (MAPKK) or of the guanine nucleotide binding protein Ras, both of which activate MAPK, enhanced estrogen-induced and antiestrogen (tamoxifen)-induced transcriptional activity of wild-type ER, but not that of a mutant ER with an alanine in place of Ser^{118}. Thus, the activity of the amino-terminal AF-1 of the ER is modulated by the phosphorylation of Ser^{118} through the Ras-MAPK cascade of the growth factor signaling pathways.

The ER belongs to a superfamily of ligand-inducible transcription factors that includes receptors for steroid hormones, thyroid hormones, vitamin D_3, and retinoic acid, as well as peroxisome proliferator–activated receptors and orphan receptors (*1*). The ER has two transcriptional activation functions, AF-1 and AF-2, which are located in the NH_2-terminal A/B region and in the ligand-binding domain (region E), respectively (*2*–*6*). Like other steroid hormone receptors, the ER is phosphorylated (*7*–*9*). The ER becomes phosphorylated at several sites when transfected COS-1 cells are treated with estradiol (E_2), and Ser^{118}—the main residue in the A/B region to be phosphorylated (*7*, *8*)—is required for full activity of AF-1 (*7*). The five amino acids located around Ser^{118} (PQLSP) (Fig. 1) (*7*, *10*) are conserved among vertebrate species

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(human, mouse, rat, chick, *Xenopus*, and trout) and correspond to the consensus phosphorylation site [PX_n(S or T)P] (where X is a neutral or basic amino acid and n = 1 or 2) for MAPK (*11*). The finding that the ligand-induced transcriptional activity of ER can be further enhanced by growth factors such as EGF and IGF (*12*) suggests a possible cross talk between the E_2 signaling pathway and growth factor signaling pathways. MAPK is activated by growth factors such as EGF, insulin, and IGF, through membrane-associated receptor tyrosine kinases, which in turn activate Ras followed by activation of the protein kinase Raf. Activated Raf then mediates signal transduction to MAPK through the MAPKK or extracellular signal–regulated protein kinase (ERK) kinases (MEKs) (*13*). Taken together, these observations suggested that phosphorylation of the ER Ser^{118} residue through the growth factor–Ras-Raf-MAPK cascade may modulate the activity of AF-1.

To determine whether Ser^{118} was phosphorylated by MAPK in vitro, we expressed the human ER (hER) truncated mutant HE15, which contains regions A through C (*2*), and its Ser^{118} mutants HE15/457 and HE15/458 (Fig. 1) (*7*) as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* (Fig. 2A). Purified recombinant HE15 (Fig. 2A) was phosphorylated by MAPK purified from *Xenopus* oocytes (*14*), under conditions in which myelin basic protein (MBP) was a good substrate for MAPK (Fig. 2B). Mutation of Ser^{118} to Ala^{118} (HE15/457) or to Glu^{118} (HE15/458) abolished phosphorylation (Fig. 2B). MAPK purified from either human cervix epithelioid carcinoma HeLa cells, African green monkey kidney COS-1 cells, or rat osteosarcoma Ros cells, with a polyclonal antibody directed against *Xenopus* MAPK, gave similar results (*15*). In contrast, purified recombinant HE15, HE15/457, and HE15/458 proteins were not phosphorylated by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA), although PKA did phosphorylate histones (Fig. 2B). We used an electrophoretic mobility-shift assay to show that *E. coli*–produced or in vitro translated HE15 and HE15/457 efficiently bound to an estrogen response element (ERE) and that their binding was not affected by MAPK phosphorylation (*15*). The mutation of Ser^{118} did not affect ERE binding by ER produced in cultured cells (*7*), and our findings are in agreement with this result.

To examine the ability of growth factors to induce phosphorylation of hER Ser^{118}, we transfected COS-1 cells with HE15 or HE15/457 (Fig. 1) and incubated them with [^{32}P]orthophosphate in the presence or absence of EGF or IGF. Immunoprecipitation of cell extracts showed that phosphorylation of HE15 was enhanced in cells treated with EGF or IGF (Fig. 2, C and D), whereas no enhancement was observed when Ser^{118} was mutated (HE15/457). Similarly, expression of a dominant active form of MAPKK (*16*) induced phosphorylation of HE15, but not of HE15/457, in transfected COS-1 cells (Fig. 2C).

To investigate whether MAPK could be involved in ER-mediated transcriptional activation, we transiently transfected COS-1 cells with the wild-type hER expression vector HEG0 (Fig. 3A) and with an expression vector for either c-Ki-Ras (k-Ras) or the dominant active mutant c-Ki-Ras^{Val12} (k-Ras^{Val12}) (*17*), together with the ERE-G-CAT or ERE-TATA-CAT reporter genes, which contain promoter elements of the rab-

Mutant	99 PLNSVSPSPLMLLHPPPQLSPF 120	Induction by k-Ras^{Val12}
HE15	.....	3.1 ± 0.1
HE15/451	.....P.A.....	2.9 ± 0.2
HE15/452	...N.P.A.....	2.8 ± 0.1
HE15/453	.....A.....	2.9 ± 0.2
HE15/454	.....P.E.....	2.7 ± 0.1
HE15/455	...N.P.E.....	2.9 ± 0.1
HE15/456	.....E.....	3.1 ± 0.2
HE15/457	.....A..	1.1 ± 0.1
HE15/458	.....E..	1.2 ± 0.1

Fig. 1. Abrogation of k-Ras–induced activation of AF-1 by mutation of Ser^{118}, but not of Ser^{102}, Ser^{104}, and Ser^{106} (*10*). Amino acids 99 to 120 of the hER and the point mutations that were created in the A/B region (*7*) are shown. COS-1 cells were cotransfected with ERE-G-CAT and each of the HE15 mutants (*7*) in the absence or presence of the expression vector for k-Ras^{Val12}, and the relative amounts of chloramphenicol acetyltransferase (CAT) activity were determined as in Fig. 3. Values for induction by k-Ras^{Val12} (average ± SEM) for six independent experiments are given.

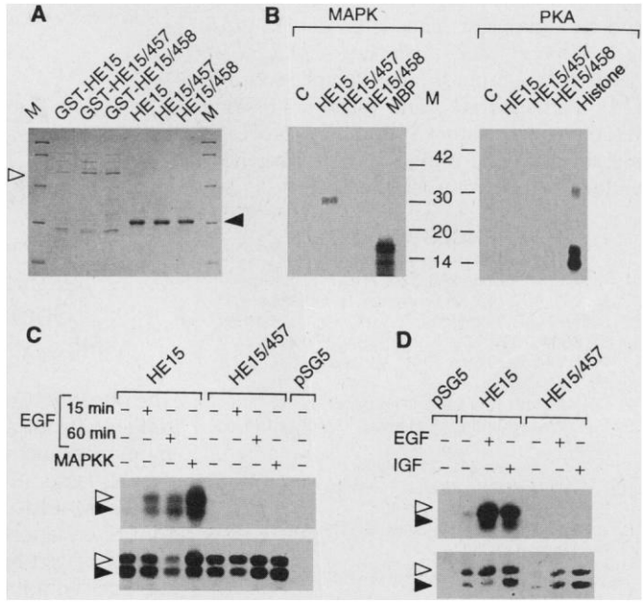

Fig. 2. Phosphorylation of the COOH-terminal truncated human ER mutant (HE15) at Ser^{118} by EGF, IGF, MAPKK, and MAPK. (**A**) Analysis of purified bacterially produced GST fusion proteins and purified HE15, HE15/457, and HE15/458 recombinant proteins. Purified GST fusion proteins (500 ng) and purified HE15 and derivatives (500 ng) (*28*) were analyzed by electrophoresis on an SDS gel (12% polyacrylamide) and stained with Coomassie blue. Positions of GST fusion proteins and recombinant proteins after cleavage are indicated by open and solid arrowheads, respectively. The molecular size markers (M) were rabbit phosphorylase (97 kD), bovine serum albumin (66 kD), rabbit aldolase (42 kD), bovine carbonic anhydrolase (30 kD), and soybean trypsin inhibitor (20 kD). (**B**) In vitro phosphorylation of hER Ser^{118} by MAPK. Purified bacterially produced HE15, HE15/457, or HE15/458 (1 μg) was incubated for 30 min at 30°C with MAPK (0.5 U/μl) [purified from nonfertilized *Xenopus* eggs (*14*)], in 50 mM tris-HCl (pH 8.0), 0.5 mM EDTA, 25 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM adenosine triphosphate (ATP) (0.05 μCi of [γ-^{32}P]ATP), and 10% glycerol or with PKA (0.5 U/μl) [from bovine heart (Sigma)], in 20 mM tris-HCl (pH 6.8), 10 mM magnesium acetate, 1 μM cAMP, and 20 μM ATP (0.05 μCi of [γ-^{32}P]ATP). MBP (Sigma; 1.25 μg) and histones (type II-S, Sigma; 2 μg) were phosphorylated by MAPK and PKA, respectively. Phosphorylation of substrates was analyzed by SDS-PAGE (10 to 20% gradient gel) and autoradiography. C, control lacking HE15. (**C** and **D**) EGF-, IGF-, and MAPKK-induced phosphorylation of hER Ser^{118} in vivo. COS-1 cells were transfected with HE15, HE15/457, or the parental expression vector pSG5 (*7*). In (C), the expression vector for a dominant active form of MAPKK (400 ng) (*15, 16*) was cotransfected as indicated. Starvation with phosphate- and serum-free Dulbecco's modified Eagle's medium (DMEM) was done 40 hours after transfection. After 5 hours of starvation, in vivo labeling was done for 4 hours in phosphate- and serum-free DMEM containing 50 μM vanadate, 50 nM okadaic acid, and 1 mCi of [^{32}P]orthophosphate. In (C) and (D), EGF (100 ng/ml) was added as indicated; in (D), EGF and IGF (100 ng/ml) were added 15 min before harvesting. ^{32}P-labeled extracts of HE15 and HE15/457 were immunoprecipitated with the monoclonal antibody (mAb) B10, followed by SDS-PAGE analysis and immunoblotting, as described (*7*). Upper lanes, autoradiograph signal from the nitrocellulose membrane; lower lanes, HE15 and HE15/457 protein revealed by chemiluminescence after the membrane was probed with mAb B10. Open and closed arrowheads denote the positions of HE15 and a proteolysed form, respectively.

bit β-globin promoter (−119 to +10) and the minimal promoter region (−34 to +33) of the adenovirus-2 major late promoter, respectively. Coexpression of k-Ras or k-Ras^{Val12} led to increases in E_2-induced transactivation by HEG0 (by 1.8 and 2.1 times, respectively, with ERE-G-CAT; by 1.8 and 2.3 times, respectively, with ERE-TATA-CAT) (Fig. 3, B and C). Similar results were obtained in HeLa cells (*15*).

Because there are two transcriptional activation functions (AF-1 and AF-2) in the ER, we investigated whether k-Ras could activate AF-1 or AF-2 with the hER truncated mutants HE15 and HEG19, respectively (Fig. 3A). Transactivation by AF-2 (HEG19) was not enhanced by cotransfection of k-Ras or k-Ras^{Val12}, whereas the activity of AF-1 (HE15) was clearly enhanced, as measured with either of the two reporter genes in COS-1 cells (Fig. 3, B and C). These observations indicate that enhancement by k-Ras of E_2-induced transactivation by hER is essentially mediated through AF-1.

An S118A substitution in HEG0 (HE457) (Fig. 3A) not only decreased the transcriptional activity of the hER, but also abolished k-Ras–mediated enhancement of transcription from both the ERE-TATA-CAT and ERE-G-CAT reporter genes (Fig. 3, B and C). In the absence of AF-2 (HE15) (Fig. 3A), the S118A mutation also abolished k-Ras–mediated transcriptional activation of both reporter genes (Fig. 4A). In sharp contrast, mutations of Ser^{102}, Ser^{104}, or Ser^{106} to nonphosphorylatable residues did not affect k-Ras–induced transactivation (Fig. 1), which indicated a specific role for Ser^{118} in this induction.

We used chimeric proteins in which the A/B region was linked to the GAL4 DNA binding domain (AB-GAL) (Fig. 4B) to confirm that MAPK-induced activation of hER was the result of enhanced AF-1 activity. In cells transfected with the 17M$_2$-G-CAT reporter gene, which contains two GAL4 17M binding sites located upstream of the globin promoter region (*3*), expression of k-Ras or of a dominant active form of MAPKK (*16*) enhanced the activation of transcription by AB-GAL (Fig. 4B), and the S118A mutation (AB/457-GAL) abolished these effects. In contrast, expression of CL100, a MAPK-specific phosphatase (*18*), decreased the activation of transcription by AB-GAL (Fig. 4B). Thus, it appears that MAPK increases the activity of AF-1 by inducing the phosphorylation of Ser^{118}. To determine whether PKA also affects the activity of AF-1, we transfected COS-1 cells with the AB-GAL expression vector and then either cotransfected these cells with a PKA expression vector or

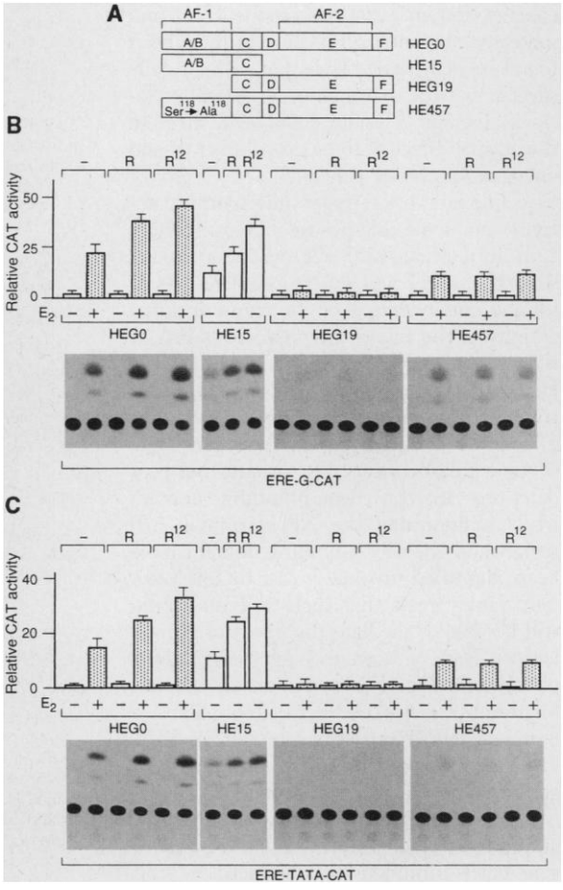

Fig. 3. Enhancement of the transcriptional activity of the hER by overexpressed k-Ras. (**A**) Representations of the hER (with regions A through F shown) and of the mutants used in (B) and (C). (**B** and **C**) Enhanced estrogen-induced transcriptional activity of the hER through AF-1 in cells overexpressing k-Ras. ERE-G-CAT was constructed by inserting a synthetic oligonucleotide containing a perfect palindromic ERE into pG-CAT (*29*). Transient transfections and CAT assays were done as described (*7, 29*). COS-1 cells were cotransfected with either 1 μg of ERE-G-CAT (B) or 2 μg of ERE-TATA-CAT (C) and 0.5 μg of receptor expression vector HEG0, HE15, HEG19, or HE457, in the presence and absence of 10 nM E_2, as indicated. Expression vectors (0.5 μg) for k-Ras (R) and k-Ras^{Val12} (R^{12}) (*17*) were also cotransfected as indicated. Representative CAT assays and graphs corresponding to means and SEM for three independent experiments are shown.

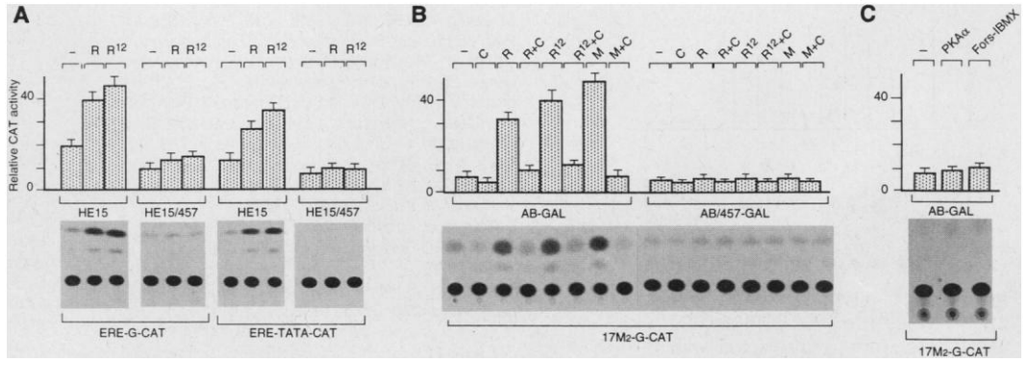

Fig. 4. Requirement of Ser^{118} for k-Ras–induced transactivation. (**A**) Transcriptional activity of HE15, but not of HE15/457, is enhanced by k-Ras overexpression. COS-1 cells were cotransfected with either ERE-G-CAT or ERE-TATA-CAT and either HE15 or HE15/457 (*7*). The expression vectors for k-Ras (R) or k-Ras^{Val12} (R^{12}) were cotransfected as in Fig. 3, as indicated. Representative CAT assays and graphs corresponding to means and SEM for three independent experiments are shown. (**B**) Transcriptional activity of AB-GAL, but not of AB/457-GAL, is enhanced in cells overexpressing k-Ras or MAPKK. AB/457-GAL was constructed by replacing the A/B region of AB-GAL (*5*) with the A/B region of HE15/457 (*7*). COS-1 cells were cotransfected with 17M$_2$-G-CAT (*3*) and with AB-GAL or AB/457-GAL expression vectors. The expression vectors for k-Ras (R), k-Ras^{Val12} (R^{12}), MAPKK (M) (400 ng), or CL100 (C) (20 ng) (*15, 18*) were cotransfected as indicated. (**C**) Lack of effect of PKA on the activity of AF-1. COS-1 cells were cotransfected with 17M$_2$-G-CAT and with AB-GAL or AB/457-GAL expression vectors, as in (B). An expression vector for the cAMP-dependent protein kinase catalytic subunit isoform α (PKAα; 0.5 μg) (*30*) was also cotransfected; alternatively, the cells were treated with 50 μM forskolin and 500 nM IBMX (Fors-IBMX), as indicated.

treated them with forskolin and isobutylmethylxanthine (IBMX) to induce accumulation of cAMP. In both instances, there was no effect on transcription of the $17M_2$-G-CAT reporter (Fig. 4C), in agreement with the absence of phosphorylation of purified HE15 protein by PKA in vitro (Fig. 2B).

The antiestrogen 4-hydroxytamoxifen (OHT) has mixed agonistic and antagonistic activities, whereas ICI 164,384 acts as a pure antiestrogen (*4*, *7*, *19*). Although OHT and ICI 164,384 do not appear to inhibit the DNA binding of the receptor, OHT blocks the activity of AF-2 but not of AF-1, whereas ICI 164,384 blocks the activities of both AF-1 and AF-2 (*4*, *19*). We investigated the effect of k-Ras on transcriptional activation by HEG0 in COS-1 cells with the use of the ERE-G-CAT reporter gene in the presence of E_2, OHT, or ICI 164,384. The partial agonistic activity of OHT was enhanced in cells transfected with k-Ras, whereas k-Ras had no effect in the presence of ICI 164,384 (Fig. 5).

Taken together, our results indicate that transcriptional activation by a member of the nuclear receptor superfamily (ER) can be induced by growth factors (EGF and IGF). This effect is mediated through the membrane-associated receptor tyrosine kinase–Ras-Raf-MAPK cascade, which enhances the activity of ER AF-1 by stimulating phosphorylation of the Ser^{118} residue located in the NH_2-terminal A/B region. Although only the Ras-mediated pathway of MAPK activation was examined here, other MAPK activation pathways (*13*) may also lead to enhanced activity of ER AF-1. Also, MAPK may phosphorylate and regulate other members of the nuclear receptor superfamily.

EGF, TGFα, and IGF-I, which promote the growth of several breast cancer–derived cell lines in culture, are produced by some breast tumors in vivo (*20*). The c-ErbB2 protein (also called HER2-Neu, a proto-oncogene product related to the EGF receptor) is overexpressed in ~20% of human breast cancers, which leads to amplification of the Ras signaling pathway and may cause an aggressive tumor phenotype (*21*). Overexpression of c-ErbB3, another member of the same family, has also been observed in 13 to 29% of breast cancers (*22*). Activating Ras mutations are found at a low frequency in primary breast cancers and metastatic effusions (*23*), and increased amounts of Ras protein have been found in malignant tissues (*24*). Our results suggest that the deregulation of phosphorylation of the Ser^{118} residue could be involved in the adverse effect of these growth factors and oncogenes in breast cancer.

Tamoxifen has estrogen-like agonistic activity on some ER-positive breast cancer cells in culture (*25*). Because our results show that OHT was almost as efficient as E_2 for the enhancement of the transcriptional activity of the ER in the presence of Ras, it is possible that MAPK—activated by growth factors, "activated" Ras, or both—may contribute to an enhanced agonistic activity of OHT, and therefore to the OHT resistance of some breast cancers. ER variants that bear deletions in the ligand-binding domain while maintaining the NH_2-terminal A/B region and the DNA-binding domain have been identified in some breast tumors (*26*). Our data suggest that such mutants would still be able to mediate the effect of growth factors, Ras, or both through Ser^{118} phosphorylation. Finally, our results may account for the estrogen-like effect of tamoxifen on bones, where it prevents osteoporosis (*27*). Enhancement of transcription of bone estrogen-responsive genes may depend mainly on the growth factor–activated AF-1 of the ER, and not on AF-2, which is induced by estrogens but is inhibited by tamoxifen.

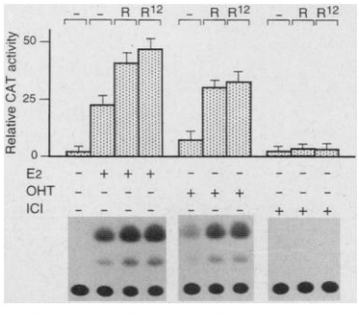

Fig. 5. Effect of k-Ras on hER transcriptional activity in the presence of E_2, OHT, and ICI 164,384. COS-1 cells were cotransfected with ERE-G-CAT, HEG0, and k-Ras (R) or k-Ras^{Val12} (R^{12}), as indicated, and were grown in the presence or absence of 10 nM E_2, 100 nM OHT, or 100 nM ICI 164,384 (ICI). CAT activities were determined as in Fig. 3. Representative CAT assays and graphs corresponding to means and SEM for three independent experiments are shown.

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28. The bacterial expression vectors for GST fusion proteins (pGEX-HE15, pGEX-HE15/457, and pGEX-HE15/458) were constructed by cloning polymerase chain reaction (PCR)–amplified fragments derived from HE15, HE15/457, and HE15/458 (*7*), respectively, into the Bam HI–Eco RI sites of pGEX-2T (Pharmacia). The GST-HE15, GST-HE15/457, and GST-HE15/458 fusion proteins were purified from *E. coli* transfected with the corresponding expression vectors, with the use of glutathione Sepharose 4B (Pharmacia) according to the manufacturer's specifications. Each GST-fused protein (500 μg) was digested with thrombin (5 U) and then sequentially passed through the glutathione Sepharose column (to remove thrombin and GST) and through a Sephadex G200 column.
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