Epidermal Growth Factor (EGF) Receptor Kinase-independent Signaling by EGF*

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The ErbB family of receptors, which includes the epidermal growth factor receptor (EGFR), ErbB2, ErbB3, and ErbB4, mediate signaling by EGF-like polypeptides. To better understand the role of the EGFR tyrosine kinase, we analyzed signaling by a kinase-inactive EGFR (K721M) in ErbB-devoid 32D cells. K721M alone exhibited no detectable signaling capacity, whereas coexpression of K721M with ErbB2, but not ErbB3 or ErbB4, resulted in EGF-dependent mitogen-activated protein kinase (MAPK) activation. The kinase activity, but not tyrosine phosphorylation, of ErbB2 was required for EGF-induced MAPK activation. The presence of tyrosine phosphorylation sites in K721M was not a requisite for signaling, indicating that transphosphorylation of K721M by ErbB2 was not an essential mechanism of receptor activation. Conversely, the mutated kinase domain of K721M (residues 648–973) and tyrosine phosphorylation of at least one of the receptors were necessary. EGF was found to activate the pro-survival protein kinase Akt in stable cell lines expressing K721M and ErbB2 but, unlike cells expressing wild-type EGFR, was incapable of activating signal transducers and activators of transcription (STAT) or driving cell proliferation. These results demonstrate that EGFR-ErbB2 oligomers are potent activators of MAPK and Akt, and this signaling does not require EGFR kinase activity.

The epidermal growth factor receptor (EGFR,[1] ErbB1, HER1) is the prototypical member of the ErbB family of receptors, which includes ErbB2 (HER2, *neu*), ErbB3 (HER3), and ErbB4 (HER4) (reviewed in Refs. 1–3). The ErbBs mediate signaling by a large number of growth factors that are structurally related to EGF such as transforming growth factor-α or amphiregulin. This family of receptors plays critical roles in the proliferation, migration, survival, and differentiation of target cells, and dysregulation of signaling by ErbBs has been implicated in the pathogenesis and progression of human cancers (2,

4). ErbBs are activated via a process of receptor homo- and heterodimerization, which is initiated by engagement of ligand by the extracellular domain of an ErbB, and almost every possible dimeric combination of ErbBs have now been observed (reviewed in Refs. 1–3, 5). Unlike EGFR, ErbB3, and ErbB4, no ligand has been identified for ErbB2 to date. Recent work has demonstrated that binding of EGF to EGFRs initiates a ligand-independent lateral propagation of receptor activation in the plasma membrane (6).

ErbBs consist of an extracellular ligand binding domain, a transmembrane spanning segment, an intracellular tyrosine kinase domain, and a C-terminal region that contains multiple tyrosines, which upon phosphorylation provide docking sites for signal transducers such as GRB2 and SHC (7). Unlike other family members, ErbB3 does not possess kinase activity (8). Early research on the EGFR indicated that the kinase activity was required for EGF-induced biological responses (9, 10). However, subsequent studies have demonstrated that kinase-inactive forms of the EGFR have the capacity to signal in an EGF-dependent manner. EGF-stimulated kinase-inactive EGFRs activate MAPK, c-*fos* gene expression, and/or DNA synthesis (11–16). Targeted inactivation of the *EGFR* gene in mice results in strain-dependent phenotypes that range from death *in utero* to postnatal abnormalities in skin, lung, kidney, gastrointestinal tract, and brain (17–19). *Waved-2* (*wa-2*) mice, which harbor a V743G mutation in the EGFR kinase domain exhibit mild skin and eye abnormalities but are healthy and fertile (20, 21). Interestingly, the V743G mutation results in a kinase-defective EGFR whose ability to phosphorylate exogenous substrate has been reduced by >90% relative to its wild-type counterpart (20). The homologous mutation in the human EGFR (V741G) significantly impairs EGFR kinase activity, and ectopic expression of V741G EGFR in myeloid BaF/3 cells confers the ability for EGF to induce survival but not proliferation (22, 23). Conversely, kinase-impaired V741G EGFR can elicit EGF-dependent mitogenesis when expressed in NIH 3T3 fibroblasts (21). Taken together, these findings raise the possibility that the EGFR kinase activity may not be required for certain aspects of EGFR function.

To better understand the role of the EGFR kinase activity in mediating EGF-stimulated cellular responses we studied signaling by a kinase-inactive EGFR in which a lysine to methionine mutation (K721M) abrogates ATP binding. Because most cell types express one or more endogenous ErbBs, an elucidation of the mechanism of EGF-induced signaling by ectopically expressed kinase-inactive EGFRs has been elusive. To circumvent this problem, we have utilized the ErbB-devoid myeloid 32D cells as a model system into which we reconstituted K721M signaling. Our studies demonstrate a specific require-

[1] The abbreviations used are: EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; PAGE, polyacrylamide gel electrophoresis; IL, interleukin.

ment for ErbB2 in K721M signaling and indicate that signaling is mediated by a K721M-ErbB2 oligomer. The EGF-stimulated hetero-oligomer is a strong activator of MAPK and the pro-survival kinase Akt, and EGFR kinase activity is not required for this signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—The 32D cell line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% medium conditioned by WEHI-3B cells (24). Cells stably expressing human EGFR were provided by J. Pierce (NCI, National Institutes of Health; Ref. 25).

Antibodies and cDNAs—Antibodies to EGFR and ErbB2 were obtained from NeoMarkers. Biotinylated PY-20 and 4G10 antibodies were purchased from ICN Biomedical and Upstate Biotechnology, respectively. Antibodies directed against the phosphorylated activated forms of MAPK, STATs, and Akt were obtained from New England BioLabs. Antibodies against ErbB3 and Akt were obtained from Santa Cruz Biotechnology. ErbB cDNAs were provided by J. Pierce and subcloned into pcDNA3.1 or pcDNA3.1/Zeo. Mutations within cDNAs constructs were made using standard molecular biology techniques, and all constructs were DNA-sequenced.

Transfections and Generation of Stable Cell Lines—Cells were transfected with 20 μg of total DNA by electroporation (960 μF, 0.25 kV) using a Bio-Rad Gene Pulser II. Stable transfectants were generated using the neomycin resistance marker in pcDNA3.1 or the Zeocin resistance marker in pcDNA3.1/Zeo. Clonal populations were generated by serial dilution and characterized by flow cytometry and Western blotting.

Cell Proliferation Assays—Assays were performed as previously described (27). Briefly, cells were washed, resuspended into 60 × 15 mm dishes (100,000 cells/ml) in RPMI 1640 medium containing 15% fetal bovine serum (basal medium) with or without 10 nM EGF. Cells were stained with trypan blue, and viable cells were counted every day using a hemocytometer.

Immunoprecipitations—Whole cell lysates were generated, and protein concentration was determined as described (34). One μg of antibody was added to 0.5 mg of lysate, incubated for 1 h at 4 °C and 5 μl of protein G-agarose was added. After 1 h at 4 °C, the immune complexes were centrifuged at 15,000 × g for 2 min and washed three times with ice-cold lysis buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer for 4 min.

Western Blotting—Proteins were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were probed with 0.3 μg/ml of primary antibody, and detection was performed using the Vectastain ABC Elite Kit (Vector Labs), enhanced chemiluminescence, and exposed to film.

RESULTS

Acquisition of Signaling by Kinase-inactive EGFR by Coexpression of ErbB2 but Not ErbB3 or ErbB4—To explore the signaling potential of a kinase-inactive EGFR, we studied signaling by a mutant human EGFR in which a lysine to methionine mutation has been introduced into the ATP binding site (K721M). Work by us (data not shown) and others have demonstrated that this mutation abolishes the kinase activity of the EGFR (9, 10). To determine the intrinsic signaling capacity of K721M, we investigated EGF-dependent signaling by ectopically expressed K721M in the interleukin-3 (IL-3)-dependent myeloid 32D cell line (24). This ErbB-devoid cell line has proved to be a powerful tool in the study of ErbB signaling (25–27). 32D cells were transiently transfected with K721M cDNA and stimulated with EGF (Fig. 1A). Lysates were analyzed for MAPK activation using an antibody specific for phosphorylated Thr-202/Tyr-204 in the activated form of MAPK (extracellular signal-regulated kinase 1 and 2; *top panel*). No activation of MAPK was detected under these conditions even though expression of K721M was confirmed by Western blotting (*lanes 1–2*).

To test the hypothesis that kinase-devoid forms of the EGFR can signal in the presence of additional human ErbB family members, we simultaneously expressed K721M along with ErbB2, ErbB3, or ErbB4. Interestingly, although ErbB2 by
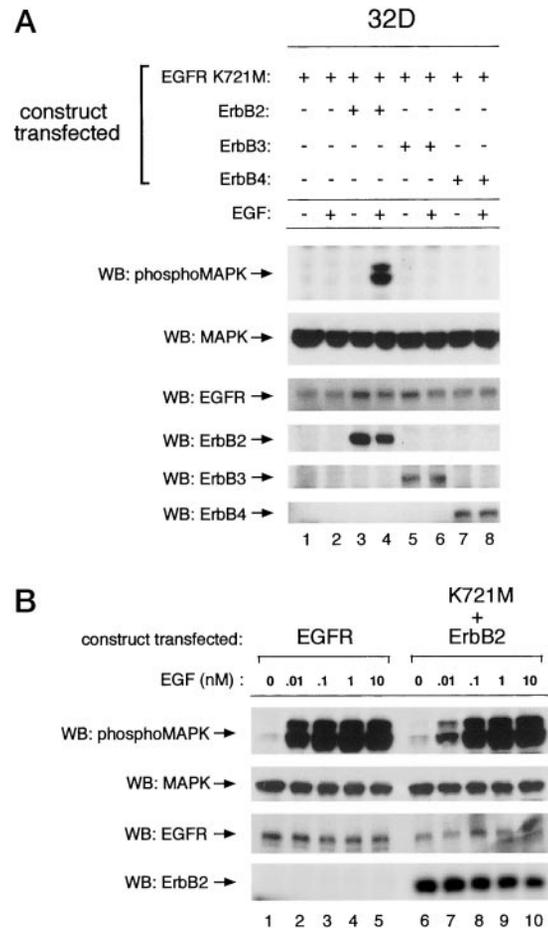
FIG. 1. **Analysis of EGF-induced MAPK activation by coexpression of K721M and ErbBs in 32D cells.** *A,* 32D cells were transfected with either K721M cDNA alone (*lanes 1–2*) or with ErbB2 (*lanes 3 and 4*), ErbB3 (*lanes 5–6*), or ErbB4 cDNA (*lanes 7–8*). Serum-starved cells were stimulated for 5 min at 37 °C with 10 nM EGF and 15 μg of whole cell lysates were separated in an 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The activation of MAPK was monitored by Western blotting (*WB*) using antibodies against the phosphorylated form of MAPK (*phosphoMAPK*). Consistent loading of each lane was confirmed by stripping the blot and reprobing for MAPK. Expression of ErbBs was confirmed by probing the blot with appropriate antibodies**.** *B,* cells were transfected with either EGFR alone (*lanes 1–5*) or a combination of K721M and ErbB2 cDNA (*lanes 6–10*). Cells were stimulated with indicated concentrations of EGF for 5 min at 37 °C.

itself was unable to promote MAPK phosphorylation in response to EGF (data not shown), coexpression of K721M with ErbB2 (*lanes 3–4*) resulted in EGF-induced MAPK stimulation. In contrast, coexpression of K721M with either ErbB3 or ErbB4 could not reconstitute EGF-dependent signaling by K721M (*lanes 6 and 8*). No differences in the magnitude of MAPK activation were observed when the ratio of transfected K721M: ErbB2 cDNAs was varied from 3 to 0.33 (data not shown), suggesting that signaling to MAPK was independent of the relative levels of the receptors. EGF dose-response studies (Fig. 1B) revealed that both EGFR or coexpressed K721M-ErbB2 activate MAPK at 10 pM EGF, with the EGFR-induced signal being slightly stronger (compare *lanes 2 and 7*). Maximal MAPK stimulation was observed at 100 pM EGF for both EGFR and K721M-ErbB2 (*lanes 3 and 8*), indicating that physiologically relevant concentrations of EGF can activate MAPK in cells coexpressing K721M and ErbB2.

ErbB2 Kinase Activity but Not Its Tyrosine Phosphorylation Is Essential for EGF-induced MAPK Activation—To determine whether ErbB2 tyrosine kinase activity was obligatory for this signaling, we introduced an aspartic acid to asparagine muta-

Signaling by an EGFR-ErbB2 Oligomer
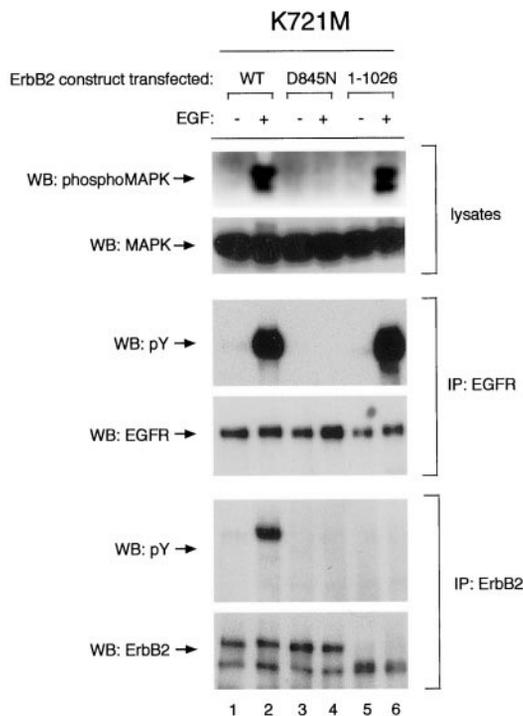
K721M

FIG. 2. **The role of ErbB2 kinase activity and tyrosine phosphorylation sites in EGF-stimulated MAPK activation by coexpressed K721M-ErbB2.** Cells were cotransfected with K721M and either wild-type ErbB2 (*WT*) (*lanes 1–2*), ErbB2 D845N (*lanes 3–4*), or ErbB2-(1–1026) cDNA (*lanes 5–6*). Cells were stimulated with EGF, and MAPK activation was evaluated by Western blotting (*WB*) as in Fig. 1. Phosphotyrosine content (*pY*) and expression of ErbBs were analyzed by immunoprecipitation (*IP*) followed by Western blotting.

tion into the catalytic domain of ErbB2 (D845N). This mutation abolished the ability of ErbB2 to support MAPK stimulation by coexpressed K721M (Fig. 2, *lanes 3–4*), demonstrating the requirement for the ErbB2 kinase activity. The C-terminal region of ErbB2 contains 6 potential tyrosine phosphorylation sites positioned at 1023, 1127, 1139, 1196, 1222, and 1249, which can function as docking sites for signal-transducing proteins (1). To explore the role of ErbB2 tyrosine phosphorylation in supporting K721M signaling, a C-terminal-truncated form of ErbB2 comprising residues 1–1026 was coexpressed with K721M. This truncated ErbB2 was able to mediate EGF-induced MAPK activation and tyrosine phosphorylation of K721M while not being tyrosine phosphorylated itself (Fig. 2, *lane 6*). Identical results were obtained when the remaining tyrosine at position 1023 was converted to phenylalanine in this truncated ErbB2 (data not shown). Further, the D845N ErbB2 mutant did not elicit phosphorylation of either K721M or itself in response to EGF (*lane 4*).

A Requirement for the Mutated Kinase Domain of K721M but Not for K721M Tyrosine Phosphorylation, in EGF-stimulated MAPK Activation—The C-terminal region of EGFR contains 6 potential tyrosine phosphorylation sites positioned at 992, 1068, 1086, 1114, 1148, and 1173 (1). C-terminal truncations of K721M at either residue 1000 or 973 had no detectable effect on ErbB2-dependent MAPK activation by EGF, but did eliminate tyrosine phosphorylation of the truncated K721M receptors (Fig. 3, compare *lane 2* with *4* and *6*). In contrast, ErbB2 tyrosine phosphorylation was unaffected by the truncation of K721M at residues 1000 or 973. However, truncation of the EGFR on the cytoplasmic side of the transmembrane region obliterated all detectable signaling (*lane 8*). c-Src has been shown to phosphorylate the EGFR at Tyr-845 (28). Nevertheless, mutation of Tyr-845 to Phe within K721M-(1–973) had no
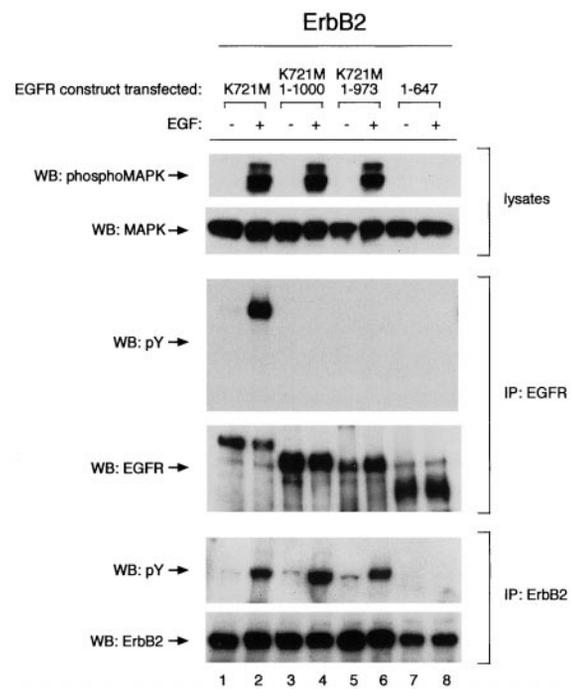
ErbB2

FIG. 3. **EGF-dependent signaling by ErbB2 and truncated K721M.** *A*, 32D cells were cotransfected with ErbB2 cDNA and either K721M (*lanes 1–2*), K721M-(1–1000) (*lanes 3–4*), K721M-(1–973) (*lanes 5–6*), or EGFR-(1–647) cDNA (*lanes 7–8*). Cells were stimulated with EGF, and MAPK activation and phosphotyrosine content (*pY*) were evaluated by Western blotting (*WB*) as in Fig. 2.

detectable effect on the EGF-induced MAPK activation, which is mediated by ErbB2 (data not shown). Thus, whereas tyrosine phosphorylation of K721M is not a requisite for the EGF-stimulated MAPK activation via ErbB2, the mutated kinase domain of K721M (residues 648–973) is required.

Tyrosine Phosphorylation of at least One of the Receptors Is Necessary for Maximal MAPK Stimulation by EGF—As shown in Figs. 2 and 3, removal of the C-terminal region of either K721M or ErbB2 did not influence EGF-stimulated MAPK activation. Thus, we tested the effect of simultaneous deletions of the C-terminal regions of K721M and ErbB2 in this signaling system (Fig. 4). Truncation of K721M at residue 973 resulted in a complete loss of its tyrosine phosphorylation and a dramatic reduction in MAPK activation in cells coexpresssing ErbB2-(1–1026) (*lanes 2* and *4*). The tyrosine residue in oncogenic *neu* (rat ErbB2), which corresponds to position 1023 in human ErbB2, is a negative regulator of *neu*-mediated transformation (29). We tested whether mutation of Tyr-1023 to Phe in ErbB2-(1–1026) increased EGF-induced MAPK stimulation by K721M-(1–973). However, this mutation did not restore MAPK activation by EGF (*lane 6*). These data demonstrate that tyrosine phosphorylation of either K721M or ErbB2 is required for maximal MAPK activation by EGF.

EGF Is a Potent Activator of MAPK and Akt in 32D Cell Lines Stably Coexpressing ErbB2 and Kinase-inactive EGFR—To further investigate the EGF-stimulated signaling by coexpressed K721M and ErbB2, we generated cell lines that stably expressed these receptors. 32D cells were transfected with K721M cDNA, selected in G418, and the clones obtained by serial dilution were screened for surface expression of K721M by flow cytometry analysis (data not shown) and Western blotting (Fig. 5A, *lower panel*). Three independent clones, namely 9.10, 9.11, and 9.16, were selected, and consistent with the results obtained in transient expression assays displayed in Fig. 1, these K721M clones were incapable of activating MAPK in response to EGF (Fig. 5A, *lanes 6, 8,* and *10*). However,
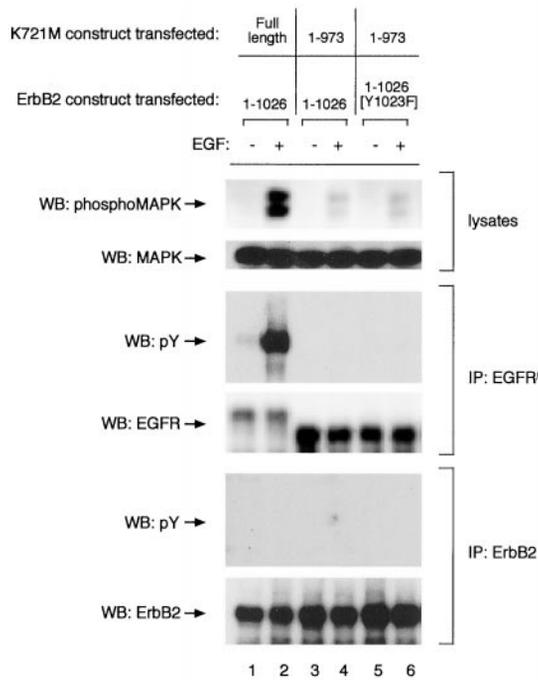
FIG. 4. **EGF-induced signaling by ErbBs lacking tyrosine phosphorylation sites.** 32D cells were cotransfected with K721M and ErbB2-(1–1026) cDNA (*lanes 1–2*), or K721M-(1–973) with either ErbB2-(1–1026) (*lanes 3–4*), or [Y1023F]ErbB2-(1–1026) (*lanes 5–6*). Cells were stimulated with EGF, and MAPK activation and phosphotyrosine content (*pY*) were evaluated by Western blotting (*WB*) as described in the legend to Fig. 2.

transient expression of ErbB2 resulted in reconstitution of MAPK stimulation by EGF in all three clones (*lanes 12, 14,* and *16*), demonstrating that K721M in these clones was functional. As a control for these experiments, we utilized 32D cells stably expressing human EGFR (*lanes 1–2*; Refs. 25–27).

Clone 9.10 was then transfected with a vector containing ErbB2 cDNA and a zeocin-resistance marker to generate 5 stable clones that simultaneously expressed K721M and varying amounts of ErbB2 (Fig. 5*B*, *lanes 7–16*). EGF activated MAPK in all five cell lines consistent with results obtained in transient expression assays (Figs. 1–3). We then evaluated the EGF-induced activation of the protein kinase Akt, which plays an important role in cell survival (30). Because activation of Akt results from phosphorylation at Thr-308 and Ser-473, we monitored activation using antibodies that specifically recognize these two phosphorylated sites (Fig. 5*B*). Treatment of all stable cell lines coexpressing K721M and ErbB2 with EGF elicited phosphorylation of Akt on Thr-308 and Ser-473 to levels comparable with cells stably expressing EGFR (*lanes 2, 8, 10, 12, 14,* and *16*).

EGF Cannot Substitute for IL-3 in the Proliferation of 32D Cells Coexpressing K721M-ErbB2 but Delays the Onset of Apoptosis—Withdrawal of IL-3 from 32D cells leads to the accumulation of cells in G_1 and rapid apoptosis (31). However, as seen in Fig. 6*A* and as previously demonstrated (25), expression of EGFR in 32D cells allows EGF to supplant the IL-3 requirement. Thus, it was important to evaluate the mitogenic and anti-apoptotic potential of EGF in cells expressing K721M alone or in the presence of ErbB2. EGF had no discernable effect on 32D cells expressing K721M alone (clone 9.10), which, like the parental cells, underwent rapid apoptosis upon IL-3 deprivation (Fig. 6*B*). Identical findings were obtained with clones 9.11 and 9.16, which also harbor K721M (data not shown). Treatment of clone 7.1, which coexpresses K721M and ErbB2, with EGF resulted in a delayed onset of apoptosis
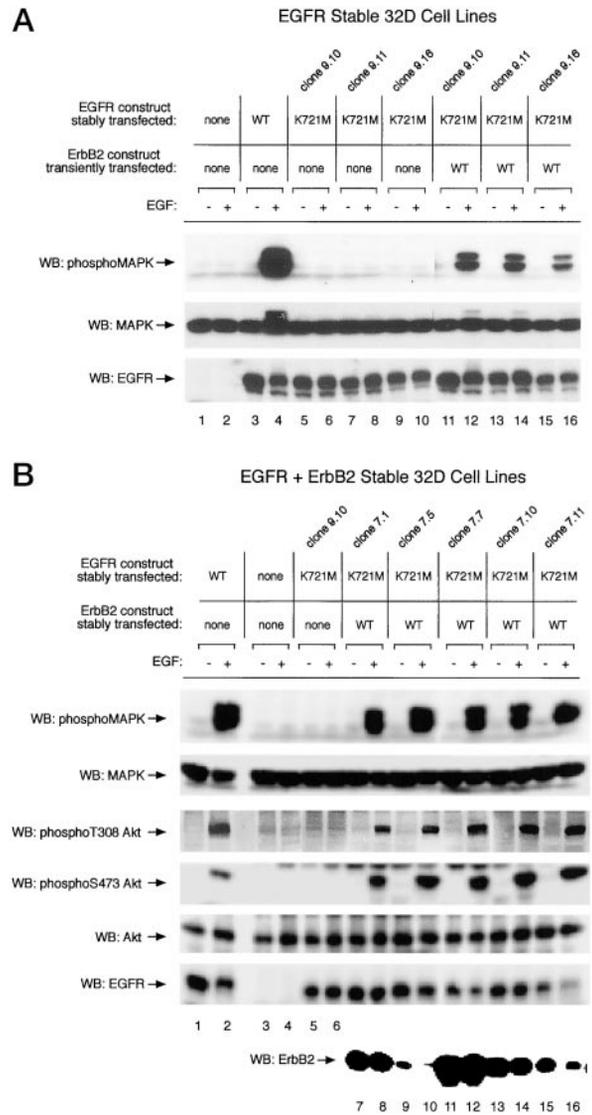
FIG. 5. **Evaluation of EGF-dependent signaling in stable 32D cells expressing K721M alone or coexpressing K721M and ErbB2.** *A,* 32D cells (*lanes 1–2*) or cells stably expressing wild-type EGFR (*WT*) (*lanes 3–4*) or clones expressing K721M (*lanes 5–10*) were exposed to 10 nM EGF, and MAPK activation was evaluated as described in the legend to Fig. 1. In *lanes 11–16* clones were transiently transfected with ErbB2 cDNA. Expression of EGFRs was evaluated by Western blotting (*WB: EGFR*)**.** *B,* 32D cells (*lanes 3–4*) or cells stably expressing EGFR (*lanes 1–2*), K721M (*lanes 5–6*) or various clones coexpressing K721M and ErbB2 (*lanes 7–16*) were stimulated with EGF. Akt activation was monitored by Western blotting (*WB*) analysis of lysates using antibodies against the phosphorylated form of Akt (*phosphoS473* and *phosphoT308 Akt*). Consistent loading of each lane was confirmed by stripping the blot and reprobing for Akt, and expression of ErbBs was confirmed by Western blotting.

compared with untreated cells (Fig. 6*C*). After 24 h of exposure to EGF, clone 7.1 cells were still viable, but underwent apoptosis during the subsequent 24 h period. This finding was consistent in all five of the 32D clones, which express both K721M and ErbB2 (data not shown). Therefore, unlike EGFR, the activated K721M-ErbB2 receptors are incapable of generating a proliferative response in the 32D cell context.

One explanation for this difference could be that EGF induces the expression and secretion of IL-3 in cells, which express EGFR, but not in cells which express K721M and ErbB2. To test the hypothesis that IL-3 may function as an autocrine mitogen for EGF-treated EGFR 32D cells, we harvested conditioned medium from EGF-treated EGFR cells and attempted to grow the parental ErbB-devoid 32D cells in this medium. We
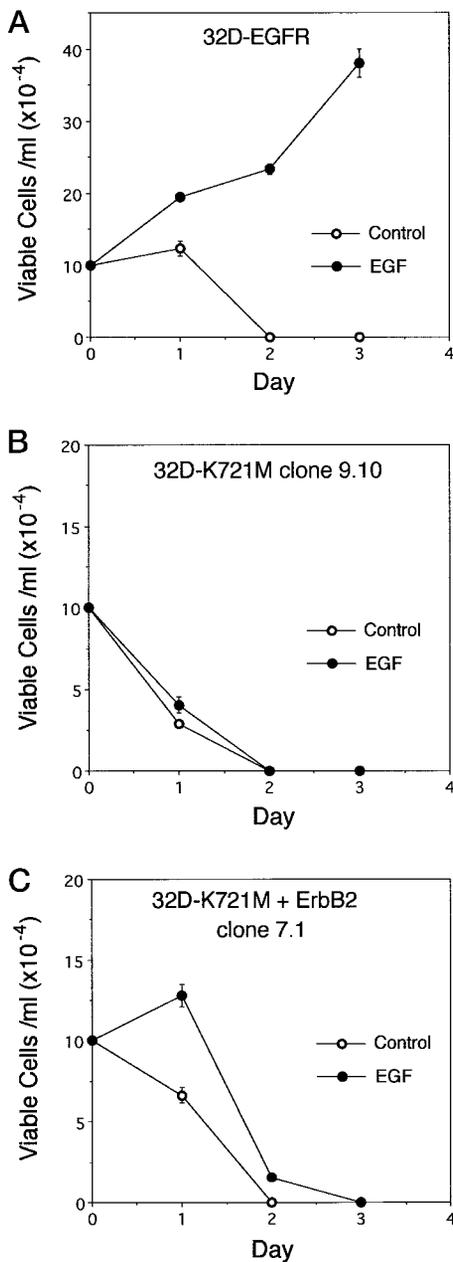
FIG. 6. **Analysis of EGF-induced proliferation of stable 32D cells.** Stable 32D cells expressing either EGFR (*A*), K721M (clone 9.10) (*B*) or K721M and ErbB2 (clone 7.1) (*C*) at a density of 100,000 cells/ml were exposed to basal medium with or without 10 nM EGF. Proliferation assays were performed as described under "Experimental Procedures." The results represent the mean ± S.E. of experiments performed in triplicate.

observed no proliferative response in 32D cells to this conditioned medium (data not shown). We also monitored the effect of anti-IL-3 neutralizing antibodies on the EGF-stimulated growth of EGFR 32D cells. Whereas these antibodies blocked the mitogenic response of the EGFR cells to exogenous IL-3, they had no effect on the EGF-induced growth (data not shown). Therefore, the proliferation of the EGFR cells by EGF is not because of an IL-3-mediated autocrine loop.

The Kinetics of MAPK and Akt Activation Is Virtually Identical in Cells Expressing EGFR or K721M-ErbB2, but EGF Is Incapable of Activating STATs in K721M-ErbB2 cells—Two additional potential explanations for the lack of an EGF-induced proliferative response in K721M-ErbB2 cells were explored. First, it was possible that the activation of MAPK and Akt in these cells was transient and thus, not sufficient to drive

cell division. Interestingly, a kinetic analysis in cells expressing EGFR or cells expressing K721M and ErbB2 (clone 7.1) revealed a strong and prolonged activation of Akt and MAPK by EGF that persisted for 24 h for MAPK and 8 h for Ser-473 of Akt (Fig. 7*A*). Phosphorylation of Akt on Thr-308 was more transient and decreased to baseline levels after 30 min of exposure to EGF (*lanes 1–4* and *11–14*). Within these parameters signaling by 32D cells possessing EGFR is virtually indistinguishable from those harboring kinase-inactive K721M simultaneously with ErbB2. Therefore, activation of MAPK and Akt does not provide an explanation for the inability of K721M-ErbB2 cells to grow in response to EGF.

Secondly, activation of STAT5 is critical to the survival and growth of myeloid cells (32, 33). Previous work from our laboratory had demonstrated that EGFR ligands activate STATs 1, 3, and 5 in an EGFR kinase-dependent, Janus kinase (Jak)1-independent manner (34). To test whether EGF was able to activate STATs in cells simultaneously expressing K721M and ErbB2, we utilized antibodies specific for the phosphorylated, activated forms of STAT1 (pY701), STAT3 (pY705), and STAT5 (pY694/pY699) (Fig. 7*B*). Evaluation of lysates derived from clone 7.1 cells treated with EGF for 10 min indicated that the K721M-ErbB2 receptors were not capable of activating any of the STATs (*lane 4*). Exposure of these cells to EGF for 5 or 30 min also did not result in any detectable STAT activation, and identical results were obtained using the clone 7.5 (data not shown). Further, IL-3 was able to activate STATs 1, 3, and 5 in both clone 7.1 and 7.5 (data not shown). Activation of STATs 1, 3, and 5 was easily detected in the 32D cells expressing EGFR (*lane 2*). Therefore, there was a correlation between EGF-induced proliferation and activation of STATs in the cell lines expressing either EGFR or K721M-ErbB2.

DISCUSSION

EGF-induced Signaling by Kinase-inactive EGFRs Is Mediated by ErbB2—The research presented here better defines the function and requirement of the EGFR kinase activity in EGF signaling. Early work indicated that the EGFR kinase was required for EGF-induced biological responses (9, 10). In contrast, additional studies revealed that kinase-inactive forms of the EGFR have the capacity to signal in an EGF-dependent manner when ectopically expressed in certain cells. Kinase-inactive EGFRs have been reported to stimulate MAPK, c-*fos* gene expression, and/or DNA synthesis (11–16). Several possible explanations for this signaling have been proposed and include the following. (i) The dimerized kinase-inactive EGFR alone has the intrinsic ability to signal, possibly through the recruitment of additional signaling proteins. (ii) Signaling by ectopically expressed kinase-inactive EGFRs occurs through a process of signal amplification, which is initiated by undetectable low levels of endogenous kinase-active EGFRs, and (iii) signaling occurs because of an interaction with another kinase, such as an ErbB-like protein. Our findings indicate that in ErbB-devoid 32D cells, kinase-inactive EGFRs have no intrinsic signaling capacity. Instead, we present strong evidence that signaling by kinase-inactive EGFRs is mediated by ErbB2 but not by the related ErbB3 and ErbB4. Further, using a signaling model of EGFR-ErbB2 oligomers in the absence of kinase-active EGFR homodimers, we demonstrate that the EGFR·ErbB2 complex is a strong activator of MAPK and the pro-survival protein kinase Akt, and that ErbB2, but not EGFR, kinase activity was a requisite for this process.

The Potential Physiological Relevance of Signaling by a Kinase-inactive EGFR—Targeted disruption of the murine *EGFR* gene results in lethality (17–19), whereas *waved-2* mice, which possess an EGFR whose kinase activity is significantly impaired, are healthy and viable (20). These findings provide
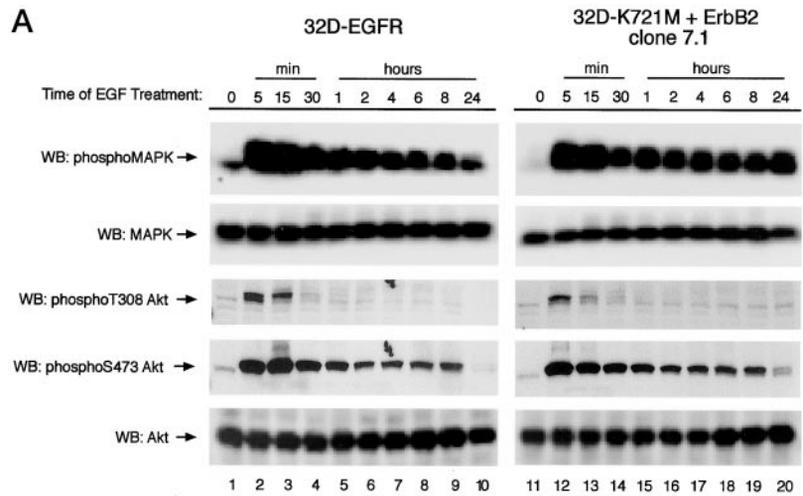
FIG. 7. **Kinetics of EGF-dependent MAPK and Akt activation by EGFR and K721M-ErbB2 32D cells.** *A,* stable 32D cells expressing EGFR or K721M and ErbB2 (clone 7.1) were stimulated with 10 nM EGF. MAPK and Akt activation were evaluated by Western blotting (*WB*) as described in the legends to Figs. 1 and 5. *B,* STAT activation by EGF in 32D cells. Cells stably expressing EGFR or coexpressing K721M and ErbB2 (*clone 7.1*) were treated with 10 nM EGF for 10 min at 37 °C, lysed, and STAT activation was monitored by Western blotting (*WB*) using antibodies against the phosphorylated form of each STAT (*phosphoSTAT 1, phosphoSTAT 3, phosphoSTAT 5*). Consistent loading of each lane was confirmed by stripping the blot and reprobing for the respective STAT.
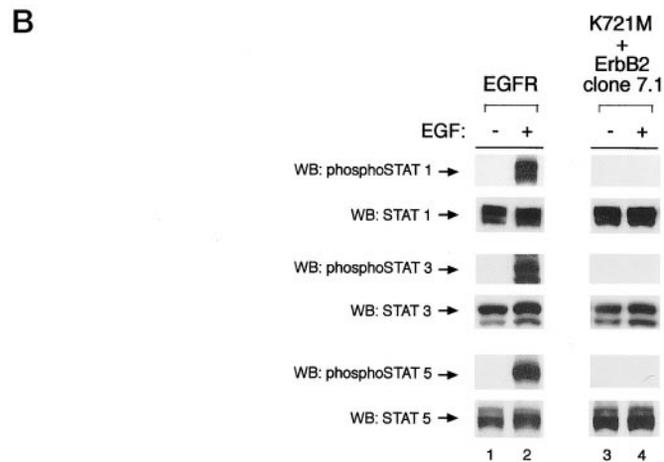
strong evidence that a significant component of EGFR signaling is independent of EGFR kinase activity. Of all ErbBs, ErbB2 is considered to be the most widely expressed and is almost always coexpressed with EGFR in epithelial cells and fibroblasts. Our *in vitro* experiments demonstrated that the K721M-ErbB2 oligomer is exquisitely sensitive to ligand because physiological concentrations of EGF (10 pM) readily activated MAPK (Fig. 1*B*). Taken together, one might expect ErbB2 to play a significant role in certain aspects of EGFR signaling *in vivo*.

EGF Cannot Drive Proliferation of 32D Cells Coexpressing K721M and ErbB2—Biochemical analysis of signaling through K721M-ErbB2 oligomers in 32D cells showed that EGF activated Ras (data not shown), MAPK and Akt, but not STATs 1, 3, and 5. However, EGF could not supplant the growth requirement of these cells for IL-3. Conversely, EGF was mitogenic in 32D cells expressing wild-type EGFR as has been previously shown (25). The finding that the K721M-ErbB2 oligomer is incapable of mediating proliferation of these cells extends previous work demonstrating that overexpression of ErbB2 was not able to induce proliferation of 32D cells, despite the fact that similar ErbB2 overexpression resulted in potent transformation of fibroblasts (26). This difference in the ability of EGFR and ErbB2 to generate a mitogenic signal in 32D cells was attributed to their respective kinase domains, demonstrating cell context specificity for mitogenic signaling by EGFR and ErbB2. In our studies, the kinetics and magnitude of activation of the MAPK and Akt pathways by 32D cells expressing either K721M-ErbB2 or EGFR were virtually indistinguishable (Fig. 7*A*) indicating that defects in these pathways could not explain the differential growth response of the cells to EGF. Additionally, the secretion of an EGF-induced autocrine growth factor, such as IL-3, was ruled out as a reason for the proliferative response in EGFR cells. The inability of the K721M-ErbB2 complex to activate STAT transcription factors in response to EGF, however, may provide an explanation for the lack of a mitogenic response in 32D cells. EGF activates STATs 1, 3 and 5 via an EGFR kinase-dependent, EGFR autophosphorylation- and Jak1-independent mechanism, which appears to require c-Src (16, 34, 35). Activation of STAT5 is essential in the survival and proliferation of myeloid cells (32). Expression of an EGFR-Jak2 chimera in 32D cells results in EGF-dependent tyrosine phosphorylation of STAT 5 and cell proliferation (33). Thus, the inability of EGF-activated K721M-ErbB2 oligomers to elicit proliferation in 32D cells may be related to the lack of STAT5 activation.

The Data Are Inconsistent with Signaling by a Kinase-inactive EGFR-ErbB2 Heterodimer—It is believed that transmodulation of ErbB2 by EGF occurs via the generation of an EGFR-ErbB2 heterodimer (1–3, 5). More recent data suggested that ErbBs may be activated by a heterotetrameric mode of receptor kinase interaction (36, 37). Biophysical studies of ligand-induced dimerization of the soluble extracellular domains of ErbBs were unable to demonstrate the existence of EGFR-ErbB2, ErbB2-ErbB3, or ErbB3-ErbB3 dimers, but were able to observe EGFR-EGFR, ErbB4-ErbB4, and ErbB2-ErbB4 dimers (38) causing the authors to question the prevailing heterodimerization model of ErbB activation. Our findings are more consistent with a model of EGF-bound K721M homodimers, which recruit and activate ErbB2 homodimers, and are inconsistent with signaling by K721M-ErbB2 heterodimers for several reasons. First, removal of all tyrosine phosphoryla-

tion sites in K721M had no effect on EGF-stimulated MAPK activation. If transphosphorylation of K721M by ErbB2 in a heterodimeric complex is critical to transmodulation, as required in the heterodimer model, then tyrosine phosphorylation acceptor sites should be required, at least to some extent. Secondly, EGF-induced tyrosine phosphorylation of ErbB2 occurs in the absence of EGFR kinase activity. Both activation of MAPK and tyrosine phosphorylation of ErbB2 by EGF requires ErbB2 kinase activity. The simplest explanation for this observation is that a K721M homodimer presents an interface for an interaction with a monomeric ErbB2, which upon engagement, recruits an additional ErbB2 to form a $[K721M]_2[ErbB2]_2$ heterotetramer. Within this complex one ErbB2 can transphosphorylate the other in the activated homodimer. Of course, we cannot rule out that an EGF-activated heterologous kinase phosphorylates ErbB2, but this kinase would require ErbB2 kinase activity for activation. Our data demonstrate a requirement for the mutated kinase domain of K721M (residues 648–973) in K721M-ErbB2 signaling. Assuming that the truncated EGFR possessing the extracellular and transmembrane domains can dimerize in response to EGF, it suggests that the mutated kinase domains of dimerized K721M contribute to the formation of an interface for ErbB2 recruitment. Tyrosine phosphorylation of at least one of the ErbBs in the oligomeric complex is necessary for activation of MAPK, presumably because this will provide docking sites for adaptor proteins such as GRB2 and SHC, which can localize the guanine nucleotide exchange factor, son-of-sevenless, to the plasma membrane where it can activate Ras. Importantly, focal stimulation of cells with EGF results in an extensive propagation of ligand-independent receptor phosphorylation over the entire cell surface (6). It is possible that EGF-bound K721M homo-oligomers inititate a similar propagation of receptor activation which involves ErbB2.

Conclusions—The work reported here provides an explanation of how a kinase-inactive EGFR can generate an EGF-dependent signal and implies that certain aspects of EGF signaling may be EGFR kinase-independent *in vivo*. Further, these results underscore the importance of ErbB2 in EGF signaling and allow us to attribute specific signaling events to the EGF-activated EGFR-ErbB2 oligomer. We found that the EGF-stimulated EGFR-ErbB2 complex is a potent activator of MAPK and Akt, and ErbB2, but not EGFR, kinase activity is required for this signaling.

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