Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8132–8136, August 1994
Biochemistry

Insect cell-expressed p180^{erbB3} possesses an impaired tyrosine kinase activity

PAMELA M. GUY*, JILL V. PLATKO*, LEWIS C. CANTLEY†, RICHARD A. CERIONE*, AND KERMIT L. CARRAWAY III†‡

*Department of Pharmacology, Cornell University, Ithaca, NY 14853; and †Department of Cell Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Hospital, Boston, MA 02115

Communicated by Leon A. Heppel, April 18, 1994 (received for review November 12, 1993)

ABSTRACT **Protein kinases share a number of highly conserved or invariant amino acid residues in their catalytic domains, suggesting that these residues are necessary for kinase activity. In p180^{erbB3}, a receptor tyrosine kinase belonging to the epidermal growth factor (EGF) receptor subfamily, three of these residues are altered, suggesting that this protein might have an impaired protein tyrosine kinase activity. To test this hypothesis, we have expressed human EGF receptor and bovine p180^{erbB3} in insect cells via baculovirus infection and have compared their autophosphorylation and substrate phosphorylation activities. We have found that, while the EGF receptor readily undergoes EGF-stimulated autophosphorylation and catalyzes the incorporation of phosphate into the model substrates $(E_4Y_1)_n$ (random 4:1 copolymer of glutamic acid and tyrosine) and GST-p85 (glutathione *S*-transferase fusion protein with the 85-kDa subunit of phosphatidylinositol 3-kinase), p180^{erbB3} autophosphorylation and substrate phosphorylation are at least 2 orders of magnitude less efficient. However, p180^{erbB3} is capable of binding the ATP analog 5′-*p*-fluorosulfonylbenzoyladenosine, indicating that the lack of observed kinase activity is probably not due to nonfunctional or denatured receptors expressed by the insect cells. On the basis of these results, we propose that p180^{erbB3} possesses an impaired intrinsic tyrosine kinase activity.**

Growth factor receptors of the class I subfamily, which include the epidermal growth factor (EGF) receptor, p185$^{erbB2/neu}$, p180^{erbB3}, and p180^{erbB4} proteins, are believed to direct cellular growth through the ligand-stimulated tyrosine phosphorylation of intracellular substrates (1, 2). Amplification of the genes encoding these proteins has been observed in a variety of human carcinomas, and it has been reported that the overexpression of p185$^{erbB2/neu}$ is associated with a poor prognosis of breast and ovarian cancer patients (3, 4). These observations suggest that members of the class I receptor subfamily may contribute to the growth or malignancy of some human tumors.

The erbB3 cDNA was originally identified in libraries from several human cancer cell lines by low-stringency screening with probes corresponding to other members of the class I receptor family (5, 6). Expression of the cloned erbB3 cDNA in COS cells followed by immunoblotting with peptide antibodies yielded a single band of ≈160 kDa (5). More recent studies indicated that *erbB3* expression in human cancer cells and transfected NIH 3T3 mouse fibroblasts produces an ≈180-kDa protein, which has been termed p180^{erbB3} (7). These studies also indicated that p180^{erbB3} is overexpressed in a number of human breast cancer cell lines and that the receptor is constitutively tyrosine-phosphorylated in a subset of these. Our very recent studies demonstrate that p180^{erbB3} is a receptor for the growth factor heregulin (HRG) and that HRG stimulates tyrosine kinase activity in NIH 3T3 cells transfected with the bovine cDNA encoding p180^{erbB3} (22).

Despite these observations, questions have arisen regarding the intrinsic protein tyrosine kinase activity of p180^{erbB3}. These questions stem from the observation that several amino acid residues that are highly conserved in the catalytic domains of protein kinases (8) are altered in the p180^{erbB3} catalytic domain (5, 6). Particularly noteworthy is the substitution of asparagine for aspartic acid at residue 834 of the predicted human p180^{erbB3} sequence (6) (residue 833 of the bovine sequence). The corresponding residue is located in the active site of protein kinase A (9) and is essential for biological activity (10). Moreover, identical point mutations in the c-*kit*- and v-*fps*-encoded tyrosine kinases abolish activity (11, 12). This raises the possibility that the EGF receptor-like protein p180^{erbB3} lacks an intrinsic protein tyrosine kinase activity (13).

In the study described here, we have expressed bovine p180^{erbB3} in insect cells via baculovirus infection and compared its autophosphorylation and tyrosine kinase activities with insect cell-expressed EGF receptor. We find that although p180^{erbB3} is capable of binding ATP and HRG, its catalytic activity is at least 2 orders of magnitude less than that of the EGF receptor.

MATERIALS AND METHODS

Antibodies. Polyclonal antiserum K1 to p180^{erbB3} was the gift of J. Koland (University of Iowa). Anti-EGF receptor monoclonal antibody 291-3A was provided by R. Schatzman (Syntex, Palo Alto, CA). Anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-EGF receptor monoclonal antibody 13A9 was obtained from Genentech. Polyclonal antiserum raised to FSBA was provided by R. Gaehlen (Purdue University). Polyclonal antiserum 3183 was raised in rabbits against the synthetic peptide Cys-Glu-Leu-Glu-Pro-Glu-Leu-Asp-Leu-Asp-Leu-Asp-Leu-Glu (from the p180^{erbB3} tail region) coupled to keyhole limpet hemacyanin through its cysteine residue. Anti-peptide antibodies were affinity-purified with immobilized peptide prior to their use in immunoprecipitation experiments.

Cloning of Bovine p180^{erbB3} and Expression of Human EGF Receptor and p180^{erbB3} in Insect Cells. An ≈500-bp fragment of p180^{erbB3} cDNA was inadvertently cloned as a side product of an independent polymerase chain reaction with a bovine brain cDNA library template. This fragment was then used to isolate a cDNA corresponding to the entire coding region of bovine p180^{erbB3}, which was sequenced and exhibited 94%

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

Abbreviations: EGF, epidermal growth factor; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; HRG, heregulin; $(E_4Y_1)_n$, a random copolymer of glutamic acid and tyrosine in a 4:1 ratio, or poly ($Glu^{80}Tyr^{20}$); GST-p85, glutathione *S*-transferase fusion protein with the 85-kDa subunits of phosphatidylinositol 3-kinase.

‡To whom reprint requests should be addressed.

amino acid identity with the human protein (5, 6) over the entire open reading frame. The human EGF receptor and bovine $p180^{erbB3}$ cDNAs were inserted into the pAcYMP1 insect-cell transfer vector (14) by standard recombinant DNA methods. Recombinant viruses encoding the EGF receptor and $p180^{erbB3}$ proteins were prepared by using the BaculoGold kit (PharMingen) according to the directions of the manufacturer and were plaque-purified prior to use in protein production. Sf9 cells were infected with various viruses and harvested after 48–72 hr. Purification of plasma membranes and phosphorylation of the polymer substrate $(E_4Y_1)_n$ [random 4:1 copolymer of glutamic acid and tyrosine, or poly($Glu^{80}Tyr^{20}$); Sigma] by membranes were carried out as described (14). Membranes obtained from cells infected with the different viruses were normalized to protein concentration.

Immunoprecipitations and Tyrosine Kinase Assays. For immunoprecipitation experiments, 10^7 infected cells were lysed in 1 ml of 20 mM sodium Hepes, pH 7.4/1% Triton X-100/150 mM NaCl/1 mM EDTA/50 μM phenylmethylsulfonyl fluoride/1 μg each of leupeptin, aprotinin, and pepstatin A per ml. One hundred microliters of the cleared lysate was removed, and the remaining 900 μl was treated with 1.6 μg of either 13A9 or 3183 antibody to form immunoprecipitates, which were either analyzed by immunoblotting or divided into four equal aliquots for use in phosphorylation reactions.

Phosphorylations by immunoprecipitates were carried out in a 100-μl volume in the presence of 5 mM $MnCl_2$ and 20 μM [γ-^{32}P]ATP (≈3000 Cerenkov cpm/pmol) for 10 min at room temperature. Reactions were stopped with the addition of 50 μl of 5× sample buffer (23) and proteins were resolved by SDS/7% PAGE. Where indicated, the model substrates $(E_4Y_1)_n$ and GST-p85 (glutathione *S*-transferase fusion protein with the 85-kDa subunit of phosphatidylinositol 3-kinase; purified with glutathione agarose as described in ref. 24) were present in the phosphorylation reactions at concentrations of 0.5 mg/ml and 0.05 mg/ml, respectively. Phosphorylated bands were excised from the dried gels, and the associated radioactivity was determined by Cerenkov counting. For $(E_4Y_1)_n$ quantification, a segment of the gel lane corresponding to the 43-kDa to 105-kDa region was excised.

Labeling. ATP binding proteins in purified plasma membranes were labeled with 100 μM 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA; Sigma) for 10 min at room temperature in the presence and absence of 1 mM ATP. Labeled proteins were detected after SDS/7% PAGE by blotting with affinity-purified antibodies to FSBA. Affinity purification of anti-FSBA antibodies with ATP-agarose was carried out as described (15).

RESULTS

The infection of insect cells with recombinant baculovirus affords the high-level expression of foreign sequences and has been used (16) in the expression of functional growth factor-responsive EGF receptor. To test the hypothesis that $p180^{erbB3}$ possesses an impaired kinase activity, we compared the autophosphorylation and tyrosine kinase activities of insect cell-expressed human EGF receptor and bovine erbB3. Using ^{125}I-labeled EGF (^{125}I-EGF) and ^{125}I-labeled HRGβ (^{125}I-HRGβ1), we have determined that the insect cell expression levels for EGF receptor and erbB3 are 55,000 (unpublished observations) and 40,000 receptors per cell (K.L.C., unpublished data), respectively, under the conditions employed. Hence, the extent of phosphorylation of receptors in purified plasma membranes and whole-cell lysates from insect cells expressing these proteins is directly comparable.

Tyrosine Kinase Activities of Receptors in Purified Plasma Membranes from Infected Insect Cells. Sf9 cells were either not infected (Fig. 1, lane 1) or infected with baculovirus encoding EGF receptor (Fig. 1, lane 2) or erbB3 (Fig. 1, lane 3). Purified plasma membranes from these cells containing equal amounts of protein were then examined for expression of the receptors by immunoblotting with anti-EGF receptor monoclonal antibody 291-3A or anti-erbB3 polyclonal antibody K1. Fig. 1*A Top* shows that the Sf9 cells infected with recombinant baculovirus encoding EGF receptor expressed this protein as a doublet of 158 kDa and 140 kDa. Previous studies have shown that Sf9 cells express the EGF receptor as a ≈155 kDa glycoprotein (16). The relative amount of the 140-kDa protein observed varied considerably from one preparation to the next (not shown), suggesting that it may be a degradation product of the full-length 158-kDa form. Cells infected with virus encoding erbB3 expressed this protein as a single band of 168 kDa when analyzed by SDS/7% PAGE (Fig. 1A *Middle*). The observed sizes of the insect cell-expressed receptors were roughly 12 kDa less than their observed sizes in mammalian cells, probably because the insect cells are incapable of transferring complex-type oligosaccharides to foreign cell-surface proteins (16). The apparent discrepancy in the levels of EGF receptor and erbB3 in

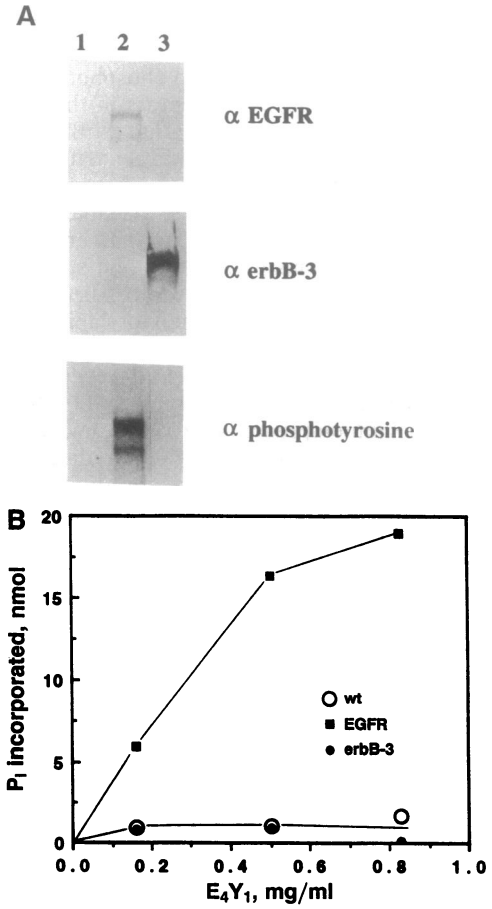

FIG. 1. Expression of EGF receptor and erbB3 in insect cells and detection of tyrosine kinase activity. (*A*) Proteins in purified plasma membranes from uninfected Sf9 insect cells (lane 1) and cells infected with recombinant baculovirus encoding human EGF receptor (lane 2) or bovine erbB3 (lane 3) were phosphorylated in the presence of ATP, resolved by SDS/PAGE, and immunoblotted with antibodies against EGF receptor (α EGFR) (*Top*), erbB3 (α erbB-3) (*Middle*) and phosphotyrosine (α phosphotyrosine) (*Bottom*). (*B*) Membranes from cells infected with wild-type (wt), EGF receptor, and erbB3 viruses were used to phosphorylate various concentrations of the polymer substrate $(E_4Y_1)_n$ in the presence of [γ-^{32}P]ATP.

Fig. 1 probably results from the relatively weak immunoblotting efficiency of the 291-3A antibody (unpublished observations).

To test the autophosphorylation activities of the EGF receptor and erbB3 proteins, we exposed the insect cell plasma membranes to 5 mM $MnCl_2$ and 20 μM ATP and analyzed the phosphotyrosine content of the receptors by immunoblotting with anti-phosphotyrosine antibodies. We observed that, while both bands of the EGF receptor doublet could be detected with anti-phosphotyrosine, the erbB3 band could not be detected (Fig. 1A *Bottom*). It should be noted that our previous studies indicate that the presence of Mn^{2+} in the phosphorylation reaction is sufficient to fully activate the autophosphorylation activities of the EGF receptor (17, 18) and erbB2/neu (14), obviating the need for growth factor activation. However, the presence of saturating levels of HRG, the ligand for $p180^{erbB3}$, was incapable of stimulating erbB3 catalytic activity (not shown).

To test the catalytic activities of the membrane receptors, we examined the phosphorylation of the model substrate $(E_4Y_1)_n$. We have observed previously that this substrate is efficiently phosphorylated by EGF receptors in purified plasma membranes from A431 cells (unpublished observations) and by the tyrosine kinase domain of $p185^{erbB2/neu}$ expressed in insect cells (14). Fig. 1*B* shows that insect cell membranes containing EGF receptor efficiently phosphorylated this substrate. However, membranes prepared from cells infected with either wild-type baculovirus or virus encoding erbB3 showed no ability to phosphorylate $(E_4Y_1)_n$. Since the amounts of the two receptors in the membranes were comparable, these data suggest that erbB3 in insect cell membranes possesses an impaired kinase activity.

Tyrosine Kinase Activities of Receptors in Immune Complex Kinase Assays. To further characterize the kinase activities of the insect cell-expressed EGF receptor and erbB3 proteins, we compared the autophosphorylation and substrate phosphorylation activities of the two receptors in immune complex kinase reactions. We first immunoprecipitated extracts from cells infected with wild-type, EGF receptor-encoding, or erbB3-encoding viruses with anti-EGF receptor (13A9) or anti-erbB3 (3183) antibodies. Substrates and [^{32}P]ATP were then added to immunoprecipitated receptors, and radiolabeled proteins were visualized by autoradiography after SDS/PAGE. Identical immunoprecipitations were performed in parallel to assess the efficiencies of immunoprecipitation by immunoblotting. We observed that both the 13A9 and 3183 antibodies precipitated >80% of their target receptors in the insect cell lysates (not shown). Moreover, staining of the blots with Ponceau S revealed that the amounts of the EGF receptor and erbB3 proteins obtained after immunoprecipitation were comparable (Fig. 2*A*).

Fig. 2*B* shows the autoradiogram corresponding to receptor autophosphorylation and the phosphorylation of $(E_4Y_1)_n$ and GST-p85 by the EGF receptor and erbB3 immunoprecipitates. In each of the 13A9 immunoprecipitates from EGF receptor-expressing cells, we observed the presence of a heavily phosphorylated band at 158 kDa (see lanes 3 and 11), which probably results from EGF receptor autophosphorylation. No phosphorylated band was observed in identical immunoprecipitates from cells infected with wild-type virus (lanes 2 and 10). Phosphorylation of GST-p85 (which migrates as a single band at ≈110 kDa; lane 11) and of $(E_4Y_1)_n$ (which runs as a smear throughout the gel lane; lane 7) was readily detectable in EGF receptor immunoprecipitates. Under identical conditions, no phosphorylation of erbB3, $(E_4Y_1)_n$ or GST-p85 in 3183 immunoprecipitates of erbB3-expressing cells was observed (lanes 4, 8, and 12, respectively). However, when vastly overexposed, a small amount of phosphorylation of each of these proteins was detected in erbB3 immunoprecipitates (not shown).

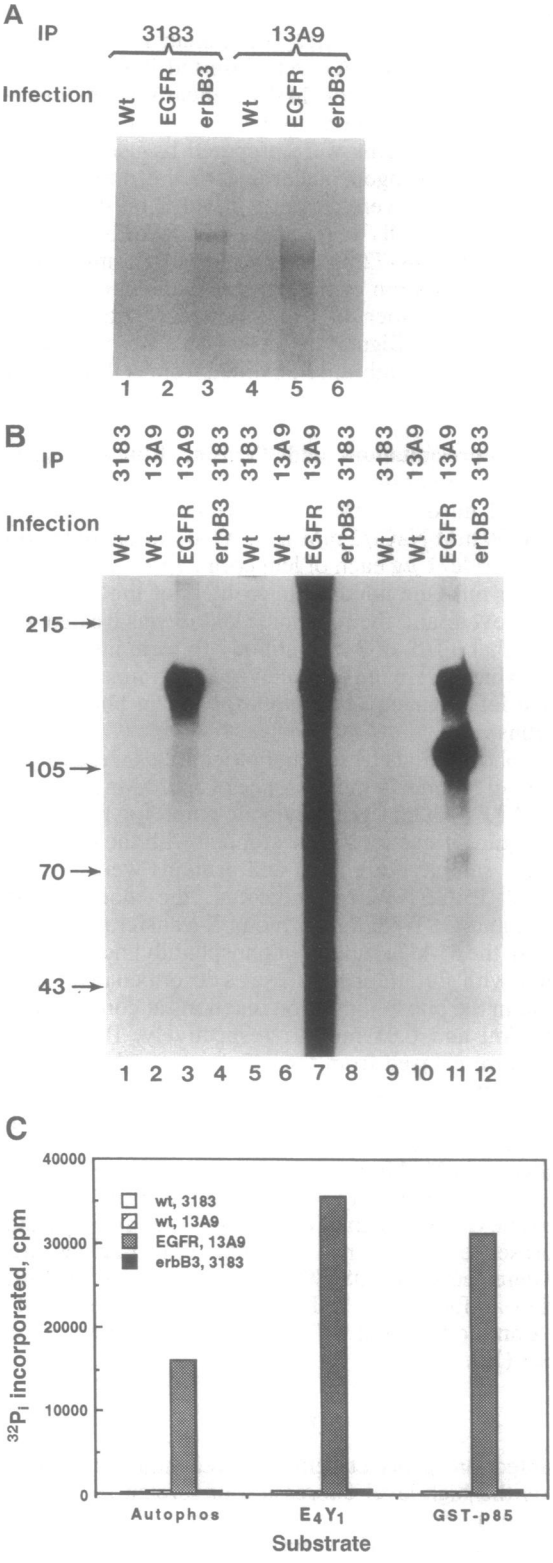

FIG. 2. Immune complex kinase assays of insect cell-expressed EGF receptor and erbB3. Lysates from cells infected with wild-type (wt), EGF receptor-encoding, or erbB3-encoding baculovirus were immunoprecipitated with anti-EGF receptor antibody 13A9 (lanes 1–3) or anti-erbB3 antibody 3183 (lanes 4–6). (*A*) The immunoprecipitated erbB3 and EGF receptor were visualized by staining with Ponceau red. (*B*) The autoradiogram depicts immune complex kinase reactions in the absence of added substrate (lanes 1–4) or in the presence of 0.5 mg of $(E_4Y_1)_n$ (lanes 5–8) or 0.05 mg of GST-p85 (lanes 9–12) per ml. Sizes are shown in kDa. The bands depicted in *A* were excised, and the associated radioactivity was determined by Cerenkov counting. IP, immunoprecipitate.

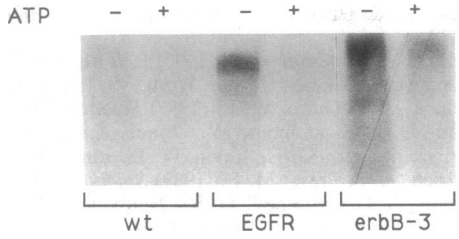

FIG. 3. FSBA labeling of insect cell-expressed EGF receptor and erbB3. Membranes from cells infected with wild type (wt), EGF receptor-encoding, and erbB3-encoding baculovirus were treated with 100 μM FSBA in the presence and absence of 1 mM ATP as indicated. Labeled proteins were resolved by SDS/PAGE, and ATP-binding proteins were detected by immunoblotting with 1 μg of affinity-purified anti-FSBA antibodies per ml, followed by alkaline phosphatase detection.

To quantify the relative kinase activities of the two receptors, the EGF receptor, erbB3, $(E_4Y_1)_n$, and GST-p85 bands in the lanes depicted in Fig. 2*A* were excised, and the associated radioactivity was determined (Fig. 2*C*). Again, we observed that there was a very small but detectable amount of phosphorylation of erbB3, $(E_4Y_1)_n$, and GST-p85 in 3183 immunoprecipitates from erbB3-infected insect cells relative to identical immunoprecipitates from cells infected with wild-type virus. However, the autophosphorylation and substrate phosphorylation activities of the EGF receptor far exceeded those for erbB3. Given that the quantities of the receptors in the immunoprecipitates were roughly equivalent, we estimate that the stoichiometry of EGF receptor phosphorylation was 65- to 120-fold greater than that for erbB3 and that the phosphorylation of the $(E_4Y_1)_n$ and GST-p85 substrates by the EGF receptor was 125- to 160-fold greater than by erbB3. It should be noted that we estimate the stoichiometry of EGF receptor autophosphorylation in the immune complex kinase reactions to be $\approx$3 mol of P_i per mol of receptor and the turnover number for $(E_4Y_1)_n$ phosphorylation to be $\approx$0.2 min^{-1}. These parameters are very similar to those obtained for the EGF receptor in plasma membranes from human carcinoma (A431) cells (unpublished observations) and for the recombinant soluble tyrosine kinase domain of erbB2/neu expressed in insect cells (14).

Affinity Labeling of Membrane Receptors with FSBA. One possible explanation for the lack of kinase activity observed with erbB3 is that the protein was denatured or functionally inactive when expressed in the insect cells. This seems unlikely because the EGF receptor was fully functional under the conditions employed and the erbB2/neu protein was also catalytically active when expressed in these cells (14). Moreover, insect cell-expressed erbB3 bound its ligand HRG with sub-nanomolar affinity (22). However, we tested the possibility that the erbB3 catalytic domain was functionally inactive by examining the abilities of the expressed receptors to specifically bind the ATP analog FSBA. This agent has been used previously to affinity-label the EGF receptor in membranes from A431 cells (19). The labeling of receptors in insect cell membranes with FSBA was detected by immunoblotting with affinity-purified anti-FSBA antibodies. Fig. 3 shows that FSBA covalently bound to the 158-kDa EGF receptor and the 168-kDa erbB3 proteins in plasma membranes purified from insect cells expressing these proteins. In both cases, labeling was inhibited with a 10-fold excess of unlabeled ATP. These data suggest that the kinase domain of insect cell-expressed erbB3 is capable of binding ATP.

DISCUSSION

The tyrosine kinase subdomain of $p180^{erbB3}$ shares 60–62% sequence identity with the catalytic domains of the other members of the class I growth factor receptor subfamily (6) (Fig. 4). Despite this degree of homology, three residues that are critically conserved throughout the protein kinases are altered in the $p180^{erbB3}$ protein, which could potentially render the erbB3 kinase inactive. Through the characterization of the autophosphorylation and substrate phosphorylation activities of the insect cell-expressed protein, we demonstrate here that erbB3 has a suppressed tyrosine kinase activity relative to the EGF receptor. This is consistent with the notion that the substituted amino acids give rise to a functionally impaired kinase.

The most significant alteration in $p180^{erbB3}$ relative to other protein kinases is the substitution of asparagine for aspartic acid at residue 834 of the human sequence (residue 833 of the bovine sequence). On the basis of the crystal structure of protein kinase A (9) and molecular modeling of the EGF receptor tyrosine kinase domain (13), this residue is situated in the kinase active site. It has been proposed that this aspartic acid is responsible for deprotonating the substrate hydroxyl, facilitating its nucleophilic attack of the γ phosphate of ATP (9, 13). Because asparagine is incapable of deprotonating the phenolic hydroxyl of the bound substrate, the catalytic activity of $p180^{erbB3}$ should be significantly suppressed. A second alteration, the substitution of histidine for glutamic acid at residue 759 of the human $p180^{erbB3}$ sequence, is part of an α-helix that has almost completely diverged from the other members of the EGF receptor family. This residue has been proposed to indirectly participate in the coordination and stabilization of the α and β phosphates of the bound ATP. However, all of the residues involved in the binding of the adenine ring are intact, consistent with our observation that insect cell-expressed erbB3 is capable of binding FSBA.

Our data indicate that although the tyrosine kinase activity of the insect cell-expressed erbB3 protein is at least 2 orders of magnitude lower than that for the EGF receptor or erbB2/neu, some autophosphorylation and substrate phos-

```
bErbB3 714 RGSGIFGTV HKGVWIPEGE SIKIPVCIKV IEDKS  GRQ SFQAVTDHML 760
ErbB3 715 LGSGVFGTV HKGVWIPEGE SIKIPVCIKV IEDKS..GRQ SFQAVTDHML 761
EGFR 718 LGSGAFGTV YKGLWIPEGE KVKIPVAIKE LREAT..SPK ANKEILDEAY 764
Neu 731 LGSGAFGTV YKGIWIPDGE NVKIPVAIKV LRENT..SPK ANKEILDEAY 777
FAK 403 IGRGQFGDV HQGIYMS.PE NPAMAVAIKT CKNCT..SDS VREKFLQEAL 448
c-src 281 LGQGCFGEV WMGTW..... NGTTRVAIKT LKPGT....M SPEAFLQEAQ 320
wee1 572 LGEGEFGSV FQ...VEDPV EKTLKVAVKK L.KVKFSGPK ERNRLLQEVS 616
MAPK 49 IGEGAYGMV SS...AYDHV RKT.RVAIKK IS..PFEHQT YCQRTLREIQ 91
cAMPK 50 LGTGSFGRV ML...VKHK. ESGNHYAMKI LDKQKVVKLK QIEHTLNEKR 94
MLCK 303 LGKGKFGNV CT...CTEK. STGLKLAAKV IKKQTP...K DKEMVMLEIE 344
...
bErbB3 827 HGMVHRNLAA RNVLL..KSP SQVQVADFGV ADLLPPDDKQ LLYNEAK.TP 873
ErbB3 828 HGMVHRNLAA RNVLL..KSP SQVQVADFGV ADLLPPDDKQ LLYSEAK.TP 874
EGFR 831 RRLVHRDLAA RNVLV..KTP QHVKITDFGL AKLLGAEEKE YHAEGGK.VP 877
Neu 844 VRLVHRDLAA RNVLV..KSP NHVKITDFGL ARLLDIDETE YHADGGK.VP 890
FAK 514 KRFVHRDIAA RNVLV..SAT DCVKLGDFGL SRYME.DSTY YKASKGK.LP 560
c-src 388 MNYVHRDLRA ANILV..GEN LVCKVADFGL ARLIEDNE.. YTARQGAKFP 433
wee1 687 KNYVHLDLKP ANVMITFEGT ..LKIGDFGM ASVWPV.PR. GMERGDD... 729
MAPK 161 ANVLHRDLKP SNLLI..NTT CDLKICDFGL ARIADPEH.. ..DHTGFLTE 204
cAMPK 161 LDLIYRDLKP ENLLIDQQG. .YIQVTDFGF AKR..VKGR. TWTLCGTP... 202
MLCK 412 MRVLHRDLKP ENILCVNTTG HLVKIIDFGL ARRYNPNEK. LKVNFGTP... 457
bErbB3 874 IKWMAL E SIHFGKYT HQSDVWSYGV TVWG
ErbB3 875 ....IKWMAL E.SIHFGKYT HQSDVWSYGV TVWE 903
EGFR 878 ....IKWMAL E.SILHRIYT HQSDVWSYGV TVWE 906
Neu 891 ....IKWMAL E.SILRRRFT HQSDVWSYGV TVWE 919
FAK 561 ....IKWMAP E.SINFRRFT SASDVWMFGV CMWE 589
c-src 434 ....IKWTAP E.AALYGRFT IKSDVWSFGI LLTE 462
wee1 730 ....CEYIAP EV.LANHLYD KPADIFSLSI TVFE 758
MAPK 205 YVATRWYRAP EIMLNSKGYT KSIDIWSVGC ILAE 238
cAMPK 203 ....PEYLAP EI.ILSKGYN KAVDWWALGV LIYE 231
MLCK 458 ....PEFLSP EV.VNYDQIS DKTDMWSLGV ITYM 486
```

FIG. 4. Sequence alignment of the erbB3 kinase domains with other protein kinases. The kinase domains of the human and bovine erbB3 proteins, human EGF receptor, rat $p185^{erbB2/neu}$, chicken focal adhesion kinase (FAK), mouse c-src, yeast wee1, mouse MAP kinase (MAPK), mouse cAMP-dependent protein kinase (cAMPK), and the rabbit skeletal muscle myosin light chain kinase (MLCK) are compared. Residues in boxes are highly conserved or invariant throughout the known protein kinases (8). The aspartic acid residue (834 in human erbB3, 833 in bovine erbB3) has been proposed to be directly involved in the phosphotransferase catalytic mechanism (9).

phorylation is detectable. However, neither the erbB3 autophosphorylation nor substrate phosphorylation reactions are stimulated in the presence of HRG, which binds to insect cell-expressed erbB3 with an affinity of 0.85 nM (22). Hence, it is likely that an endogenous insect-cell tyrosine kinase is responsible for the observed erbB3 phosphorylation and coimmunoprecipitates with erbB3.

A previous report indicated that $p180^{erbB3}$ overexpressed in NIH 3T3 mouse fibroblasts is constitutively tyrosine phosphorylated and that a chimera consisting of the EGF receptor extracellular domain and the erbB3 intracellular domain can undergo an EGF-stimulated autophosphorylation reaction (7). On the basis of those studies, the authors concluded that $p180^{erbB3}$ possesses a ligand-stimulatable tyrosine kinase activity. Indeed, we have observed that HRG stimulates the tyrosine phosphorylation of an ≈180-kDa protein in NIH 3T3 cells stably transfected with bovine *erbB3*, but not in parental NIH 3T3 cells (22). However, our recent studies indicate that EGF treatment of A431 cells stimulates the tyrosine phosphorylation of $p180^{erbB3}$ and elicits the recruitment of phosphatidylinositol 3-kinase to a complex containing the $p180^{erbB3}$ protein (25). Moreover, we have found that there is a strong propensity for $p180^{erbB3}$ and $p185^{erbB2/neu}$ to heterodimerize upon HRG binding, and that this heterodimerization event mediates the tyrosine phosphorylation of both receptors (26).

These observations may point to the biochemical function of a kinase-suppressed receptor in transmembrane signaling. We propose that $p180^{erbB3}$ is a target for ligand-stimulated heterodimerization with other class I growth factor receptors and acts to modulate the growth regulatory input by being cross-phosphorylated. (We have observed that NIH 3T3 cells express both EGF receptor and $p185^{erbB2/neu}$, which could account for the phosphorylation of $p180^{erbB3}$ in these cells.) The cross-phosphorylation of $p180^{erbB3}$ leads, then, to the recruitment and activation of intracellular signaling molecules that would not be stimulated if this receptor were not present on the surface of the cell. For example, the tail region of the $p180^{erbB3}$ protein contains six putative tyrosine phosphorylation sites that fit the consensus sequence for interaction with phosphatidylinositol 3-kinase (20), whereas EGF receptor and $p185^{erbB2/neu}$ have none. It is possible that $p180^{erbB3}$ acts to couple phosphatidylinositol 3-kinase activation to EGF signaling in a manner analogous to the coupling of phosphatidylinositol 3-kinase to insulin signaling by IRS-1 (21).

We thank R. Schatzman for anti-EGF receptor monoclonal antibody 291-3A, J. G. Koland for anti-$p180^{erbB3}$ serum K1, R. Gaehlen for anti-FSBA serum, and M. S. Atkisson for purification of recombinant baculovirus. We also are grateful for the expert secretarial assistance of Ms. Cindy Westmiller. This research was supported by grants to R.A.C. (GM40654) and L.C.C. (GM41890) from the National Institutes of Health and a grant from the Lucille P. Markey Charitable Trust (to L.C.C.). K.L.C. is the recipient of a postdoctoral fellowship from the American Cancer Society.

1. Yarden, Y. & Ullrich, A. (1988) *Annu. Rev. Biochem.* **57,** 443–478.
2. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61,** 203–212.
3. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. & McGuire, W. L. (1987) *Science* **235,** 177–182.
4. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. & Press, M. F. (1989) *Science* **244,** 707–712.
5. Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. & Shoyab, M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4905–4909.
6. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. & Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9193–9197.
7. Kraus, M. H., Pedi, P., Starks, V., Muraro, R. & Aaronson, S. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2900–2904.
8. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241,** 42–52.
9. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-h., Taylor, S. S. & Sowadski, J. M. (1991) *Science* **253,** 407–414.
10. Gibbs, C. S. & Zoller, M. J. (1991) *J. Biol. Chem.* **266,** 8923–8931.
11. Moran, M. F., Koch, C. A., Sadowski, I. & Pawson, T. (1988) *Oncogene* **3,** 665–672.
12. Tan, J. C., Nocka, K., Ray, P., Traktman, P. & Besmer, P. (1990) *Science* **247,** 209–212.
13. Knighton, D. R., Cadena, D. L., Zheng, J., Ten Eyck, L. F., Taylor, S. S., Sowadski, J. M. & Gill, G. N. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5001–5005.
14. Guy, P. M., Carraway, K. L., III & Cerione, R. A. (1992) *J. Biol. Chem.* **267,** 13851–13856.
15. Anostario, M., Harrison, M. L. & Gaehlen, R. L. (1990) *Anal. Biochem.* **190,** 60–65.
16. Greenfield, C., Patel, G., Clark, S., Jones, N. & Waterfield, M. D. (1988) *EMBO J.* **7,** 139–146.
17. Koland, J. G. & Cerione, R. A. (1988) *J. Biol. Chem.* **263,** 2230–2237.
18. Koland, J. G. & Cerione, R. A. (1990) *Biochim. Biophys. Acta* **1052,** 489–498.
19. Buhrow, S. A., Cohen, S. & Staros, J. V. (1982) *J. Biol. Chem.* **257,** 4019–4022.
20. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B. & Cantley, L. C. (1993) *Cell* **72,** 767–778.
21. Sun, X. J., Miralpeix, M., Myers, M. G., Jr., Glasheen, E. M., Backer, J. M., Kahn, C. R. & White, M. F. (1992) *J. Biol. Chem.* **257,** 4019–4022.
22. Carraway, K. L., Sliwkowski, M. X., Akita, R. W., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C. & Cerione, R. A. (1994) *J. Biol. Chem.* **269,** 14303–14306.
23. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
24. Koland, J. G., O'Brien, K. M. & Cerione, R. A. (1990) *Biochem. Biophys. Res. Commun.* **166,** 90–100.
25. Soltoff, S. P., Carraway, K. L., III, Prigent, S. A., Gullick, W. G. & Cantley, L. C. (1994) *Mol. Cell. Biol.* **14,** 3550–3558.
26. Sliwkowski, M. X., Shaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendley, B. M., Cerione, R. A., Vandlen, R. L. & Carraway, K. L. (1994) *J. Biol. Chem.* **269,** 14661–14665.