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A single amino acid substitution is sufficient to modify the mitogenic properties of the epidermal growth factor receptor to resemble that of gp185$^{erb\,B\text{-}2}$

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The epidermal growth factor (EGF) receptor (EGFR) and the *erb*B-2 gene product, gp185$^{erb\,B\text{-}2}$, exhibit distinct abilities to stimulate mitogenesis in different target cells. By using chimeric molecules between these two receptors, we have previously shown that their intracellular juxtamembrane regions are responsible for this specificity. Here we describe a genetically engineered EGFR mutant containing a threonine for arginine substitution at position 662 in the EGFR juxtamembrane domain, corresponding to threonine 694 in gp185$^{erb\,B\text{-}2}$. This mutant, designated EGFR^{Thr662}, displayed affinity for EGF binding and catalytic properties that were indistinguishable from those of the wild type EGFR. However, EGFR^{Thr662} behaved much as gp185$^{erb\,B\text{-}2}$ in a number of bioassays which readily distinguish between the mitogenic effects of EGFR and gp185$^{erb\,B\text{-}2}$. Moreover, significant differences were detected in the pattern of intracellular proteins phosphorylated on tyrosine *in vivo* by EGFR and EGFR^{Thr662} in response to EGF. Thus, small differences in the primary sequence of two closely related receptors have dramatic effects on their ability to couple with mitogenic pathways.

Key words: EGFR/*erb*B-2/substrates/signal transduction/tyrosine kinases

Introduction

Tyrosine kinase receptors (TKRs) signal through tyrosine-specific phosphorylation of a number of intracellular substrates which are thought to be intermediates in biochemical pathways eventually leading to mitosis. While several substrates have been identified (reviewed in Ullrich and Schlessinger, 1990; Aaronson, 1991), comparatively little is known about the coupling between the latter and effector pathways.

The mechanisms of mitogenic signal transduction by the structurally related EGFR (Ullrich *et al.*, 1984) and gp185$^{erb\,B\text{-}2}$ (Coussens *et al.*, 1985) molecules appear to differ (Di Fiore *et al.*, 1990b). Under comparable conditions of expression and enzymatic activity, these two proteins display striking differences in their ability to elicit mitogenic responses in different target cells. In NIH-3T3 cells, gp185$^{erb\,B\text{-}2}$ is 100-fold more potent than the EGFR as a transforming protein (Di Fiore *et al.*, 1987a, 1990b). Conversely, in the hematopoietic line 32D, EGFR is a more potent mitogenic transducer than gp185$^{erb\,B\text{-}2}$ (Pierce *et al.*, 1988; Di Fiore *et al.*, 1990b). By using chimeric molecules between EGFR and gp185$^{erb\,B\text{-}2}$, we have previously identified the juxtamembrane regions of these two proteins (comprising roughly the first 150 amino acids of the intracellular portions, also referred to as TK1 region) as being responsible for this phenotypic dichotomy (Di Fiore *et al.*, 1990b; Segatto *et al.*, 1991). More detailed structure-function analysis of the EGFR TK1 region identified a short domain, encompassing amino acid position 660-667 in the juxtamembrane region, whose deletion markedly reduced the mitogenic ability of the EGFR, but left enzymatic and binding properties unaltered (Segatto *et al.*, 1991).

Based on the studies described above, the region corresponding to amino acid residues 660-667 is likely to play a major role in determining substrate recognition by the EGFR kinase (Segatto *et al.*, 1991). Amino acid alignment of EGFR and gp185$^{erb\,B\text{-}2}$ (Figure 1) in this region reveals a single amino acid difference consisting of a threonine for arginine substitution in gp185$^{erb\,B\text{-}2}$ (positions 662 and 694 of the EGFR and gp185$^{erb\,B\text{-}2}$ sequences, respectively). Since this region is contained within the TK1 domain, which confers specificity to the EGFR and gp185$^{erb\,B\text{-}2}$ (Segatto *et al.*, 1991), the single Arg → Thr change might contribute to cell specificity for mitogenic signalling by these two molecules.

In the present study, we endeavored to test this hypothesis directly. To this end, we took advantage of comparative bioassays in NIH-3T3 fibroblasts and 32D cells, which readily distinguish between EGFR-like and *erb*B-2-like phenotypes (Di Fiore *et al.*, 1990b). Here we report that an EGFR mutant bearing a single Arg → Thr substitution at position 662 (EGFR^{Thr662}) exhibited an *erb*B-2-like phenotype in such bioassays. We propose, therefore, that in the *erb*B subfamily of receptors this single determinant contributes significantly to the routing of the mitogenic signal in a receptor-specific fashion.

Results

We engineered the Arg → Thr mutation at the codon corresponding to amino acid 662 in the EGFR cDNA (see Materials and methods) and expressed it in the LTR-2 vector (Di Fiore *et al.*, 1987b) which also contains the *Ecogpt* selectable marker conferring resistance to mycophenolic acid (Mulligan and Berg, 1981). The resulting EGFR mutant expression vector, designated LTR-EGFR^{Thr662} (Figure 1), was transfected into NIH-3T3 cells to compare its transforming efficiency with that of wild type EGFR and gp185$^{erb\,B\text{-}2}$.

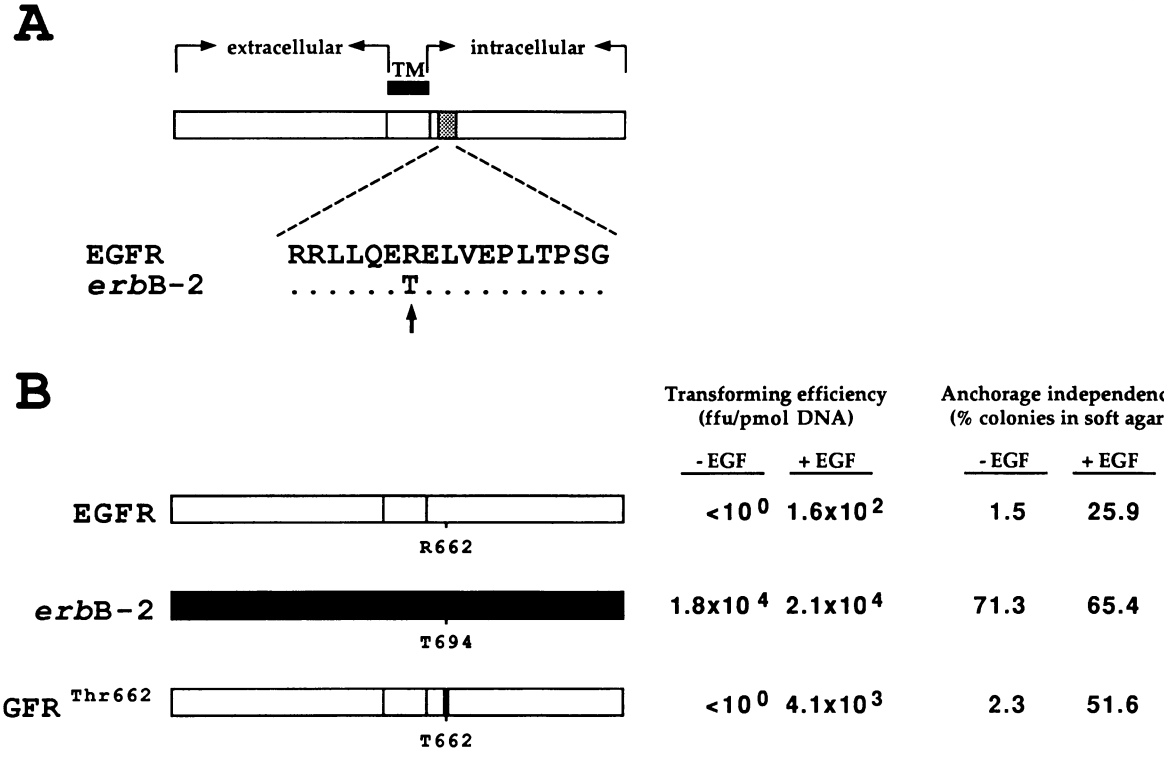

Fig. 1. Transforming activity in NIH-3T3 cells of EGFR^{Thr662} in comparison with EGFR and *erb*B-2. (**A**) An ideal *erb*B subfamily receptor is depicted with its juxtamembrane region highlighted (shaded box). The predicted amino acid sequence of that region is shown underneath for EGFR and *erb*B-2. The EGFR and *erb*B-2 sequences encompass amino acid positions 656–672 and 688–704, respectively (numbered according to Ullrich *et al.*, 1984 and Coussens *et al.*, 1985 for EGFR and gp185$^{erb\text{-}B2}$, respectively). Identical residues are indicated with a dot in the *erb*B-2 sequence. The single amino acid difference (Arg^{662} and Thr^{694} in EGFR and *erb*B-2, respectively) is indicated with an arrow. (**B**) The structures and biological activities of the EGFR^{Thr662}, EGFR and *erb*B-2 are compared. The EGFR^{Thr662} mutant and wild type EGFR and *erb*B-2, engineered in the LTR-2 expression vector (Di Fiore *et al.*, 1987b) were transfected into NIH-3T3 cells as indicated in Materials and methods. Transforming efficiency was calculated by scoring the foci of morphological transformation after 3 weeks and is expressed as focus forming efficiency per pmol of transfected DNA (ffu/pMol). Anchorage independence was tested in semisolid medium, as described in Materials and methods. Values are the mean of three independent experiments.

As previously shown, the transforming activity of gp185$^{erb\text{B-}2}$ was ~100-fold higher than that of EGFR; furthermore, the latter exhibited transforming ability only in the presence of EGF (Figure 1). EGFR^{Thr662} also displayed EGF-dependent transformation, but exhibited greatly increased transforming efficiency, ~25-fold higher than that of EGFR and only 4- to 5-fold lower than that of gp185$^{erb\text{B-}2}$ (Figure 1). Similarly in a soft agar assay, marker-selected (Mulligan and Berg, 1981) mass populations of NIH-EGFR^{Thr662} exhibited increased anchorage-independent growth in comparison with NIH-EGFR transfectants (Figure 1). The increase in transforming potential of EGFR^{Thr662} also correlated with an enhanced stimulation of DNA synthesis when compared with EGFR in a standard [^{3}H]thymidine incorporation assay (see below).

Marker-selected mass populations of NIH-EGFR^{Thr662} and NIH-EGFR were obtained for biochemical studies. As shown in Figure 2, both wild type and mutant EGFR were expressed at comparable levels (panel A) and no significant differences between the two receptors were detected in the time-course of dose dependence of EGF-stimulated autophosphorylation *in vivo* (panels B and C, respectively). *In vitro* immunocomplex kinase assays also indicated similar kinetic parameters for the two molecules (Table I). [^{125}I]EGF binding studies showed that both EGFR^{Thr662} and EGFR were expressed as both high (K_d ~10^{-10} M) and low (K_d 10^{-8} M) affinity receptors, with similar proportions of high and low affinity binding sites (high affinity sites representing ~5% of the total receptor pool, see Table I). In addition, TPA treatment caused the disappearance of [^{125}I]EGF high affinity binding sites in both NIH-EGFR^{Thr662} and NIH-EGFR, as expected (Davis, 1988; see also Table I). All these results indicated that the different mitogenic properties of EGFR^{Thr662} and EGFR could not be accounted for by differences in their levels of expression, EGF binding or intrinsic kinase activity.

The qualitative nature of these differences was established by gene transfer experiments performed in 32D hematopoietic cells. This myeloid precursor cell line is absolutely dependent on interleukin-3 (IL-3) for survival and proliferation and does not express either EGFR or gp185$^{erb\text{B-}2}$. Adoptive expression of EGFR in these cells resulted in their ability to be mitogenically stimulated by EGF in the absence of IL-3 (Pierce *et al.*, 1988; Di Fiore *et al.*, 1990b). Conversely, expression of gp185$^{erb\text{B-}2}$ had little effect on the growth characteristics of this cell line, despite high levels of gp185$^{erb\text{B-}2}$ expression and constitutive kinase activity (Di Fiore *et al.*, 1990b). We transfected the EGFR^{Thr662} mutant into 32D cells to compare its biological properties with the wild type EGFR. We also transfected an EGFR–*erb*B-2^{TK1} chimera in which the entire juxtamembrane region of gp185$^{erb\text{B-}2}$ was substituted for the analogous region of EGFR and that has been shown previously to exhibit an *erb*B-2-like behavior (Segatto *et al.*, 1991). In

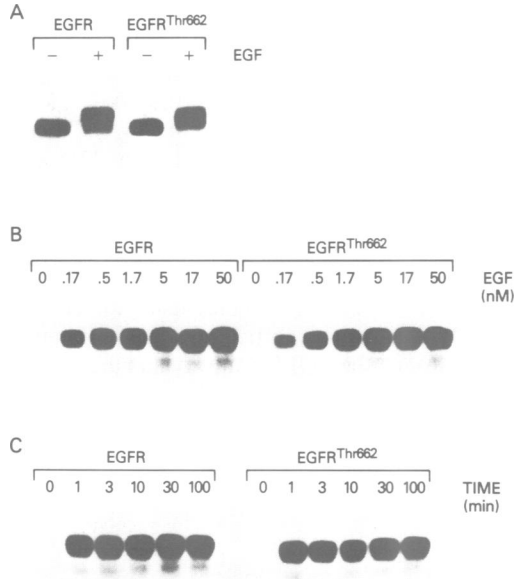

Fig. 2. Levels of expression and autophosphorylation activity of EGFR^{Thr662} and EGFR in NIH-3T3 transfectants. Cellular protein extracts were prepared from serum-starved marker-selected mass populations of NIH-3T3 transfectants. Where indicated, cells were treated with EGF at 37°C prior to lysis. Cellular proteins (100 μg) were subjected to SDS−PAGE and analyzed by immunoblotting with either the EGFR-specific antiserum E7 (Di Fiore *et al.*, 1990a, panel A) or with an anti-phosphotyrosine monoclonal antibody (anti-pTyr mAb, Upstate Biotechnology, panels B and C). (**A**) Levels of expression of EGFR^{Thr662} and EGFR in NIH-3T3 in the absence (− lanes) or presence of EGF (+ lanes, 17 nM EGF for 5 min at 37°C). (**B**) EGF dose−response for autophosphorylation *in vivo*. NIH-EGFR^{Thr662} and NIH-EGFR transfectants were either mock-treated (− lanes) or treated with EGF, at the indicated molar concentrations, for 5 min at 37°C prior to lysis. (**C**) Time-course of EGF-induced autophosphorylation *in vivo*. NIH-EGFR^{Thr662} and NIH-EGFR transfectants were either mock-treated (0 lanes) or treated with EGF (17 nM at 37°C) for the indicated lengths of time, prior to lysis. Results are typical and representative of at least three experiments.

a standard [^{3}H]thymidine incorporation assay, both 32D-EGFR^{Thr662} and 32D-EGFR−*erb*B-2^{TK1} transfectants exhibited reduced responsiveness to EGF as compared with the EGFR (Figure 3A). This contrasted sharply with their increased mitogenic signalling in an NIH-3T3 background (Figure 3B).

Analysis of the EGF dose−response curves revealed additional interesting features. EGFR, EGFR^{Thr662} and EGFR−*erb*B-2^{TK1} NIH-3T3 transfectants exhibited the same ED_{50} for EGF, despite different levels of maximal mitogenic stimulation achieved at saturating doses (Figure 3B). Since EGFR, EGFR^{Thr662} and EGFR−*erb*B-2^{TK1} were expressed at similar levels and displayed comparable EGF binding and enzymatic properties (Table I and Segatto *et al.*, 1991), we concluded that at the same level of receptor occupancy EGFR^{Thr662} and EGFR−*erb*B-2^{TK1}, like gp185^{erbB-2} (Di Fiore *et al.*, 1990b; Fazioli *et al.*, 1991), signal through a more potent mitogenic pathway than the EGFR in NIH-3T3 cells.

In 32D cells, conversely, EGFR, EGFR^{Thr662} and EGFR−*erb*B-2^{TK1} were able to deliver comparable maximal mitogenic stimulation (Figure 3A). However, both EGFR^{Thr662} and EGFR−*erb*B-2^{TK1} displayed a significantly increased ED_{50} for EGF, when compared with the EGFR. In particular, at EGF concentrations in the range of the K_d for high affinity receptors, the mitogenic response of either 32D-EGFR^{Thr662} or 32D-EGFR−*erb*B-2^{TK1} was negligible, while it was half maximal for 32D-EGFR. Thus, supraphysiological levels of receptor occupancy are needed for EGFR^{Thr662} and EGFR−*erb*B-2^{TK1} to deliver a mitogenic signal comparable with the EGFR in 32D cells. A possible explanation for this phenomenon is that critical substrates for the *erb*B-2-activated pathway are missing in 32D cells (Di Fiore *et al.*, 1990b), and the mitogenic response elicited by EGFR^{Thr662} and EGFR−*erb*B-2^{TK1} is due to promiscuous (and low efficiency) recruitment of substrates from other pathways. In any case, these results are consistent with our previous finding that the *erb*B-2 kinase is relatively inefficient in mitogenic signalling in 32D cells (Di Fiore *et al.*, 1990b). All these results, taken together, establish that the Arg^{662} → Thr^{662} confers to the EGFR kinase mitogenic properties resembling those of gp185^{erbB-2}.

A simple model to account for the effects of the EGFR^{Thr662} is that the Arg^{662} → Thr^{663} mutation influences the interactions of the mutated EGFR with some intracellular

Table I. Analysis of [^{125}I]EGF binding properties *in vivo* and catalytic properties *in vitro* of EGFR and EGFR^{Thr662} expressed in NIH-3T3 fibroblasts

	Binding properties[a]	
	EGFR	EGFR^{Thr662}
Receptors/cell		
high affinity	15 000	10 000
low affinity	320 000	260 000
K_d (nM)		
high affinity	0.07	0.05
low affinity	8.2	11.1
Receptors/cell + PMA		
high affinity	−	−
low affinity	340 000	250 000
K_d (nM) + PMA		
high affinity	−	−
low affinity	7.2	9.6
	Kinetic properties[b]	
	EGFR	EGFR^{Thr662}
K_m ATP (autophosphorylation) (μM)	3−4	3−6
K_m ATP (peptide phosphorylation) (μM)	4−8	6−10
K_m peptide (peptide phosphorylation) (μM)	180−200	170−220

[a][^{125}I]EGF binding was assessed by Scatchard analysis over a range of concentrations from 0.0017 to 55.6 nM in triplicate wells. Specificity of binding was controlled in parallel competition experiments using a 100-fold molar excess of unlabeled EGF. Where indicated, binding experiments were performed in the presence of 100 nM PMA. Data were analyzed with the LIGAND software (Munson and Rodbard, 1980).

[b]Cell lysates were prepared from NIH-3T3 transfectants and equal amounts of receptor were immunoprecipiated for the *in vitro* immunocomplex kinase assays. Phosphorylation reactions were performed as described in Materials and methods. Reactions were carried out at the initial rate of 1 min at 4°C. The synthetic peptide KGSTAENAEYLRV, containing the Y^{1173} autophosphorylation site of the EGFR, was used for the reactions of peptide−substrate phosphorylation. K_ms were calculated from Lineweaver−Burk plots of the enzymatic kinetics. Results are reported as the range of values obtained in two independent experiments performed in duplicate.

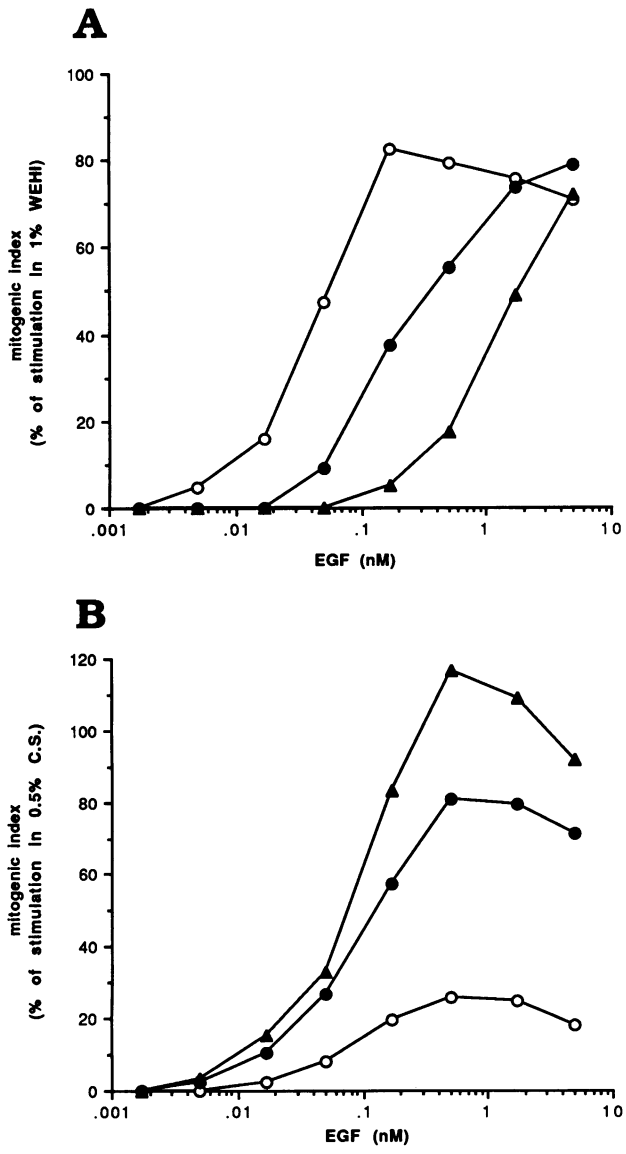

Fig. 3. Dose–response analysis of [^{3}H]thymidine incorporation upon EGF stimulation of 32D and NIH-3T3 transfectants. Eukaryotic expression vectors for EGFR, EGFR^{Thr662} or EGFR–*erb*B-2^{TK1} were transfected (1 μg of cloned DNA) into either 32D cells (**A**) or NIH-3T3 fibroblasts (**B**) and mass populations were selected into mycophenolic acid-containing medium. [^{3}H]thymidine incorporation assays were performed as previously described (Pierce *et al.*, 1988; Fazioli *et al.*, 1991). Data are expressed as a mitogenic index calculated as indicated in Materials and methods. Cell lines expressing comparable numbers of receptors were selected for the mitogenic assays and the [^{125}I]EGF binding of the cell lines was also tested in parallel to the [^{3}H]thymidine incorporation assays. The numbers of [^{125}I]EGF binding sites per cell, in the experiment shown, were: (A), 32D-EGFR [○] 4.8 × 10^5; 32D-EGFR^{Thr662} [●] 4.4 × 10^5; 32D-EGFR–*erb*B-2^{TK1} [▲] 5.7 × 10^5. (B), NIH-EGFR [○] 3.2 × 10^5; NIH-EGFR^{Thr662} [●] 2.8 × 10^5; NIH-EGFR–*erb*B-2^{TK1} [▲] 3.5 × 10^5. Calculated ED_{50} for mitogenic response to EGF are: (A), 32D-EGFR [○] 0.05 nM; 32D-EGFR^{Thr662} [●] 0.25 nM; 32D-EGFR–*erb*B-2^{TK1} [▲] 1.0 nM. (B), NIH-EGFR [○] 0.08 nM; NIH-EGFR^{Thr662} [●] 0.08 nM; NIH-EGFR–*erb*B-2^{TK1} [▲] 0.09 nM. Results are typical and representative of at least three experiments.

substrates, possibly shifting the specificity (or the affinity) of interaction, at least in part, towards *erb*B-2 substrates. If so, one should expect qualitative and/or quantitative differences in the pattern of proteins whose tyrosine phosphorylation is induced by the EGFR^{Thr662} in comparison with the wild type EGFR. Indeed, in NIH-3T3 transfectants we could show a higher stoichiometry of phosphorylation of two proteins in the 30–35 kDa range by the EGFR^{Thr662} mutant (Figure 4A), along with other minor differences. Conversely, in 32D cells, the EGFR^{Thr662} mutant was less efficient than the wild type EGFR at phosphorylating a number of intracellular substrates (Figure 4B), despite similar levels of receptor expression and EGF-induced receptor tyrosine phosphorylation *in vivo* (Figure 4B). These results are consistent with the hypothesis that EGFR^{Thr662} cannot efficiently route its mitogenic signalling through the EGFR pathway and that some critical component(s) of its own pathway are missing in 32D cells. Thus, the biochemical analysis provides a potential mechanism for the altered biological properties of the EGFR^{Thr662} mutant.

Discussion

By comparing the signalling properties of EGFR and gp185^{erbB-2} in different target cells, we have been able to show marked differences in the ability of these two closely related receptors to couple with mitogenic pathways (Di Fiore *et al.*, 1990b; Fazioli *et al.*, 1991; Segatto *et al.*, 1991). Based on our bioassays, an EGFR-like phenotype was defined by potent activation of mitogenic signals in 32D cells, relative to weaker stimulation of NIH-3T3 fibroblasts (Di Fiore *et al.*, 1990b). The reciprocal phenotype was defined as *erb*B-2-like. Our present studies establish that a single amino acid change is capable of converting the EGFR to an *erb*B-2-like phenotype. Our results further indicate that other determinants in the TK1 (or juxtamembrane) region may also contribute to the differences between the two receptors. In fact, the substitution of the TK1 region of gp185^{erbB-2} into EGFR in the EGFR–*erb*B-2^{TK1} chimera had an even greater impact than the Arg → Thr mutation (see Figure 3).

The distinct biological properties of the EGFR^{Thr662} mutant, compared with wild type EGFR, could not be ascribed to differences in the levels of expression, affinity for EGF binding or alterations in the intrinsic phosphotransferase activity. Therefore, they must reside ultimately in the diverse ability of the two receptors to recruit and phosphorylate intracellular substrates. Indeed, in the NIH-3T3 system, the stronger mitogenic ability of the EGFR^{Thr662} mutant, compared with wild type EGFR, correlated with higher stoichiometry of tyrosine phosphorylation of at least two putative substrates in the 32–35 kDa region. Interestingly, this substrate doublet was not phosphorylated by the EGFR^{Thr662} in 32D cells. A possible explanation for this phenomenon is that the 32–35 kDa proteins are not expressed in 32D cells, a hypothesis that would provide a structural basis for the decreased mitogenic potency of EGFR^{Thr662} in these cells, compared with EGFR. Whether the increased phosphorylation of the 32–35 kDa proteins is responsible for (or contributes to) the *erb*B-2-like phenotype of EGFR^{Thr662} remains to be ascertained. Our evidence indicates that proteins in the same molecular weight area are efficiently phosphorylated in stable *erb*B-2 transfectants (data not shown). In addition the size of the proteins in question is reminiscent of that of a 37 kDa protein which

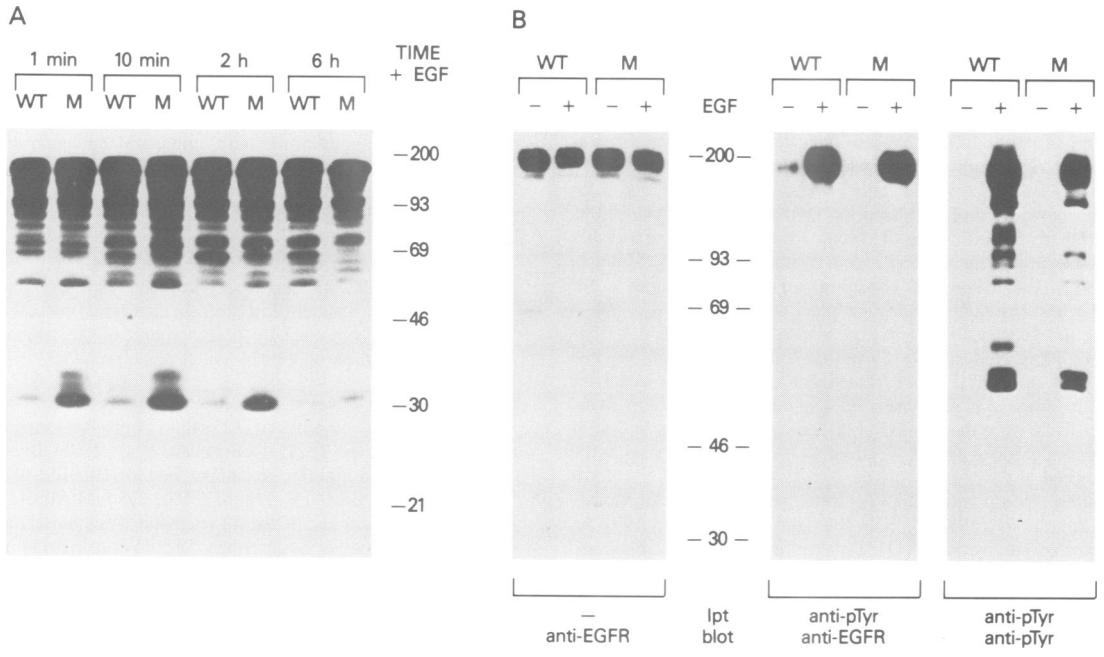

Fig. 4. *In vivo* tyrosine phosphorylation of cellular proteins by EGFR^{Thr662} and EGFR in NIH-3T3 (panel A) or 32D (panel B) transfectants. The transfectants used are described in the legend to Figure 3 and expressed comparable number of receptors. (**A**) NIH-EGFR^{Thr662} (M) and NIH-EGFR (WT) were serum-starved for 16 h and then treated with EGF (5.0 or 0.5 nM at 37°C) for the indicated lengths of time prior to lysis. Cellular proteins (2.0 mg) were precleared through absorption on Gamma Bind G–agarose (Genex Corp.), immunoprecipitated with the described anti-pTyr mAb and then fractionated by SDS–PAGE for subsequent immunoblot analysis with the anti-pTyr mAb. The reported results refer to the treatment with EGF at 5.0 nM, but similar kinetics of phosphorylation were obtained with 0.5 nM EGF. (**B**) 32D-EGFR^{Thr662} (M) and 32D-EGFR (WT) were serum-starved for 2 h and then either mock-treated (– lanes) or treated with EGF 5.0 nM for 5 min at 37°C, prior to lysis. In the experiment shown on the left, cellular proteins (100 μg) were analysed by SDS–PAGE followed by immunoblot with anti-EGFR antibody E7. In the experiment shown on the right, cellular proteins (2.0 mg) were immunoprecipitated with the anti-pTyr mAb and then analyzed by immunoblot with either the E7 antibody or the anti-pTyr mAb, as indicated. The lack of recognition of any of the 'putative' substrates, immunoprecipitated with the anti-pTyr mAb, by the E7 Ab demonstrates that they are not degradation products of the EGFR (a complete characterization of the E7 antibody is reported in Di Fiore *et al.*, 1990a; Fazioli *et al.*, 1991). WT: wild type EGFR; M, mutant EGFR^{Thr662}. Results are typical and representative of at least three experiments. Molecular mass markers are indicated in kDa.

has been reported to be specifically phosphorylated by an EGFR–*erb*B-2 chimera (Lee *et al.*, 1989). We are presently generating monoclonal antibodies against several proteins whose tyrosine phosphorylation is induced by the *erb*B-2 kinase. The availability of these reagents should help address these issues.

The question arises as to the molecular mechanisms by which the Arg/Thr determinants impart substrate specificity. One possibility is that the arginine and threonine residues perform different structural functions in creating 'pockets' responsible for substrate recognition. However, other explanations can be envisaged. The newly engineered Thr^{662} in the EGFR falls in the middle of a cluster of Ser/Thr phosphorylation sites, including Thr^{654} (Hunter *et al.*, 1984; Davis and Czech, 1985; Livneh *et al.*, 1988), Thr^{669} (Heiserman and Gill, 1988; Countaway *et al.*, 1990) and Ser^{671} (Heiserman and Gill, 1988), all of which are conserved in gp185$^{erb\,B\text{-}2}$. If the newly engineered Thr^{662} were a phosphorylation site as well, then it might alter the catalytic properties of EGFR by a simple shift in charge of this critical region or by interfering with the phosphorylation of neighboring sites. It is important to note, however, that the effect of the Arg^{662} → Thr^{662} mutation seems to be quite specific. In fact, a Glu-Arg to Asp-Val mutation of residues 661 and 662, which also brings about a change in charge, did not alter the biochemical or biological properties of the EGFR (Segatto *et al.*, 1991).

Previous work has focused primarily on the role of the highly divergent C-terminal domains of the EGFR and gp185$^{erb\,B\text{-}2}$ in directing the binding of intracellular substrates (Margolis *et al.*, 1990; Vega *et al.*, 1992). Our present data show that the highly conserved juxtamembrane (or TK1) region also participates in dictating mitogenic action and possibly substrate specificity of these two related receptors. We have previously identified 'microheterogeneity' in the TK1 regions of pairs of related receptors belonging to different subfamilies of TKRs (Segatto *et al.*, 1991). In particular, we found that clusters of sequence divergence in the TK1 domains fall into similar positions (Segatto *et al.*, 1991), suggesting that evolutionary pressure favored the selection of a restricted number of topological variations and that minimal changes in these 'hypervariable' regions might have a profound impact on the signalling specificity of several TKRs. Indeed, there is accumulating evidence implicating the juxtamembrane (or TK1) region of TKRs in the transduction of mitogenic signals (White *et al.*, 1988; Cochet *et al.*, 1991; Segatto *et al.*, 1991). Most previous studies, however, including our own, have focused on the identification of regions whose alteration abolished certain biological or biochemical responses to growth factors. Here, we describe a molecular determinant that influences the qualitative nature of cellular responses to the activation of TKRs. Such knowledge should provide the molecular genetic tools necessary to identify the substrates responsible for growth factor-specific effects on cellular metabolism and possibly to intercede selectively with their functions.

Materials and methods

Transfection and mitogenic assays

All of the LTR-2-based expression vectors were transfected into NIH-3T3 cells by the calcium phosphate method (Wigler *et al.*, 1977), at 10-fold serial dilutions of plasmid DNA. Where indicated, EGF (20 ng/ml) was added at day 14. Transforming efficiency was calculated by scoring the foci of morphological transformation after 3 weeks and is expressed as focus forming efficiency per pmol of transfected DNA (ffu/pMol). The results are normalized for the efficiency of colony formation in parallel dishes subjected to selection in mycophenolic acid-containing medium (Mulligan and Berg, 1981). Under this latter condition, we typically obtained $\sim 10^4$ mycophenolic acid-resistant colonies per pmol of DNA.

Anchorage independence was tested in semisolid medium as described previously (Di Fiore *et al.*, 1987a). The transfectants used were isolated from plates that received 1 μg of cloned DNA and were selected for their ability to grow in a mycophenolic acid-containing medium (mass populations). Cells were then plated at 10-fold serial dilutions in 0.45% sea-plaque agarose medium plus 10% calf serum. Colonies comprising >100 cells were scored at 14 days.

[^{3}H]thymidine incorporation assays were performed as previously described (Pierce *et al.*, 1988 for 32D cells and Fazioli *et al.*, 1991 for NIH-3T3 transfectants, respectively). Data are expressed as a mitogenic index calculated as the fraction of stimulation obtained in the presence of EGF with respect to the stimulation obtained with non-saturating concentrations of an optimal mitogen (WEHI cell conditioned medium containing IL-3, and calf serum for 32D and NIH-3T3, respectively). The mitogenic index was calculated as follows: [(EGF c.p.m. − background c.p.m.)/(optimal mitogenic c.p.m. − background c.p.m.)] × 100; the background values were obtained in the absence of any mitogens.

Engineering of eukaryotic expression vectors

The LTR−*erb*B-2 (Di Fiore *et al.*, 1987b) expression vector has been described previously. The engineering of the EGFR−*erb*B-2^{TK1} chimera and its characterization have been reported in Segatto *et al.* (1991). Briefly, the EGFR−*erb*B-2^{TK1} chimera encodes a protein which corresponds to the EGFR except for a stretch of 151 amino acids in the intracellular juxtamembrane region, which was substituted with the analogous region of gp185^{erbB-2}. The amino acid boundaries of the TK1 regions are from position 689 to 840 and 657 to 808 for *erb*B-2 and EGFR, respectively (numbering is according to Ullrich *et al.*, 1984 and Coussens *et al.*, 1985 for EGFR and gp185^{erbB-2}, respectively).

To engineer the LTR−EGFR^{Thr662} expression vector, we started from a modified version of the LTR−EGFR vector (the original LTR−EGFR is described in Di Fiore *et al.*, 1987a, the modified version, henceforth referred to as LTR−EGFR 5M is described in Segatto *et al.*, 1991). The LTR−EGFR 5M contains unique *Sal*I and *Spe*I sites engineered by site-directed mutagenesis at positions 2225−2230 and 2682−2687, respectively in the EGFR open reading frame. The creation of these two sites did not alter the predicted protein sequence of EGFR. Two oligonucleotides: 5′-CTGCGTCGACTGCTGCAGGAGACGGAGCTCGTGGAGCCT-3′ (oligo 1) and 5′-GCGGTGCACTAGTCTACG-3′ (oligo 2) were used for PCR amplification of an EGFR fragment in the region corresponding to nucleotide position 2221−2694, using the LTR−EGFR 5M as a template. The first oligonucleotide encompassed the *Sal*I site (positions 5−10 of oligo 1) and also contained a single point mutation at position 23 consisting of a C for G transversion, with respect to the original EGFR sequence. This transversion changed the triplet AGG, encoding arginine, to ACG (underlined in the sequence of oligo 1, above), encoding threonine. Thus, the close proximity of the *Sal*I site to the codon to be mutagenized enabled us to use oligo 1 as a mutagenizing oligo. Oligo 2 encompassed the *Spe*I site (positions 8−13 of oligo 2). The 474 bp PCR-amplified fragment corresponded therefore to the EGFR 5M coding sequence between positions 2221 and 2694, except for the single base mutation causing the Arg^{662} → Thr^{662} mutation. After digestion with *Sal*I and *Spe*I, the PCR-amplified fragment was cloned into the LTR−EGFR 5M vector which had been depleted of the corresponding *Sal*I−*Spe*I fragment, to generate the LTR−EGFR^{Thr662} expression vector. This vector was sequenced in both strands in the regions which underwent genetic manipulations, including the entire 474 bp cloned fragment. In all of the described transfection experiments the LTR−EGFR 5M was used as the control vector encoding a wild type EGFR.

Protein studies

Cellular proteins were detergent extracted from mass cell populations derived after transfection and marker selection. Where indicated, cell lysis was performed after incubating cells at 37°C with EGF. Lysates were obtained in a buffer containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid, pH 7.5), 150 mM NaCl, 1% Triton, 10% glycerol, 5 mM EGTA (ethylene glycol tetraacetic acid), phosphatase inhibitors (10 mM sodium pyrophosphate and 400 μM sodium orthovanadate) and protease inhibitors [1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg/ml aprotinin]. Immunoprecipitation and immunoblot analysis were performed as described previously (Fazioli *et al.*, 1991; Segatto *et al.*, 1991). Immunodetection was carried out with either the EGFR-specific antiserum E7 (Di Fiore *et al.*, 1990a) or with an anti-phosphotyrosine monoclonal antibody (anti-pTyr mAb, Upstate Biotechnology). The specificity of immunodetection for the peptide antiserum was controlled by performing parallel staining of identical blots with antibodies preabsorbed with the specific peptides (2 mg/ml). In the case of the anti-pTyr antibody, specificity was controlled by preabsorption of the antibody with either phosphotyrosine, phosphoserine or phosphothreonine.

In vitro kinase assays

In vitro immunocomplex kinase assays were performed as described previously (Honegger *et al.*, 1988; Segatto *et al.*, 1991). Briefly, cell extracts were prepared in a buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin (lysis buffer) and normalized for amount of receptor as described (Segatto *et al.*, 1991). For the *in vitro* autophosphorylation assay, lysates containing equal amounts of receptor were immunoprecipitated with the Ab-1 monoclonal antibody directed against the extracellular domain of the EGFR (Oncogene Science) and the specific immunoprecipitates, recovered with protein G−agarose, resuspended in 30 μl of HNTG (0.1% Triton X-100, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol) supplemented with 15 mM $MgCl_2$, 15 mM $MnCl_2$, 20 μCi of [γ-^{32}P]ATP (3000 Ci/mM, Amersham) and various concentrations of unlabeled ATP (final ATP concentration 0.5−15 μM). Kinase reactions were performed for 1 min at 4°C (initial conditions) and ^{32}P incorporation into EGFR was measured by precipitation with trichloroacetic acid as described (Segatto *et al.*, 1991).

The synthetic peptide KGSTAENAEYLRV, containing the Y1173 autophosphorylation site of the EGFR, was used as an *in vitro* substrate of the EGFR kinase. Conditions were as described for the autophosphorylation reaction; each reaction contained 20 μCi of [γ-^{32}P]ATP and in addition, either 1 mM peptide and varying concentration of unlabeled ATP (for determining K_m ATP) or 0.0033−1 mM peptide in the presence of 15 μM ATP (for determining the K_m for peptide substrate). Reactions were analyzed by SDS−PAGE using a 20% acrylamide separation gel overlaid with a 7.5% gel. After autoradiography, the peptide bands were excised and counted as described (Segatto *et al.*, 1991). Under our conditions of analysis, no significant difference was observed in the *in vitro* kinase assays performed in the absence of EGF or by pretreating the cell lysates with EGF 50 mM at 22°C for 10 min, prior to immunoprecipitation (Segatto *et al.*, 1991).

EGF binding assay

EGF binding to intact cells was assessed as described previously (Segatto *et al.*, 1991), with [^{125}I]EGF (New England Nuclear, 150−200 μCi/μg) over a range of concentrations from 0.0017 to 55.6 nM for at least 6 h at 4°C. Assays were performed in triplicate wells and specificity of binding was determined by parallel experiments in which a 100-fold molar excess of unlabeled EGF was used to compete out the tracer. Where indicated, cells were incubated for 1 h with 100 nM phorbol myristate acetate (PMA, Sigma) at 37°C before performing the assay and [^{125}I]EGF binding was measured in the presence of 100 nM PMA. The number of receptors per cell and their dissociation constant (K_d) for EGF were determined from Scatchard plots. Analysis of the binding was performed by the LIGAND software (Munson and Rodbard, 1980).

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