The EMBO Journal vol.13 no.12 pp.2831–2841, 1994

Identification of c-erbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-erbB-3 chimera

Sally A.Prigent[1] and William J.Gullick[2]

Molecular Oncology Laboratory, ICRF Oncology Group, Hammersmith Hospital, Du Cane Road, London W12 0HS, UK

[1]Present address: Departments of Medicine and Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636, USA
[2]Corresponding author

Communicated by J.Brockes

c-erbB-3 is a member of the type I (EGF receptor-related) family of growth factor receptors for which no ligand has been identified. To facilitate ligand stimulation we have constructed a chimeric receptor which possesses an activatable kinase and promotes the growth of NIH 3T3 fibroblasts. In this study we have shown that SHC and phosphatidylinositol 3′-kinase bind to the activated EGF receptor/c-erbB-3 chimera. Whereas p85 is not phosphorylated to a significant extent, SHC appears to be a major substrate for phosphorylation on tyrosine. In contrast to EGF receptor and c-erbB-2, we were unable to detect binding of activated c-erbB-3 to GRB2. Using synthetic peptides corresponding to each of 13 potential phosphorylation sites on c-erbB-3, we have shown that tyrosine 1309 is responsible for SHC binding. Peptides containing the motif YXXM inhibit p85 association. By comparison with recently reported SHC binding sites on Middle T antigen and Trk we have identified a SHC binding motif, NPXY.

Key words: c-erbB-3/growth factor receptors/ phosphorylation

Introduction

The gene encoding c-erbB-3 was cloned almost simultaneously by two groups (Kraus *et al.*, 1989; Plowman *et al.*, 1990). It encodes a 160–180 kDa glycoprotein which has all the structural features of the EGF family of tyrosine kinase receptors [which also includes EGF receptor (EGFR), c-erbB-2 and c-erbB-4 (Plowman *et al.*, 1993)]. The highest proportion of sequence identity with c-erbB-3 is within the catalytic domains of EGFR (60%), c-erbB-2 (62%) and c-erbB-4 (63%), although EGFR, c-erbB-2 and c-erbB-4 are somewhat more similar to each other than to c-erbB-3 (~80% sequence identity between receptor pairs). Moreover, c-erbB-3 is the only family member to contain unusual residues within the kinase domain, including His740 and Asn815 which are present as glutamate and aspartate, respectively, in other kinases (Plowman *et al.*, 1990). Mutation of the corresponding aspartate residue to asparagine in the v-*fps* tyrosine kinase completely abolished kinase activity (Moran *et al.*, 1988). Similarly, the murine white spotting (W^{42}) phenotype results from the same amino acid replacement in the proto-oncogene c-*kit* which also encodes a defective kinase (Tan *et al.*, 1990). Finally, c-erbB-3 lacks potential regulatory threonine sites which are present in EGFR, c-erbB-2 and c-erbB-4, suggesting that c-erbB-3 may not be subject to negative regulation by protein kinase C (Plowman *et al.*, 1990). These data suggest that c-erbB-3 may possess slightly different properties to other family members.

Great advances have been made recently in understanding the mechanism by which tyrosine kinase receptors interact with intracellular signalling components. Following ligand binding, autophosphorylation on specific tyrosine residues facilitates the recruitment of proteins to the activated receptor. This association is achieved by the high-affinity interaction of characteristic elements called src homology 2 (SH2) domains with phosphotyrosine residues (Pawson and Schlessinger, 1993). A large number of proteins containing SH2 domains have now been identified, including those with enzymatic activity such as the $p21^{ras}$ GTPase-activating protein (Trahey *et al.*, 1988), phosphotyrosine phosphatases (Shen *et al.*, 1991; Freeman *et al.*, 1992; Feng *et al.*, 1993; Vogel *et al.*, 1993) and phospholipase Cγ (Stahl *et al.*, 1988), and those with no obvious enzymatic activity including GRB2 (Lowenstein *et al.*, 1992), which is the mammalian homologue of the *Caenorhabditis elegans* protein sem-5 (Clark *et al.*, 1992) and SHC (Pelicci *et al.*, 1992). Additional motifs called src homology 3 (SH3) domains have also been identified in a number of these proteins which direct cellular localization of signalling molecules (Bar-Sagi *et al.*, 1993). Those SH2-containing proteins which do not possess enzymatic activity act as adaptor molecules linking other functional elements with the activated receptors. An example of this is the p85 protein which interacts with the p110 subunit of phosphatidylinositol (PI) 3′-kinase which possesses the enzymatic activity (Escobedo *et al.*, 1991). Other examples include GRB2 which has been shown to couple the EGF receptor with the $p21^{ras}$ signalling pathway by binding both activated EGF receptor and the guanine nucleotide exchange protein sos (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). SHC is phosphorylated by and physically associates with activated EGF receptor and c-erbB-2 (Segatto *et al.*, 1993). Following phosphorylation it appears to then interact with GRB2 (Rozakis-Adcock *et al.*, 1992). The precise role of this protein is less well defined than that for GRB2, but it may also be involved in $p21^{ras}$ signalling, at least in the case of non-receptor tyrosine kinases such as v-Src which cannot couple directly to GRB2.

By mapping the specific phosphotyrosine residues responsible for binding SH2 domain-containing proteins, it has been possible to identify phosphotyrosine-containing consensus sequences responsible for binding different signalling molecules, and it is now possible to predict to a certain extent which effector molecules are likely to be

© Oxford University Press

involved in the signalling of a novel receptor (Songyang *et al.*, 1993). Analysis of the c-erbB-3 amino acid sequence reveals several potential binding sites for PI3-kinase, a phosphotyrosine phosphatase (SH-PTP2) binding site (Freeman *et al.*, 1992; Feng *et al.*, 1993; Vogel *et al.*, 1993) and two possible binding sites for GRB2.

In this work, we investigated the function of the c-erbB-3 protein and its effect on the growth of indicator cells. In particular, its interaction with specific second messenger pathways was explored.

Results

Comparison of wild-type and mutant c-erbB-3 in vitro and in vivo

The kinase activity of c-erbB-3 was assessed by immune-complex assay, using transiently transfected COS-1 cells as a source of c-erbB-3 protein and an anti-peptide antibody (49.3) specific for c-erbB-3 for immunoprecipitation (Figure 1). Since this antibody reacts with the intracellular domain of c-erbB-3 and could potentially inhibit kinase activity, the c-erbB-3 protein was eluted with the corresponding peptide prior to the kinase assay. This peptide contains no tyrosine residues and therefore could not act as a substrate. c-erbB-3 showed a very low level of autophosphorylation in immune-complex assays which was dependent on the presence of 2–4 mM Mn^{2+} ions and independent of Mg^{2+} ion concentration (data not shown). Phosphoamino acid analysis revealed that phosphorylation was on tyrosine and serine residues (data not shown). To determine whether basal autocatalytic activity could be enhanced by replacing His740 and Asn815 by the potentially more favourable residues Glu and Asp, mutants were constructed and analysed similarly. The double mutant showed, if anything, a reduced activity relative to the wild-type protein (Figure 1).

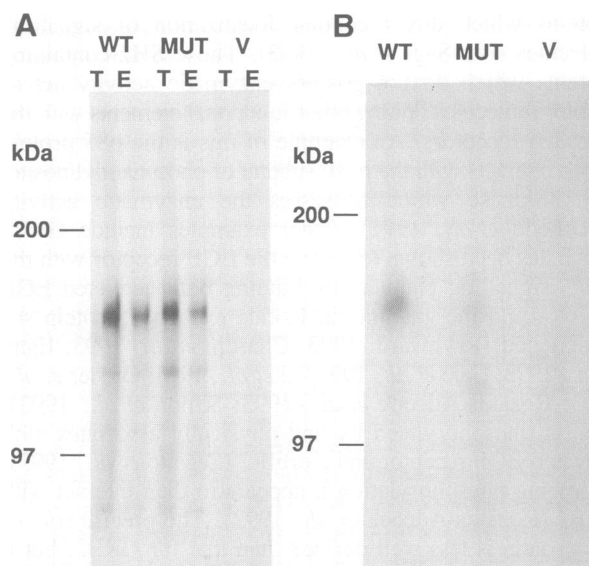

Fig. 1. Comparison of wild-type and mutant c-erbB-3 kinase activity *in vitro*. The wild-type (WT) and mutant (MUT) c-erbB-3 proteins were expressed at similar levels as determined by immunoprecipitation from metabolically labelled COS-1 cells using serum 49.3 (**A**). T and E refer to the total precipitated protein released by SDS, and that which may be eluted by addition of 49.3 peptide, respectively. Kinase assays performed on immune-complexes from COS-1 cells transfected with wild-type c-erbB-3 (WT), mutant c-erbB-3 (MUT) and vector (V) are shown (**B**).

The autocatalytic activity of wild-type and mutant c-erbB-3 was also compared *in vivo* (Figure 2). COS-1 cells, which express ~50 000 EGF receptors per cell (Livneh *et al.*, 1986), were transfected with wild-type and mutant c-erbB-3. The transfected cells expressed a similar quantity of endogenous EGF receptors and c-erbB-3 protein, as determined by immunoprecipitation from [^{35}S]methionine-labelled cells (data not shown).

c-erbB-3 immunoprecipitates were analysed by immunoblotting with an anti-phosphotyrosine antibody. The extent of phosphorylation of the mutant c-erbB-3 protein was

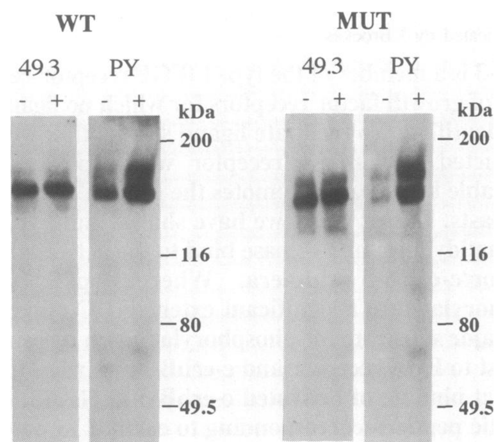

Fig. 2. Comparison of wild-type and mutant c-erbB-3 kinase activity *in vivo*. Wild-type (WT) and mutant (MUT) c-erbB-3 proteins were transiently expressed in COS-1 cells. c-erbB-3 protein was precipitated from unstimulated cells (−) or cells which had been stimulated with EGF (+) using polyclonal antibody 49.3. Bound proteins were eluted with 49.3 peptide (2.5 mg/ml), and analysed by immunoblotting with antibody 49.3 to compare the expression levels of the constructs (49.3), and with an anti-phosphotyrosine antibody (PY).

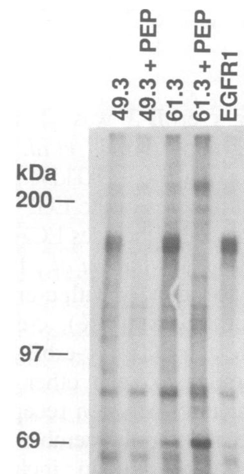

Fig. 3. Expression of EGF receptor/c-erbB-3 chimera in NIH 3T3 cells. To test for correct expression of the EGFR/c-erbB-3 chimera, protein was precipitated from a 9 cm plate of [^{35}S]methionine-labelled EGFR/c-erbB-3-3T3 cells (clone 18) using an antibody against the EGFR extracellular domain (EGFR1), an anti-peptide antibody reacting with the juxtamembrane domain of c-erbB-3 (61.3) and an anti-peptide antibody reacting downstream of the kinase domain of c-erbB-3 (49.3). Reaction with anti-peptide antibodies was inhibited by prior incubation with the corresponding peptide (PEP).

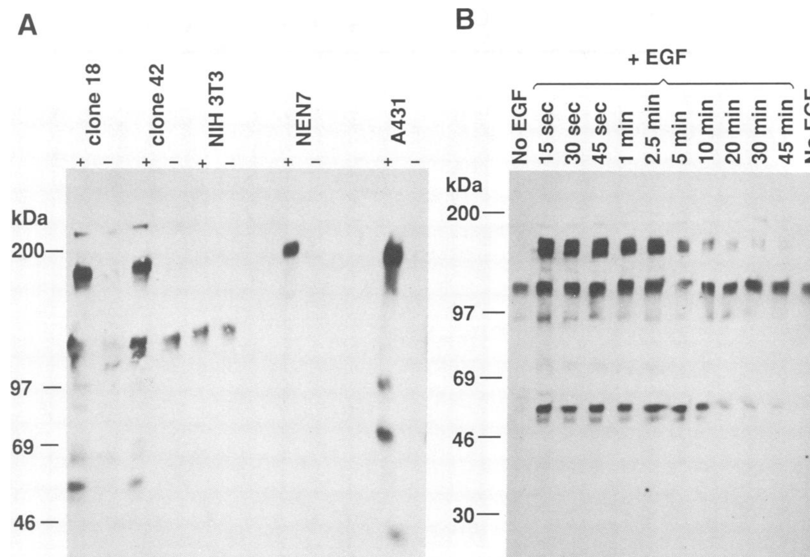

Fig. 4. EGF stimulation of phosphorylation. (**A**) Whole cell lysates of stimulated EGFR/c-erbB-3-3T3 cells were analysed by SDS–PAGE and immunoblotting with anti-phosphotyrosine antibodies. (+) and (−) refer to EGF-stimulated and unstimulated cells, respectively. In the case of NEN7 and A431 cells, one tenth of the amount of protein was used. (**B**) EGFR/c-erbB-3-3T3 cells were incubated with EGF for the indicated times. Phosphorylation was terminated rapidly by the addition of boiling sample buffer to cells which were analysed by SDS–PAGE and immunoblotting with anti-phosphotyrosine antibody.

reduced relative to the wild-type. Phosphorylation of both mutant and wild-type c-erbB-3 was increased by treatment of the cells with EGF prior to lysis.

Construction and expression of EGFR/c-erbB-3 chimera in COS-1 and NIH 3T3 cells

To generate a ligand-activatable system, the EGFR/c-erbB-3 chimera was fused just inside the cell membrane at a common *Nar*I site. When expressed in COS-1 cells, the protein was correctly located at the cell surface as deduced by precipitating the EGFR/c-erbB-3 protein from intact and lysed cells with antibodies specific for the intracellular and extracellular domains (Kumar *et al.*, 1991). The ability of the chimeric protein to bind EGF was confirmed by cross-linking of [^{125}I]EGF to intact cells followed by immunoprecipitation (data not shown). Stable NIH 3T3 cell lines expressing EGFR/c-erbB-3 were created and analysed by binding assays using [^{125}I]EGF to determine the number of receptors per cell (Aharonov *et al.*, 1978). Clones 18 and 42, possessing ~130 000 and 70 000 receptors per cell, respectively, were selected for functional studies. These receptor numbers were verified by comparative FACS analysis using the EGFR1 mAb to the common EGFR extracellular domain to label the cells, and NEN7 cells expressing 2×10^6 EGFR/c-erbB-2 chimeric molecules per cell as a standard (Lehväslaiho *et al.*, 1989). To check that the expressed protein contained the complete c-erbB-3 cytoplasmic domain, antibodies recognizing two distinct sites within the c-erbB-3 kinase domain (Prigent *et al.*, 1992) were used to precipitate the EGFR/c-erbB-3 protein, and the peptides against which the antibodies were raised were used to specifically compete for c-erbB-3 binding (Figure 3). The EGFR/c-erbB-3 chimera migrated at ~180 kDa on SDS–polyacrylamide gels.

Table I. Anchorage-independent growth of EGFR/c-erbB-3-3T3 cells

	Colonies per 30 000 cells		
	0 nM EGF	3 nM EGF	30 nM EGF
Chimera clone 18	1 ± 1	86 ± 16	78 ± 1
Chimera clone 42	50 ± 16	205 ± 2	1011 ± 69
NIH 3T3 mock transfected	1 ± 1	2 ± 1	3 ± 1

Clones 18 and 42 expressing the EGFR/c-erbB-3 chimera and mock-transfected NIH 3T3 cells were suspended in 1.2% Methocel in the presence and absence of EGF (3 and 30 nM). Colonies >0.1 mm diameter were scored after 2 weeks. Data shown are the results of one representative experiment.

EGF stimulation of phosphorylation in NIH 3T3 cells expressing the EGF receptor/c-erbB-3 chimera

EGF stimulated the phosphorylation of the chimeric protein in clones 18 and 42 as deduced by immunoblotting of whole cell lysates with anti-phosphotyrosine antibodies (Figure 4A). The identity of the stimulated 180 kDa chimeric protein was confirmed by immunoprecipitation of the phosphoprotein with antibodies specific for EGFR and c-erbB-3 (data not shown). For comparison, NEN7 cells expressing the EGFR/c-erbB-2 chimera and A431 cells expressing 2×10^6 EGF receptors per cell were used. When 10-fold less total protein was loaded in the case of NEN7 and A431 cells, a similar signal on the anti-phosphotyrosine immunoblot was obtained, suggesting that the degree of receptor stimulation obtained for the three family members is comparable. Stimulation of c-erbB-3 autophosphorylation was maximal after 15 s and decreased to almost basal level after 45 min, probably due to dephosphorylation by phosphatases and/or down-regulation of the receptor (Figure 4B). Moreover, an

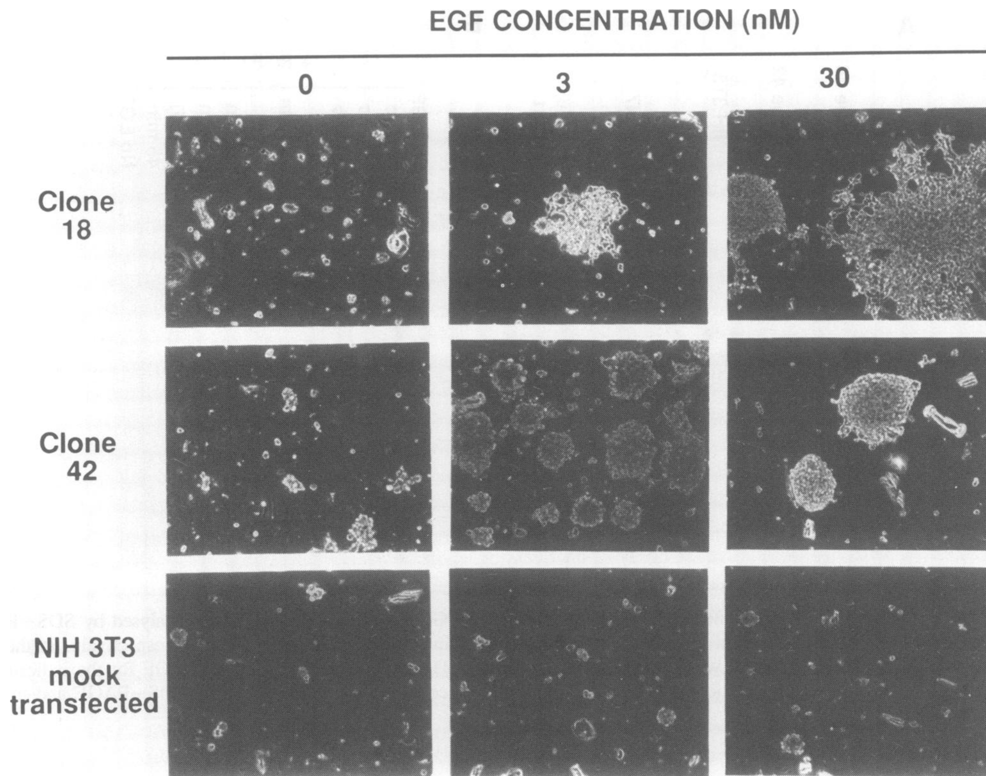

Fig. 5. Growth of colonies in 1.2% Methocel. Clones 18 and 42 expressing the EGFR/c-erbB-3 chimera and mock-transfected NIH 3T3 cells were suspended and grown in 1.2% Methocel. Colonies were photographed after 3 weeks. The panels correspond to 0.5×0.6 mm fields.

additional band of ~54 kDa was seen consistently in the EGFR/c-erbB-3-3T3 cells on repeated experiments (Figure 4B). This phosphoprotein was absent in EGF-stimulated NIH 3T3 cells and unstimulated EGFR/c-erbB-3-3T3 cells, and appeared in parallel to the activated c-erbB-3 protein, suggesting that it may be a major substrate for c-erbB-3.

Anchorage-dependent and -independent growth of NIH 3T3 cell lines expressing the EGF receptor/ c-erbB-3 chimera

EGF addition to EGFR/c-erbB-3-3T3 cells enabled them to grow in an anchorage-independent manner, as determined by their ability to grow in 1.2% Methocel. Clone 42 showed a higher basal level of transformation than clone 18, which only formed colonies in the presence of EGF (Table I). Clones 18 and 42 formed larger colonies in the presence of 30 nM EGF as compared with 3 nM EGF (Figure 5). For comparison, NEN7 and mock-transfected NIH 3T3 cells were also cultured in 1.2% Methocel. Although some small colonies were observed for mock-transfected NIH 3T3 cells, these did not increase in number or size in response to EGF. In the case of NEN7 cells, colonies were only observed in the presence of EGF as described previously (Lehväslaiho *et al.*, 1989). The growth-promoting effect of EGF on clones 18 and 42 was confirmed by [^{3}H]thymidine incorporation studies. Using cells growing attached as monolayers, EGF stimulation resulted in a 3.9-fold increase in thymidine incorporation at 0.5−5.0 nM EGF relative to the unstimulated cells. NIH 3T3 cells showed a maximal 2.7-fold stimulation in response to EGF at the same concentrations (data not shown).

Association of activated EGFR/c-erbB-3 with SHC

Since the EGF receptor/c-erbB-3 chimera was able to promote a growth response in NIH 3T3 cells, we were interested to determine which elements of established SH2 signalling pathways were involved. To determine whether SHC associated with activated c-erbB-3, NIH 3T3 cells expressing an EGFR/c-erbB-3 chimera (clone 18 as described above) were stimulated with EGF. To specifically precipitate EGFR/c-erbB-3 protein, lysates from $\sim 2\times10^6$ cells were incubated with an antibody raised against the intracellular portion of c-erbB-3 (49.3). This eliminated the possibility of cross-reaction with the small amount of EGF receptor present in the NIH 3T3 cells. Since the p52 and p46 forms of SHC migrate at the same position as immunoglobulin heavy chain, total precipitates could not readily be analysed by immunoblotting due to the cross-reaction of immunoglobulin heavy chain with the second layer of the immunoblot. To avoid this cross-reaction, EGFR/c-erbB-3 chimera protein was eluted from the antibody−protein A−Sepharose complex using a solution of peptide against which antibody 49.3 was raised. Eluates were analysed by SDS−PAGE and immunoblotting using a rabbit polyclonal antibody recognizing the three forms of SHC (Figure 6A). For comparison, total lysate from $\sim 10^5$ cells was analysed similarly (Figure 6B). The three forms of SHC (p66, p52 and p46) associated specifically with activated c-erbB-3, although the molecular weights of p46 and p52 appeared to be slightly higher on this gel system. The predominant form present was p52, which also appeared to be the most abundant form present in total cell lysates. No SHC protein was present in precipitates from the parental NIH 3T3 cell line, even when using the EGFR1 antibody

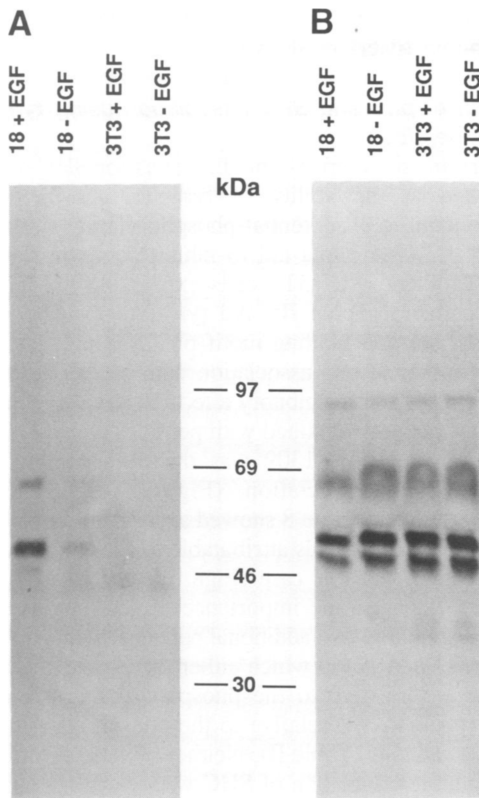

Fig. 6. Association of activated c-erbB-3 with SHC. (**A**) NIH 3T3 cells and transfected NIH 3T3 cells expressing $\sim 10^5$ EGF receptor/c-erbB-3 chimeric receptors per cell (clone 18) were incubated for 2 min with 10^{-6} M EGF. Cells were lysed and EGFR/c-erbB-3 protein was precipitated with an antibody specific for c-erbB-3 (49.3). Precipitated proteins were eluted with the corresponding 49.3 peptide against which the antibody was raised, and eluates were analysed by SDS–PAGE and immunoblotting using an antibody recognizing SHC. (**B**) For comparison, total cell lysate from 10^5 cells was also analysed.

specific for the extracellular domain of EGF receptor (data not shown), and only a very small amount of the p52 form was found associated with EGFR/c-erbB-3 in the basal state. This probably represents association with a small proportion of EGFR/c-erbB-3 which is phosphorylated in the unstimulated state.

Association of EGFR/c-erbB-3 with tyrosine phosphorylated proteins

Immunoblots which had been analysed for the presence of SHC as described above were stripped and reprobed with an anti-phosphotyrosine antibody (Figure 7). The major phosphorylated protein comigrated exactly with the p52 form of SHC, and bands corresponding to p46 and p66 were also detectable. Some other proteins of apparent molecular weights 85, 110 and 125 kDa were detectable.

Association of EGFR/c-erbB-3 with PI3-kinase.

Immunoprecipitates prepared from EGF-stimulated cells using antibody 49.3 were analysed for the association of PI3-kinase by immunoblotting with an antibody specifically reacting with the p85 subunit of the enzyme. As was found in the case of SHC, p85 interacts with the activated EGFR/c-erbB-3 receptor, and to a greatly reduced extent with the chimeric receptor from unstimulated cells (Figure 8A). p85 was also found associated with EGF receptor precipitated

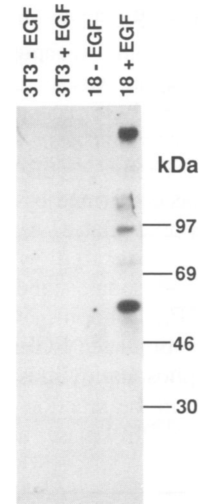

Fig. 7. Association of tyrosine-phosphorylated proteins with activated c-erbB-3. Immunoprecipitations were performed on lysates from EGF-stimulated cells exactly as described in Figure 6A. Eluted proteins were analysed by SDS–PAGE and immunoblotting with an anti-phosphotyrosine antibody.

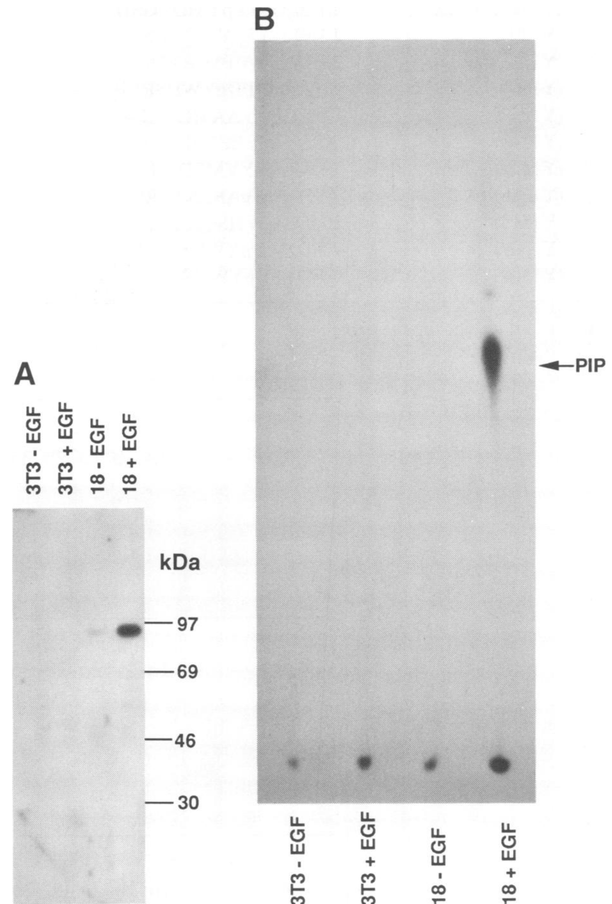

Fig. 8. Association of PI3-kinase with activated c-erbB-3. (**A**) Immunoprecipitations were performed on lysates from EGF-stimulated cells exactly as described in Figure 6A. Eluted proteins were analysed by SDS–PAGE and immunoblotting with a rabbit polyclonal antibody recognizing the p85 subunit of PI3-kinase. (**B**) Immunoprecipitations were performed on lysates from EGF-stimulated cells exactly as described in Figure 5A. Precipitated proteins bound to protein A–Sepharose were incubated with PI and [γ-^{32}P]ATP as described and analysed by TLC. A PI phosphate standard (PIP) prepared by phosphorylating PI with purified PI3-kinase migrated at the position indicated.

from EGF-stimulated EGFR-3T3 cells, which express an equivalent number of EGF receptors, using antibody EGFR1; however, the signal was several-fold less than that obtained with the activated EGF receptor/c-erbB-3 chimera, and was comparable with that seen in unstimulated cells expressing the EGF receptor/c-erbB-3 chimera (data not shown). This result was confirmed by assay for PI3-kinase activity in the immunoprecipitates. A phosphorylated lipid (R_f: 0.57), which comigrated with a ^{32}P-labelled phosphatidylinositol 3-phosphate standard (provided by Dr R.Woschowlski, ICRF, London), was produced when precipitates from EGF-stimulated EGFR/c-erbB-3-3T3 cells were incubated with phosphatidylinositol and [γ-^{32}P]ATP (Figure 8B). Inclusion in the reaction buffer of 1% Triton X-100, which inhibits PI3-kinase activity and favours PI4-kinase activity, inhibited the formation of this phospholipid (data not shown).

Table II. Sequences of c-erbB-3 phosphopeptides

1a	(Y1241, Y1243)	TTPDEDpYEpYMNRQR
1b	(Y1243)	TTPDEDYEpYMNRQR
2a	(Y1178, Y1180)	DEDEEpYEpYMNRRRR
2b	(Y1180)	DEDEEYEpYMNRRRR
3a	(Y1203, Y1205)	LEELGpYEpYMDVGSD
3b	(Y1205)	LEELGYEpYMDVGSD
4	(Y1035)	SPSSGpYMPMNQGNL
5	(Y1309)	DSAFDNPDpYWHSRLF
6	(Y1257)	GPGGDpYAAMGACPA
7	(Y1270)	ASEQGpYEEMRAFQG
8	(Y1140)	EDVNGpYVMPDTHLK
9	(Y1288)	APHVHpYARLKTLRS
10	(Y1113)	RGDSApYHSQRHSLL
11[a]	(Y1309)	AFDNADpYWHS
12[a]	(Y1309)	AFDAPDpYWHS

[a]Data are not shown for these peptides.

Inhibition of p85 and SHC association using tyrosine phosphopeptides

To determine the c-erbB-3 binding sites for SHC and p85, we looked at the ability of tyrosine phosphopeptides corresponding to all potential phosphorylation sites within the c-erbB-3 C-terminal tail to inhibit complex formation in permeabilized NIH 3T3 cells expressing the EGFR/c-erbB-3 chimera (Table II). All tyrosine phosphopeptides containing the p85 binding motif pYXXM (1a, 2a, 3a, 4, 6 and 7) inhibited p85 association with c-erbB-3, whereas other peptides had no inhibitory effect (Figure 9B). Complete inhibition was only obtained with peptides 4 and 7. Peptide 5 corresponding to Y1309 was the only peptide which inhibited SHC association (Figure 9C). The lane corresponding to peptide 8 showed a slightly reduced signal for SHC; however this is attributable to reduced loading of EGFR/c-erbB-3 in this case (Figure 9A).

To further assess the importance of residues in peptide 5 for SHC binding, two additional peptides were synthesized (peptides 11 and 12) in which either the asparagine residue (position −3 relative to the phosphotyrosine) or proline residue (position −2 relative to the phosphotyrosine) was changed to alanine (Table II). Neither of these peptides was able to inhibit association of SHC with c-erbB-3 under the conditions tested (data not shown).

Comparison between EGF receptor, EGFR/c-erbB-2 and EGFR/c-erbB-3 with respect to binding to a GRB2−GST fusion protein

EGFR/c-erbB-3 protein from EGF-stimulated EGFR/c-erbB-3-3T3 cells failed to associate with GRB2−GST fusion

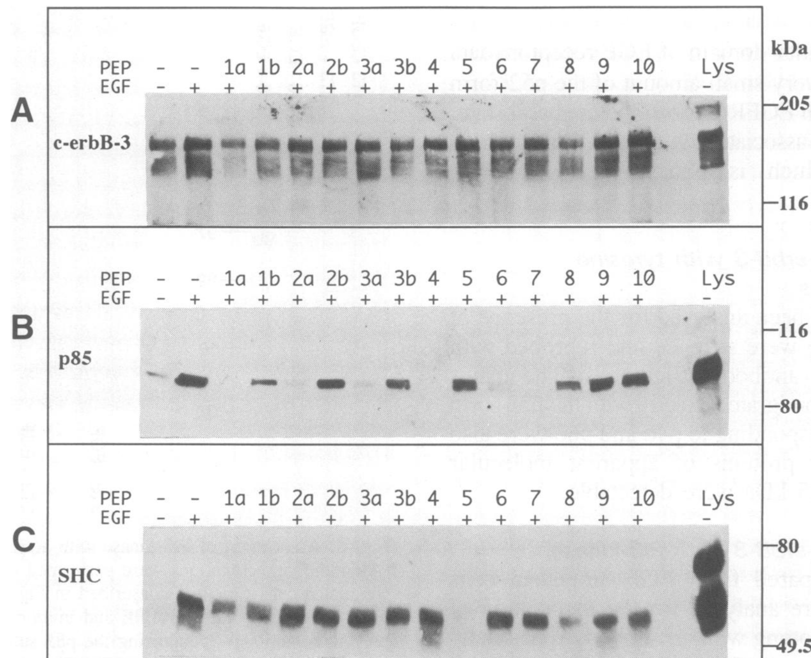

Fig. 9. Inhibition of p85 and SHC association with c-erbB-3 using synthetic phosphopeptides. NIH 3T3 cells expressing the EGF receptor/c-erbB-3 chimera were permeabilized in the presence of 70 μM phosphopeptides as indicated. Cells were stimulated for 4 min with EGF (5×10^{-7} M), protein from cell lysates was precipitated with the 49.3 antibody, eluted with peptide 49.3 and analysed by SDS−PAGE and immunoblotting with antibodies specific for c-erbB-3 (A), p85 (B) and SHC (C). Peptide sequences are as indicated in Table II.

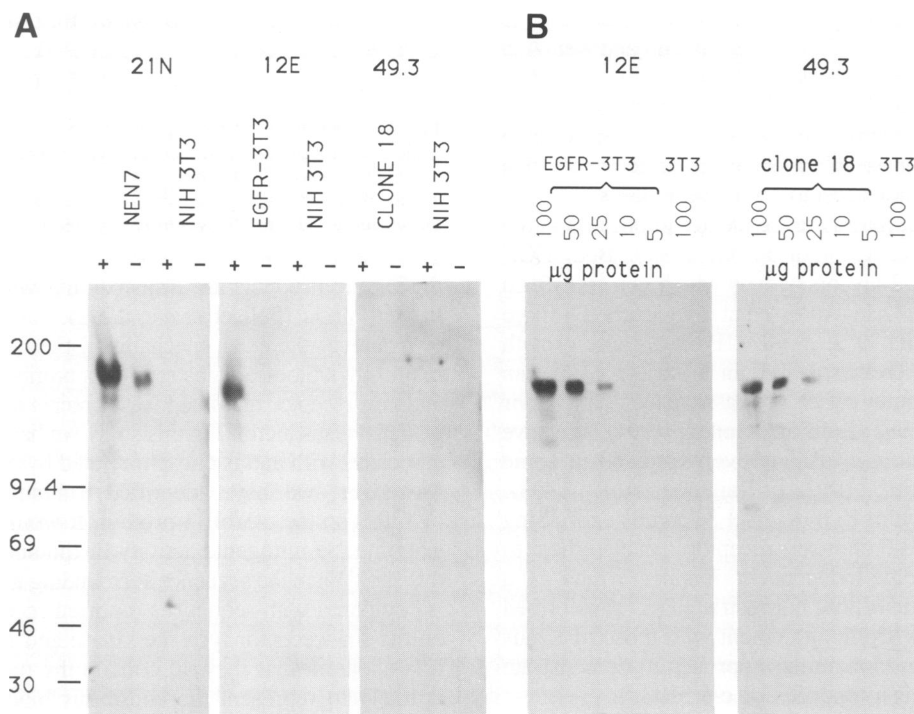

Fig. 10. Association of EGFR/c-erbB-3 with a GRB2−GST fusion protein. (**A**) Cell lysates from EGF-stimulated (+) and unstimulated (−) cells (NEN7, EGFR-3T3 and EGFR/c-erbB-3-3T3; clone 18) were incubated with a GRB2−GST fusion protein immobilized on glutathione-agarose. Bound proteins were analysed by SDS−PAGE and immunoblotting with antibodies 21N, 12E and 49.3, which specifically recognize c-erbB-2, EGF receptor and c-erbB-3, respectively. (**B**) Cell lysates were prepared from EGFR-3T3, clone 18 and NIH 3T3 cells. Antibodies 12 E and 49.3 were used for immunoblotting of varying quantities of total cellular protein, as indicated.

protein immobilized on glutathione-agarose as determined by immunoblotting with polyclonal antibody 49.3 (Figure 10A). For comparison, EGF receptor protein and EGFR/c-erbB-2 chimera protein were precipitated from EGF-treated EGFR-3T3 and NEN7 cells, respectively, with the immobilized GRB2−GST fusion protein, and in these cases a prominent band was observed by immunoblotting with antibodies 12E and 21N, respectively. No immunoreactive protein could be precipitated from EGF-stimulated NIH 3T3 cells, or from unstimulated EGFR-3T3 cells, although a small amount of EGFR/c-erbB-2 protein was precipitated from unstimulated NEN7 cells suggesting a somewhat elevated basal level of phosphorylation of this protein in these cells. The sensitivity of antibodies 12E and 49.3 was compared by immunoblotting of lysates prepared from EGFR-3T3 and EGFR/c-erbB-3-3T3 cells (clone 18). Both cell lines express ~100 000 receptors per cell, and when equivalent amounts of total cell protein were analysed, similar signals were obtained by immunoblotting (Figure 10B).

Discussion

It has been recognized for many years that all tyrosine kinases possess certain conserved features, the most evident of these being a Gly-X-Gly-X-X-Gly motif in subdomain I and a lysine residue in subdomain II (Hanks *et al.*, 1988). In addition, both serine/threonine and tyrosine kinases contain a number of invariant or almost invariant residues, which include a glutamate residue in subdomain III and an aspartate residue in subdomain VI, both of which are different in c-erbB-3 (Kraus *et al.*, 1989; Plowman *et al.*, 1990). Evidence from the crystal structure of cAMP-dependent protein kinase (cAPK) suggests that the substituted aspartate in c-erbB-3 (corresponding to Asp166 of cAPK) normally forms part of the catalytic loop and may in fact function as the catalytic base (Knighton *et al.*, 1991). The carbonyl group of the glutamate residue which is absent in c-erbB-3 (Glu91 in cAPK) is in close proximity to the MgATP binding site. In the light of the differences in these apparently critical residues, it was of interest to determine whether or not c-erbB-3 possesses catalytic activity.

Our findings indicate that c-erbB-3 possesses a very low level of autocatalytic activity in the unstimulated state, and that by replacing Asn815 with aspartate, and His740 with glutamate, no increase in catalytic activity is observed. By replacing the extracellular domain with a ligand-activatable EGF receptor domain, one can, however, clearly detect phosphorylation of the chimeric receptor upon EGF stimulation. This approach has been used previously to characterize receptors for which no ligands were available (Riedel *et al.*, 1984; Lehväslaiho *et al.*, 1989; Seedorf *et al.*, 1991). It is unlikely that this phosphorylation could be due to cross-phosphorylation by EGF receptor as NIH 3T3 fibroblasts express a very low number (~3000) of EGF receptors per cell (Di Fiore *et al.*, 1987a), and EGF-stimulated autophosphorylation of endogenous EGF receptor in NIH 3T3 cells could not be detected. It is, however, possible that a proportion could be attributed to *trans*-phosphorylation by EGF receptor. These observations demonstrate that the aspartate residue found to be essential for the function of other kinases (Moran *et al.*, 1988; Tan *et al.*, 1990) may be altered to an asparagine in c-erbB-3

without inhibiting autocatalytic activity. It would be of some interest to investigate the effect of the His/Glu and Asn/Asp mutations on the catalytic activity of the activatable EGFR/c-erbB-3 chimera. Since the catalytic site of c-erbB-3 may be structurally different from other kinases, it may be possible to design tyrosine kinase inhibitors more selective for this receptor than for other receptor tyrosine kinases.

Tyrosine kinase receptors are frequently implicated in the growth of human cancers (Aaronson, 1991). Both EGF receptor and c-erbB-2 are oncogenes which can transform NIH 3T3 fibroblasts when expressed at high levels (Di Fiore *et al.*, 1987b; Hudziak *et al.*, 1987). Both of these growth factor receptors are overexpressed in a variety of human cancers, and in some cases are associated with poor prognosis (Gullick, 1991; Lofts and Gullick, 1991). We have shown, likewise, that c-erbB-3 is overexpressed in some cancers (Lemoine *et al.*, 1992a,b; Rajkumar *et al.*, 1993). In this study, addition of EGF to NIH 3T3 cells expressing EGF receptor/c-erbB-3 chimeric receptors enabled them to grow in an anchorage-independent manner which is a property characteristic of, but not restricted to, transformed cells. These findings are in agreement with those of Kraus *et al.* (1993), who used a similar approach to demonstrate the growth-promoting properties of c-erbB-3.

Since c-erbB-3 was able to couple with the signalling elements in NIH 3T3 cells to promote a growth response, we were interested in identifying some of the proteins involved. The identification of src homology domains has facilitated rapid advances in our understanding of the mechanism by which tyrosine kinases signal (Pawson and Schlessinger, 1993). Signalling pathways are now being defined by interactions of SH2 domains with distinct phosphotyrosine residues within defined consensus sequences. It now seems possible that at least part of the network of events following receptor activation might be predicted from the primary amino acid sequence of the receptor. Most receptors appear to signal through p21^{ras}. A number of reports have shown that GRB2 links the activated EGF receptor to the nucleotide exchange factor of p21^{ras}, sos, thereby localizing it close to the membrane. This is achieved by interaction of the SH2 domain of GRB2 with Y1068 of the EGF receptor, and the SH3 domain with a proline-rich motif of *sos* (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993).

An additional protein, SHC, has been shown to be phosphorylated in response to EGF and to associate with GRB2. However, it has been shown that SHC does not form part of the complex of sos, EGF receptor and GRB2 (Buday and Downward, 1993), suggesting that the complex formed between GRB2 and SHC, in the case of EGF receptor, forms part of a different signalling pathway which may or may not converge with the p21^{ras} signalling pathway as it is currently perceived. Some evidence that SHC may be involved in p21^{ras} signalling is provided by the observation that the differentiation effect of SHC on PC12 cells is prevented by expression in these cells of a dominant negative *Ras* mutant (Pelicci *et al.*, 1993). SHC is phosphorylated in v-Src transformed cells (McGlade *et al.*, 1992), resulting in its binding to the GRB2−sos complex. This association is proposed to stimulate guanine−nucleotide exchange on Ras through an unknown mechanism. In contrast to GRB2,

c-erbB-3	DSAFD**NPDY**WHSRLF
Trk	IE**NPQY**FSDA
Middle T	PSLLS**NPTY**SVMRSH

Fig. 11. Comparison of binding sites for SHC on c-erbB-3, Trk and Middle T antigen. The SHC binding site on Trk was determined using the peptide indicated (Obermeier *et al.*, 1993). Tyrosine 250 of Middle T was identified using site-directed mutagenesis and is contained within the sequence indicated (Dilworth *et al.*, 1994).

SHC gene products are transforming when overexpressed in fibroblasts (Pelicci *et al.*, 1992).

Whatever the exact mechanism, the fact that SHC is the major physiological substrate for a number of both receptor and non-receptor tyrosine kinases points to an important role in signal transduction. In this study we have shown that SHC associates with and is phosphorylated by activated c-erbB-3. Moreover, we have identified a binding site for SHC (Y1309) on the c-erbB-3 protein. It would be of interest to examine the mitogenic activity of mutant c-erbB-3 lacking this site. When the sequence surrounding this tyrosine residue is compared with sequences reported recently to bind SHC on the Trk receptor tyrosine kinase and Middle T antigen, it is apparent that they all contain the motif NPXY which is likely to represent the consensus binding site for SHC (Figure 11). In this study, peptides lacking either Asn(−3) or Pro(−2) were unable to inhibit the association between SHC and c-erbB-3, suggesting that both residues are important for SHC binding. This is the first SH2-containing protein which appears to require the strict conservation of residues N-terminal to the phosphotyrosine residue for specificity.

In contrast to EGF receptor and c-erbB-2, we could not detect binding of c-erbB-3 to GRB2. In this respect, c-erbB-3 shows some similarity to Trk receptors which phosphorylate SHC but do not bind GRB2 (Suen *et al.*, 1993). We conclude that the two potential binding sites predicted by Songyang *et al.* (1993) are (i) not autophosphorylated, (ii) do not bind GRB2, or (iii) they are occupied by another protein. Clearly it is possible that c-erbB-3 employs the pathway thought to be used by v-Src, in that phosphorylation of SHC by c-erbB-3 promotes binding of SHC to the GRB2−sos complex and subsequent activation of p21^{ras}. Indeed, we could precipitate a small amount of SHC from EGF-stimulated 3T3 cells expressing the EGF receptor/c-erbB-3 chimera using the GRB2−GST fusion protein bound to glutathione-agarose (data not shown). The amount of SHC precipitated from these cells with GRB2−GST was less than that observed for EGF-stimulated EGFR-3T3 cells, but significantly more than that precipitated from NIH 3T3 cells. We have not yet investigated whether Ras is activated in these cells in response to EGF.

In addition to SHC association with activated c-erbB-3, we also observed association of the p85 subunit of PI3-kinase. Like SHC and GRB2, p85 has no catalytic domain and acts as an adaptor molecule which links the activated tyrosine kinase with the catalytic p110 subunit of PI3-kinase. p85 possesses two SH2 domains and one SH3 domain. The presence of the p110 subunit in the immune complexes from EGF-stimulated cells was confirmed by an assay for PI3-kinase activity. A protein comigrating with p85 was phosphorylated in response to EGF, but to a lesser extent than SHC. Phosphorylation of p85 has only been observed under conditions where the tyrosine kinase is overexpressed,

and it seems that it is not essential for function (Hu *et al.*, 1992). The role of PI3-kinase in cellular transformation was first suggested by Kaplan *et al.* (1985). PI3-kinase has been shown subsequently to interact with a number of growth factor receptors, including PDGF receptor, CSF-1 receptor and the insulin receptor substrate IRS1. PI3-kinase also associates to some extent with EGF receptor, although it has been shown that p85 alone binds more avidly to EGF receptor than the p85–p110 active complex (Hu *et al.*, 1992). Relatively low levels of PI3-kinase activity have been found associated with activated EGF receptor as compared with PDGF receptor, suggesting that PI3-kinase plays a minor role in EGF receptor signal transduction. The consensus binding site for p85 to phosphorylated tyrosine kinases is now well defined as the sequence YMXM or possibly YXXM. In contrast to EGF receptor, c-erbB-3 contains six potential PI3-kinase binding sites in its C-terminal domain, and one in its kinase domain. Of these, only one is conserved in EGF receptor, c-erbB-2, and c-erbB-4 which is within the kinase domain and is not thought to be phosphorylated in EGFR. We have shown that synthetic peptides corresponding to these sites (Y1035, Y1178, Y1203, Y1241, Y1257 and Y1270) inhibited association of p85 with c-erbB-3, suggesting that they could indeed act as docking sites for p85 if phosphorylated *in vivo*. These data suggest that PI3-kinase may play a more important role in the direct signalling of c-erbB-3 than other family members. The possibility clearly exists, however, for cross-talk via heterodimerization with other type I family members, since we have shown that EGF receptor is able to phosphorylate c-erbB-3 *in vivo*, at least under conditions where they are co-expressed at similar high levels. Other differences in the signalling pathways of the type I family of growth factor receptors may exist, resulting in signalling diversity. Whereas both EGF receptor and c-erbB-2 use phospholipase $C\gamma$ as a substrate, c-erbB-3 contains no potential phospholipase $C\gamma$ binding site (Songyang *et al.*, 1993). Indeed, we were unable to detect binding of $PLC\gamma$ to c-erbB-3 by immunoblotting (data not shown).

It has been shown for PDGF receptor that if the tyrosine residues within the p85 binding motif are mutated to prevent PI3-kinase interaction, the ability of the PDGF receptor to stimulate Ras activity is lost, as is its ability to activate raf and MAP kinase and to stimulate DNA synthesis (Fantl *et al.*, 1992). Others have shown that if the tyrosine residues which bind PI3-kinase (740/751), $PLC\gamma$ (1021), GAP (771) and SH-PTP2 (1009) are all mutated, PDGF is unable to activate Ras or stimulate DNA synthesis. When binding of either PI3-kinase or $PLC\gamma$ was restored, Ras activation and mitogenesis were normal (Valius and Kazlauskas, 1993). These results suggest that the GRB2–sos complex is not the only mechanism for stimulating Ras.

Clearly the processes of signal transduction cannot simply be explained by the interaction of receptors with GRB2. PI3-kinase plays an important role in cell signalling, although the precise targets for the phosphatidylinositol products phosphorylated on the D3 position remain elusive. The role of SHC in signal transduction is even less well defined, but its extensive use as a primary target for tyrosine kinases suggests a vital function for this protein. We have shown that both SHC and PI3-kinase are targets for c-erbB-3. Moreover, we have identified distinct differences in the proteins which interact with closely related tyrosine kinases, in that c-erbB-3 fails to interact with GRB2 in contrast to EGF receptor and c-erbB-2, whereas PI3-kinase appears to interact more strongly with c-erbB-3 than EGF receptor. This suggests a mechanism for achieving signalling diversity since heterodimerization between related family members, which has been shown to occur between EGF receptor and c-erbB-2 (Goldman *et al.*, 1990), could potentially activate a distinct network of events. It will be interesting to investigate the role of cross-talk between c-erbB-3 and other type I growth factor receptors.

Materials and methods

DNA constructs and vectors

The cDNA clone for c-erbB-3 contained in the vector pCDM8 (Invitrogen) was a gift from Dr G.Plowman, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA. This was used for transient expression in COS-1 cells. The EGFR cDNA was a gift from Dr G.Gill, UCSD, CA. For construction of the EGFR/c-erbB-3 chimera, two restriction fragments encoding the extracellular and transmembrane domains of EGF receptor (*Not*I–*Afl*III, encoding amino acids 1–154; *Afl*III–*Nar*I, encoding amino acids 155–647) and a restriction fragment encoding the intracellular portion of c-erbB-3 (*Nar*I–*Xba*I, encoding amino acids 651–1323) were inserted into the vector pRc/CMV for evaluation by transient transfection in COS-1 cells. The chimera construct was then subcloned into an LTR-based vector pLTRpoly (Lehväslaiho *et al.*, 1990) (provided by Dr K.Alitalo, Helsinki, Finland) for transfection into NIH 3T3 fibroblasts.

Cell culture and transfections

COS-1 (Gluzman, 1981), NIH 3T3 (Jainchill *et al.*, 1969) and A431 (Giard *et al.*, 1973) cells were obtained from the American Tissue Culture Collection and NIH 3T3 cells transfected with an EGFR/c-erbB-2 chimera (NEN-7 cells; Lehväslaiho *et al.*, 1989) were a gift from Dr K.Alitalo, University of Helsinki, Finland. NIH 3T3 cells expressing $\sim 10^5$ EGF receptors per cell (EGFR-3T3) were kindly provided by Dr D.Hills, ICRF Oncology Group, Hammersmith Hospital, UK. Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, UK) containing 10% fetal calf serum. 10 μg of plasmid with 1 μg of selection plasmid pRc/CMV (Invitrogen) were used to transfect 9 cm plates of NIH 3T3 fibroblasts using the calcium phosphate method (Graham and van der Eb, 1973). For selection of transfected cells, 2.5 mg/ml G418 (Gibco) was included in the growth medium. Nine centimetre plates of COS-1 cells were transfected with 10 μg of DNA by the DEAE/Dextran method (Selden, 1992).

Selection of NIH 3T3 chimera clones

Transfected NIH 3T3 fibroblasts were labelled with the EGFR1 antibody (10 μg/ml) (Waterfield *et al.*, 1982) and fluorescein-conjugated $F(ab')_2$ rabbit anti-mouse immunoglobulins (1:20 dilution) (DAKOPATTS) and subjected to two rounds of FACS sorting using a Becton Dickinson FACStar^{PLUS} sorter. At each sort, 5% of the most strongly fluorescent cells were selected. Cells were then single-cell cloned and analysed for their ability to bind ^{125}I-labelled EGF (Amersham, UK) as described previously (Aharonov *et al.*, 1978).

Site-directed mutagenesis

A *Sac*I fragment of c-erbB-3 (site in vector to base 3522) was subcloned into M13mp19. Site-directed mutagenesis was performed using the Amersham oligonucleotide-directed mutagenesis kit using the oligonucleotides CGGGCAGCCAGGTCTCTATGCAC (for the Asn to Asp mutation) and CTGCCAATGGCCAGCATTTCATCTG (for the His to Glu mutation) together in the same reaction. The mutant clones were sequenced and the mutated *Bam*HI–*Bgl*II fragment (bases 2382–3000) was replaced in the full-length c-erbB-3 cDNA.

Immune-complex kinase assays

9 cm plates of COS-1 cells transiently transfected with c-erbB-3 or mutant c-erbB-3 containing the His/Glu740 and Asn/Asp815 mutations were lysed and c-erbB-3 protein was immunoprecipitated with polyclonal antibody 49.3 as described previously (Prigent *et al.*, 1992). Immunoprecipitates were washed twice with phosphorylation buffer (50 mM HEPES pH 7.4, 5% glycerol, 0.2% Triton X-100, 150 mM NaCl, 2 mM $MnCl_2$, 12 mM $MgCl_2$, 100 μM sodium orthovanadate) and 49.3 peptide (Prigent *et al.*, 1992) was added to a concentration of 2.5 mg/ml and incubated at 37°C for 10 min to elute c-erbB-3 from the antibody. 10 μCi [γ-^{32}P]ATP

(Amersham, UK) was added for 10 min at room temperature (100 μM final concentration, 1 Ci/mmol). Precipitates were boiled in sample buffer and analysed by SDS−PAGE (Schägger and von Jagow, 1987). Proteins were transferred to Immobilon-P PVDF membranes (Millipore) and subjected to autoradiography. Bands of interest were excised and analysed for phosphoamino acid content as described previously (Kamps and Sefton, 1989). To compare the level of expression of the wild-type and mutant proteins, transfected cells were metabolically labelled in parallel with [^{35}S]methionine, proteins were immunoprecipitated with antibody 49.3, eluted with peptide and electrophoresed on SDS−polyacrylamide gels which were treated with Amplify (Amersham) for fluorography, as described previously (Prigent *et al.*, 1992).

EGF stimulation of whole cells

Cell monolayers in six-well dishes were washed and routinely incubated for 2 min at 37°C with DMEM in the presence or absence of synthetic EGF 1-48 (10^{-6} M), supplied by Dr R.Goodlad, ICRF, London, UK. The medium was removed and cells were lysed in lysis buffer containing phosphatase inhibitors (50 mM Tris pH 7.4, 1% Triton X-100, 5 mM EGTA, 150 mM NaCl, 25 mM benzamidine, 2 mM PMSF, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate). The protein concentration of cell lysates was determined by the Bradford protein assay (Bio-Rad Laboratories Ltd, UK). Defined amounts of protein were analysed by SDS−PAGE and immunoblotting with a rabbit polyclonal anti-phosphotyrosine antibody provided by Dr T.S.Pillay, Royal Postgraduate Medical School, London, UK (Pillay and Makgoba, 1992). To determine the time course for activation of c-erbB-3, cells were incubated in six-well plates over a period of up to 45 min with EGF (10^{-6} M). Cells were lysed in boiling sample buffer at the indicated times to terminate phosphorylation reactions rapidly. Samples were subjected to SDS−PAGE and immunoblotting with anti-phosphotyrosine antibody.

Determination of anchorage-independent growth

Cells were seeded in methyl cellulose (Sigma) suspension (Methocel) as described previously (Lemoine *et al.*, 1989). 35 mm-diameter wells of six-well plates were coated with DMEM containing 0.9% agarose and duplicate wells were seeded with 30 000 cells suspended in DMEM containing 1.2% Methocel and 10% fetal calf serum, in the presence or absence of EGF (3 or 30 nM). Colonies >0.1 mm were scored after 2 weeks.

[^{3}H]Thymidine incorporation

96-well plates were seeded with 1000 cells/well and grown to 80% confluence. The medium was replaced with DMEM containing 0.5% FCS and cells were incubated for 48 h. EGF was added (0.05−50.0 nM final concentration) and cells were incubated for a further 16 h before addition of [^{3}H]thymidine (0.2 μCi/well) for 4 h. Cells were ruptured by repeated freezing and thawing and analysed on an LKB Wallac 1295-001 cell harvester and 1205 Betaplate liquid scintillation counter (LKB Pharmacia, UK).

Immunoprecipitation and immunoblotting

Following EGF stimulation, cells were lysed in 50 mM Tris pH 7.4, 1% Triton X-100, 5 mM EGTA, 150 mM NaCl, 25 mM benzamidine, 2 mM PMSF, 10 mM NaF and 1 mM Na orthovanadate. EGF receptor/c-erbB-3 chimeric protein was precipitated for 2 h with 5 μg of purified rabbit polyclonal antibody 49.3 (Prigent *et al.*, 1992) which reacts specifically with the intracellular domain of c-erbB-3, or EGFR1 which reacts with the extracellular domain of EGF receptor (Waterfield *et al.*, 1982) and 2.5 mg of protein A−Sepharose. Immunoprecipitates were washed extensively with TBS (50 mM Tris pH 7.4 and 150 mM NaCl) containing 0.1% Triton X-100. EGFR/c-erbB-3 protein was then eluted from the protein A−Sepharose at 37°C for 15 min with 40 μl of a 5 mg/ml solution of the 49.3 peptide. Eluates were analysed on 7.5% SDS−polyacrylamide gels as above. Proteins were transferred to nitrocellulose and probed with rabbit antibodies specific for c-erbB-3 (49.3), for the p85 subunit of PI3-kinase (provided by Drs L.Cantley and S.Soltoff, Tufts University School of Medicine, Boston, MA, and commercially available from UBI, New York), antibodies specific for SHC (UBI, New York) or antibodies reacting with phosphotyrosine (provided by Dr T.S.Pillay, RPMS, UK). Immunoblots were developed using the ECL system, (Amersham, UK). When probing with more than one antibody was required, blots were routinely stripped for 1 h at 60°C with 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris, pH 6.8, blocked and reprobed.

PI3-kinase assays

PI3-kinase assays were performed essentially as described previously (Morgan *et al.*, 1990). Briefly, EGFR/c-erbB-3 protein was precipitated from EGF-treated or untreated NIH 3T3 cells or EGFR/c-erbB-3-3T3 cells as described above. Precipitates were then washed twice with 20 mM HEPES pH 7.2, 5 mM $MnCl_2$, 0.13 mM Na orthovanadate and 12.5 mM NaF. Assays were performed in 100 μl of the same buffer containing 0.2 mg/ml phosphatidylinositol, 10 μCi [γ-^{32}P]ATP and 50 μM ATP for 30 min at 37°C. Lipids were extracted as described previously (Morgan *et al.*, 1990), and analysed by TLC using the borate system (Walsh *et al.*, 1991). For inhibition of PI3-kinase, 1% Triton X-100 was included in the reaction buffer.

Inhibition of p85 and SHC binding to EGFR/c-erbB-3 using phosphopeptides

Synthetic peptides, as indicated in Table II, were synthesized by Nicola O'Reilly and Elizabeth Li, Peptide Synthesis Unit, ICRF, London, UK. EGFR/c-erbB-3-3T3 cells were permeabilized in the presence of 70 μM peptide as described previously (Buday and Downward, 1993), and treated with EGF (5×10^{-7} M) for 4 min at room temperature. EGFR/c-erbB-3 protein was precipitated with 49.3 as described above, but in the presence of 70 μM peptide. Bound proteins were eluted with 49.3 peptide (2.5 mg/ml) and analysed by SDS−PAGE and immunoblotting for c-erbB-3, p85 and SHC, as described above.

Formation of receptor−GRB2 complexes in vitro

6 μg of GRB2−GST fusion protein prepared as described previously (Egan *et al.*, 1993) were bound to 35 μl of a suspension of glutathione agarose (Pharmacia, UK) for 30 min at room temperature. Lysate was prepared from 175 cm^2 monolayers of cells [NIH 3T3, NEN7, EGFR-3T3 and EGFR/c-erbB-3-3T3 (clone 18 as described in Results)], which had been treated with EGF (10^{-6} M) for 4 min, and from untreated cells. Cell lysate was incubated for 2 h at 4°C with GRB2−GST−glutathione agarose complexes, precipitates were washed extensively with TBS/0.1% Triton X-100 and bound proteins were eluted in sample buffer and analysed by SDS−PAGE and immunoblotting. Rabbit polyclonal antibodies 12 E (Gullick *et al.*, 1985), 21 N (Gullick *et al.*, 1987) and 49.3 (Prigent *et al.*, 1992) were used for detection of EGF receptor, EGF receptor/c-erbB-2 chimera protein, and EGF receptor/c-erbB-3 chimera protein, respectively. The sensitivity of antibodies 49.3 and 12 E were also compared by probing immunoblots of whole cell lysate from EGFR-3T3 and EGFR/c-erbB-3-3T3 cells (clone 18).

Acknowledgements

We are grateful to Dr G.Plowman for supplying the c-erbB-3 clone, to Dr G.Gill for supplying the EGFR clone, to Dr K.Alitalo for supplying the NEN-7 cells, to Dr D.Hills for supplying the EGFR-3T3 cells, to Drs L.Cantley and S.Soltoff for providing the anti-p85 antibody and to Dr T.S.Pillay for providing the anti-phosphotyrosine antibody. We wish to thank Dr S.Egan for providing the pGEX vector containing the GRB2−GST sequence. We are also grateful to Dereck Davis and Simon Monard for performing the FACS sorting of NIH 3T3 transfectants, and to Nicola O'Reilly and Elizabeth Li for synthesizing the tyrosine phosphopeptides. We also wish to thank Dr J.Feramisco for allowing some of the experiments to be performed in his laboratory.

References

Aaronson,S.A. (1991) *Science*, **254**, 1146−1152.

Aharonov,A., Pruss,R.M. and Herschman,H.R. (1978) *J. Biol. Chem.*, **253**, 3970−3977.

Bar-Sagi,D., Rotin,D., Batzer,A., Mandiyan,V. and Schlessinger,J. (1993) *Cell*, **74**, 83−91.

Buday,L. and Downward,J. (1993) *Cell*, **73**, 611−620.

Chardin,P., Camonis,J.H., Gale,N.W., Van Aelst,L., Schlessinger,J., Wigler,M.H. and Bar-Sagi,D. (1993) *Science*, **260**, 1338−1343.

Clark,S.G., Stern,M.J. and Horvitz,H.R. (1992) *Nature*, **356**, 340−344.

Di Fiore,P.P., Pierce,J.H., Fleming,T.P., Hazan,R., Ullrich,A., King,C.R., Schlessinger,J. and Aaronson,S.A. (1987a) *Cell*, **51**, 1063−1070.

Di Fiore,P.P., Pierce,J.H., Kraus,M.H., Segatto,O., King,C.R. and Aaronson,S.A. (1987b) *Science*, **237**, 178−182.

Dilworth,S.M., Brewster,C.E.P., Jones,M.D., Lanfrancone,L., Pelicci,G. and Pelicci,P.G. (1994) *Nature*, **367**, 87−90.

Egan,S.E., Giddings,B.W., Brooks,M.W., Buday,L., Sizeland,A.M. and Weinberg,R.A. (1993) *Nature*, **363**, 45−51.

Escobedo,J.A., Navankasattusas,S., Kavanaugh,W.M., Milfay,D., Fried,V.A. and Williams,L.T. (1991) *Cell*, **65**, 75−82.

Fantl,W.J., Escobedo,J.A., Martin,G.A., Turck,C.W., del Rosario,M., McCormick,F. and Williams,L.T. (1992) *Cell*, **69**, 413−423.

Feng,G.-S., Hui,C.-C. and Pawson,T. (1993) *Science*, **259**, 1607–1611.
Freeman,R.M.,Jr, Pluzky,J. and Neel,B.G. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11239–11243.
Gale,N.W., Kaplan,S., Lowenstein,E.J., Schlessinger,J. and Bar-Sagi,D. (1993) *Nature*, **363**, 88–92.
Giard,D.J., Aaronson,S.A., Todaro,G.J., Arnstein,P., Kersey,J.H., Dosik,H. and Parks,W.P. (1973) *J. Natl Cancer Inst.*, **51**, 1417–1423.
Gluzman,Y. (1981) *Cell*, **23**, 175–182.
Goldman,R., Ben Levy,R., Peles,E. and Yarden,Y. (1990) *Biochemistry*, **29**, 11024–11028.
Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **52**, 456–467.
Gullick,W.J. (1991) *Br. Med. Bull.*, **47**, 87–98.
Gullick,W.J., Downward,J. and Waterfield,M.D. (1985) *EMBO J.*, **4**, 2869–2877.
Gullick,W.J., Berger,M.S., Bennett,P.L.P., Rothbard,J.B. and Waterfield,M.D. (1987) *Int. J. Cancer*, **40**, 246–254.
Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42–52.
Hu,P., Margolis,B., Skolnik,E.Y., Lammers,R., Ullrich,A. and Schlessinger,J. (1992). *Mol. Cell. Biol.*, **12**, 981–990.
Hudziak,R.M., Schlessinger,J. and Ullrich,A. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7159–7163.
Jainchill,J.L., Aaronson,S.A. and Todaro,G.J. (1969) *J. Virol.*, **4**, 549–553.
Kamps,M.P. and Sefton,B.M. (1989) *Anal. Biochem.*, **176**, 22–27.
Kaplan,D., Schaffhausen,B., Cantley,L. and Roberts,T. (1985) *Nature*, **315**, 239–242.
Knighton,D.R., Zheng,J., Ten Eyck,L.F., Ashford,V.A., Xuong,N.-H., Taylor,S.S. and Sowadski,J.M. (1991) *Science*, **253**, 407–414.
Kraus,M.H., Issing,W., Miki,T., Popescu,N.C. and Aaronson,S.A. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 9193–9197.
Kraus,M.H., Fedi,P., Starks,V., Muraro,R. and Aaronson,S.A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2900–2904.
Kumar,R., Shepard,H.M. and Mendelsohn,J. (1991) *Mol. Cell. Biol.*, **11**, 979–986.
Lehväslaiho,H., Lehtola,L., Sistonen,L. and Alitalo,K. (1989) *EMBO J.*, **8**, 159–166.
Lehväslaiho,H., Sistonen,L., diRenzo,F., Partanen,J., Comoglio,P., Hölttä,E. and Alitalo,K. (1990) *J. Cell Biochem.*, **42**, 123–133.
Lemoine,N.R., Mayall,E.S., Jones,T., Sheer,D., McDermid,S., Kendall-Taylor,P. and Wynford-Thomas,D. (1989) *Br. J. Cancer*, **60**, 897–903.
Lemoine,N.R. *et al.* (1992a) *Br. J. Cancer*, **66**, 1116–1121.
Lemoine,N.R., Lobresco,M., Leung,H., Barton,C., Hughes,C.M., Prigent,S.A., Gullick,W.J. and Klöppel,G. (1992b) *J. Pathol.*, **168**, 269–273.
Li,N. *et al.* (1993) *Nature*, **363**, 85–88.
Livneh,E., Benveniste,M., Prywes,R., Felder,S., Kam,Z. and Schlessinger,J. (1986) *J. Cell Biol.*, **103**, 327–31.
Lofts,F.J. and Gullick,W.J. (1991) In Dickson,R.B. and Lippman,M.E. (eds), *Genes, Oncogenes and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer*. Kluwer Academic Publishers, Boston, USA, Vol. III, pp. 161–179.
Lowenstein,E.J. *et al.*, (1992) *Cell*, **70**, 431–442.
McGlade,J., Cheng,A., Pelicci,G., Pelicci,P.G. and Pawson,T. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8869–8873.
Moran,M.F., Koch,C.A., Sadowski,I. and Pawson,T. (1988) *Oncogene*, **3**, 665–672.
Morgan,S.J., Smith,A.D. and Parker,P.J. (1990) *Eur. J. Biochem.*, **191**, 761–767.
Obermeier,A., Lammers,R., Weismuller,K.-H., Jungs,G., Schlessinger,J. and Ullrich,A. (1993) *J. Biol. Chem.*, **268**, 22963–22966.
Olivier,J.P. *et al.* (1993) *Cell*, **73**, 179–191.
Pawson,T. and Schlessinger,J. (1993) *Curr. Biol.*, **3**, 434–442.
Pelicci,G. *et al.* (1992) *Cell*, **70**, 93–104.
Pelicci,G. *et al.* (1993) *J. Cell. Biochem.*, **Suppl. 17A**, 238.
Pillay,T.S. and Makgoba,M.W. (1992) *FEBS Lett.*, **308**, 38–42.
Plowman,G.D., Whitney,G.S., Neubauer,M.G., Green,J.M., McDonald,V.L., Todaro,G.J. and Shoyab,M. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4905–4909.
Plowman,G.D., Culouscou,J.-M., Whitney,G.S., Green,J.M., Carlton,G.W., Foy,L., Neubauer,M.G. and Shoyab,M. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1746–1750.
Prigent,S.A., Lemoine,N.R., Hughes,C.M., Plowman,G.D., Selden,C. and Gullick,W.J. (1992) *Oncogene*, **7**, 1273–1278.
Rajkumar,T., Gooden,C.S.R., Lemoine,N.R. and Gullick,W.J. (1993) *J. Pathol.*, **170**, 271–278.
Riedel,H., Dull,T.J., Schlessinger,J. and Ullrich,A. (1984) *Nature*, **324**, 68–70.
Rozakis-Adcock,M. *et al.* (1992) *Nature*, **360**, 689–692.
Rozakis-Adcock,M., Fernley,R., Wade,J., Pawson,T. and Bowtell,D. (1993) *Nature*, **363**, 83–85.
Schägger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 368–379.
Seedorf,K., Felder,S., Millauer,B., Schlessinger,J. and Ullrich,A. (1991) *J. Biol. Chem.*, **266**, 12424–12431.
Segatto,O., Pelicci,G., Giuli,S., Digiesi,G., Di Fiore,P.P., McGlade,J., Pawson,T. and Pelicci,P.G. (1993) *Oncogene*, **8**, 2105–2112.
Selden,R.F. (1992) In Ausubell,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman.J.G., Smith,J.A. and Struhl,K. (eds), *Current Protocols in Molecular Biology*. Vol. 2, John Wiley and Sons, New York, NY, pp. 9.2.1–9.2.6.
Shen,S.-H., Bastien,L., Posner,B.I. and Chretien,P. (1991) *Nature*, **352**, 736–739.
Simon,M.A., Dodson,G.S. and Rubin,G.M. (1993) *Cell*, **73**, 169–177.
Songyang,Z. *et al.* (1993) *Cell*, **72**, 767–778.
Stahl,M.L., Ferenz,C.R., Kelleher,K.L., Kriz,R.W. and Knopf,J.L. (1988) *Nature*, **332**, 269–272.
Suen,K.-L., Bustelo,X.R., Pawson,T. and Barbacid,M. (1993) *Mol. Cell. Biol.*, **13**, 5500–5512.
Tan,J.C., Nocka,K., Ray,P., Traktman,P. and Besmer,P. (1990) *Science*, **247**, 209–212.
Trahey,M. *et al.* (1988) *Science*, **242**, 1697–1700.
Valius,M. and Kazlauskas,A. (1993) *Cell*, **73**, 321–334.
Vogel,W., Lammers,R., Huang,J. and Ullrich,A. (1993) *Science*, **259**, 1611–1614.
Walsh,J.P., Caldwell,K.K. and Majerus,P.W. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9184–9187.
Waterfield,M.D., Mayes,E.L.V., Stroobant,P., Bennett,P.L.P., Young,S., Goodfellow,P.N., Banting,G.S. and Ozanne,B. (1982) *J. Cell. Biochem.*, **20**, 149–161.

Received on October 1, 1993; revised on April 15, 1994