ErbB3 (HER3) interaction with the p85 regulatory subunit of phosphoinositide 3-kinase

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ErbB3 (HER3), a unique member of the ErbB receptor family, lacks intrinsic protein tyrosine kinase activity and contains six Tyr-Xaa-Xaa-Met (YXXM) consensus binding sites for the SH2 domains of the p85 regulatory subunit of phosphoinositide 3-kinase. ErbB3 also has a proline-rich sequence that forms a consensus binding site for the SH3 domain of p85. Here we have investigated the interacting domains of ErbB3 and p85 by a unique application of the yeast two-hybrid system. A chimaeric ErbB3 molecule containing the epidermal growth factor receptor protein tyrosine kinase domain was developed so that the C-terminal domain of ErbB3 could become phosphorylated in the yeast system. We also generated several ErbB3 deletion and Tyr → Phe site-specific mutants, and observed that a single ErbB3 YXXM motif was necessary and sufficient for the association of

ErbB3 with p85. The incorporation of multiple YXXM motifs into the ErbB3 C-terminus enabled a stronger ErbB3/p85 interaction. The proline-rich region of ErbB3 was not necessary for interaction with p85. However, either deletion or mutation of the p85 SH3 domain decreased the observed ErbB3/p85 association. Additionally an ErbB3/p85 SH3 domain interaction was detected by an assay *in vitro*. These results were consistent with a model in which pairs of phosphorylated ErbB3 YXXM motifs co-operate in binding to the tandem SH2 domains of p85. Although a contributing role for the p85 SH3 domain was suggested, the N- and C-terminal SH2 domains seemed to be primarily responsible for the high-affinity association of p85 and ErbB3.

INTRODUCTION

ErbB (HER) family receptors regulate mammalian cell survival, proliferation and differentiation in response to growth factors such as epidermal growth factor (EGF) and heregulin [1,2]. ErbB3 (HER3) is unique among ErbB family members in that it is devoid of intrinsic protein tyrosine kinase activity [3,4] as well as being the only family member that contains multiple consensus sites in its C-terminal domain for binding the p85 subunit of phosphoinositide (PI) 3-kinase. Although ErbB3 lacks kinase activity, Tyr residues within its cytoplasmic tail become phosphorylated on its heterodimerization with other ErbB family receptors, such as the EGF receptor (EGFR) or ErbB2, which allows ErbB3 to associate with PI 3-kinase. The EGF-dependent phosphorylation of ErbB3 triggers ErbB3/p85 association and increases the level of PI 3-kinase activity that is co-immunoprecipitated with ErbB3 [5]. Likewise, when ErbB3 is phosphorylated by EGFR *in vitro* and added to cell lysates, ErbB3 co-immunoprecipitates with both p85 and the catalytic activity of the p110 subunit of PI 3-kinase [6]. Additionally it has been shown that in mouse fibroblasts transfected with ErbB2 and ErbB3 cDNA species, PI 3-kinase activity and mitogenesis increase in a heregulin-dependent manner, with both responses being dependent on the presence of ErbB3 [7]. PI 3-kinase activation by the ErbB2/ErbB3 co-receptor complex might also mediate heregulin-induced proliferation in mammary epithelial cells [8].

The p85 regulatory subunit of PI 3-kinase contains five distinct

domains: an SH3 domain, a Bcr-homology region, two SH2 domains, and an inter-SH2 (IS) domain. The IS domain has been shown to be the region of p85 responsible for high-affinity interaction with the p110 catalytic subunit of PI 3-kinase [9–13]. The proline-rich Bcr domain exhibits sequence similarity with the breakpoint cluster region gene product [14] and has been implicated in an intramolecular interaction with the p85 SH3 domain [15], as well as in mediating intermolecular associations with the SH3 domains of Src protein tyrosine kinase family members [16–19].

The amino acid sequence motif Tyr-Xaa-Xaa-Met (YXXM) has been shown, when phosphorylated, to specifically associate with the SH2 domains of p85 [20,21]. Six such consensus binding sites for the SH2 domains of p85 occur at the C-terminus of ErbB3; if phosphorylated, they could potentially mediate the association of ErbB3 and p85. Synthetic ErbB3-derived phosphopeptides that contain phosphorylated YXXM sequences have been shown to inhibit the association of p85 with ErbB3 in the context of a chimaeric EGFR/ErbB3 receptor [22]. However, it is not known which specific ErbB3 YXXM motifs are phosphorylated *in vivo*, and whether the C-terminal, the N-terminal, or both p85 SH2 domains account for p85 association with ErbB3.

The C-terminal tail of ErbB3 also contains a proline-rich sequence, PPRPPRP, that forms a consensus binding site for the SH3 domains of both p85 and Src-family protein tyrosine kinases [23–25] and could also contribute to the association of ErbB3 with p85. SH3 domains recognizing PXXP amino acid motifs in

Abbreviations used: cSH2, C-terminal p85 SH2 domain; EGF, epidermal growth factor; EGFR, EGF receptor; ER/B3, chimaeric EGFR/ErbB3 construct; GST, glutathione S-transferase; IS, inter-SH2 domain; n/cSH2, contiguous nSH2, IS and cSH2 domains; nSH2, N-terminal p85 SH2 domain; PI, phosphoinositide.

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signalling proteins have been shown to mediate protein–protein interactions independently as well as in co-operation with SH2 domains. Interestingly, it has been observed that the SH3 domain of p85 can interact with either of two proline-rich motifs [PPTPKRPPRP (residues 84–96) and PAPALPPKPPK (residues 303–314)] contained within the Bcr region of p85 itself [15]. This intramolecular interaction could cause conformational changes within p85 that regulate its association with other proteins or modulate the catalytic activity of the associated p110 catalytic subunit of PI 3-kinase [15].

The yeast two-hybrid system is a genetic approach for studying protein–protein interactions [26], which has been used to study the interaction between p85 and the insulin and insulin-like growth factor 1 receptors [27,28] as well as the interaction between the p85 and p110 subunits of PI 3-kinase [10]. Here we describe a novel application of the yeast two-hybrid system in mapping the interacting domains of p85 and ErbB3.

EXPERIMENTAL

Yeast strains and expression plasmids

The yeast two-hybrid system reporter strain SFY526 (MAT α ura3-52 his3- Δ 200 ade2-801 trp-901 leu2-3,112 can^r gal4-542 gal80-538 URA3::GAL1-lacZ) and the respective Gal4 DNA-binding domain (pGBT9) and transcriptional activation domain (pGAD424) vectors have been described [26]. Various pGAD-p85 vectors [10] were kindly provided by the laboratory of Dr. Jeffrey Pessin (University of Iowa, Iowa City, IA, U.S.A.). The rat ErbB3 cDNA sequence has been described previously [29]. The cDNA encoding the protein tyrosine kinase domain of the human EGFR (amino acid residues 647–972) was excised from pMMTV-ER [30] and subcloned into the yeast two-hybrid expression vector pGBT9 to yield pGBT-EGFR Δ CT. The cDNA encoding the C-terminal tail of ErbB3 (residues 938–1339) was subsequently subcloned in-frame with the EGFR protein tyrosine kinase domain sequence by use of an *Acl*I restriction site conserved in both EGFR and ErbB3 cDNA sequences to generate pGBT-ER/B3. pGBT-ER(K/M)/B3 was generated by subcloning into pGBT-ER/B3 a cDNA encoding an EGFR protein tyrosine kinase domain with a Lys-721 $\rightarrow$ Met mutation that abolished kinase activity [31]. pGBT-ER/B3 Δ 1121 was made by a *Sac*II/*Sal*I restriction digestion and subsequent religation of the plasmid. pGBT-ER/B3 Δ 1204 and pGBT-ER/B3 Δ 1237 were made by PCR amplification of truncated sequences by using the forward primer 5'-ATATATAGCGAGATCTCCTCC-3' and the reverse primers 5'-ATGTCGACGCTACCCCTTCTCTT-3' or 5'-ATGTCGACTCTCTGTGTACTGCC-3' respectively. These primers incorporated *Bgl*II and *Sal*I restriction sites into the amplified sequences that allowed them to be subcloned in-frame with the pGBT-ER/B3 protein tyrosine kinase domain plasmid. The pGBT vector conveniently provides in-frame stop codons for all three deletion mutants. The pGBT-ER/B3- Δ Pro, -1051Y, -1194Y, -1051/1194Y, -1051/1194F, -6F and pGAD-p85-D21N site mutants were generated by using the ExSite mutagenesis method (Stratagene, La Jolla, CA, U.S.A.).

β -Galactosidase activity assays

Quantitative assays of β -galactosidase activity were performed as described by Fields and Song [26], except that Chlorophenol Red β -D-galactopyranoside (Sigma) was used as the chromogenic substrate and the absorbance was determined at 574 nm. One unit of β -galactosidase activity was defined as $(1000 \times A_{574}) / [A_{600} \times \text{volume (ml)} \times \text{time (min)}]$ as described by Miller [32]. All

results are expressed as means $\pm$ S.E.M. for at least four independent assays performed on distinct colonies.

Immunoblotting

Yeast cells were grown in 50 ml of selective medium overnight; they were then pelleted, washed in 1 ml of water and resuspended in 250 μ l of 20 % (w/v) trichloroacetic acid. Glass beads (500 μ m) were added to the samples, which were then vortex-mixed six times for 30 s each time. The trichloroacetic acid precipitates were neutralized with 250 μ l of acetone/ammonium hydroxide (5:0.3, v/v), after which 400 μ l of 1 % (w/v) SDS was added and the samples were boiled for 7 min. Lysates were transferred to fresh tubes containing 100 μ l of 5 $\times$ sample buffer, and 50 μ l of each sample was subjected to SDS/PAGE. Resolved proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.), which was blocked with 5 % (w/v) dry milk in PBS then immunostained with ErbB3-specific (2C3; NeoMarkers, Fremont, CA, U.S.A.), GAL4(DBD)-specific (RK5C1, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or p85 N-terminal SH2 domain-specific (Upstate Biotechnology, Lake Placid, NY, U.S.A.) antibodies. Membranes were incubated with horseradish peroxidase-conjugated anti-(mouse Ig) and detected by an enhanced chemiluminescence method (Amersham, Chicago, IL, U.S.A.).

Binding assays *in vitro*

Several cDNA species encoding N-terminal domains of p85 were amplified by PCR and expressed as glutathione S-transferase (GST) fusion proteins, which were purified as described previously [33]. An Asp-21 $\rightarrow$ Asn amino acid substitution was introduced into the SH3 domain by use of the ExSite mutagenesis method (Stratagene). Each GST or GST-p85 peptide (2 nmol) was incubated in buffer A [20 mM sodium Hepes (pH 7.4)/50 mM NaCl/10 % (v/v) glycerol] supplemented with 1.8 mM MnCl₂, 0.02 % (w/v) Triton X-100, and 2.0 pmol of purified recombinant ErbB3 cytosolic domain (hisTKD-B3) [4] for 15 min on ice (total volume 200 μ l). The mixture was added to glutathione-agarose (1:1 suspension in buffer A) and incubated for 15 min at 4 °C. The agarose suspensions were centrifuged for 5 s at 600 g. The pellets were washed in 500 μ l of ice-cold buffer A and then suspended in 110 μ l of SDS/PAGE sample buffer. Samples (20 μ l) were resolved by SDS/PAGE and subjected to immunoblotting with ErbB3 antibody. GST-p85 peptides (910 pmol) were also analysed by SDS/PAGE with Coomassie Blue staining.

RESULTS

Generation of a chimaeric yeast two-hybrid construct to examine the interaction between ErbB3 and p85

Analysis of the deduced amino acid sequence of the ErbB3 C-terminal phosphorylation domain reveals six consensus binding sites for the N-terminal and C-terminal SH2 domains of p85, as well as a proline-rich candidate ligand for the p85 SH3 domain. To examine the interaction between p85 and ErbB3 with the two-hybrid system, we generated a pGBT9 yeast two-hybrid vector expressing the cytosolic domain of ErbB3 (residues 660–1339). The ErbB3 cytosolic domain did not seem to interact with p85 (Figure 1), presumably owing to the lack of intrinsic kinase activity in ErbB3 and the absence of an associating kinase capable of phosphorylating ErbB3 in yeast. To overcome the

		β -galactosidase (U)
ER TKD	ER TKD	0.0
B3 TKD	B3 CT	0.0
ER TKD	B3 CT	93.8 $\pm$ 1.2
ER K/M	B3 CT	0.0

Figure 1 Interaction of an EGFR/ErbB3 chimaeric construct (ER/B3) with the p85 protein in the yeast two-hybrid system

The yeast reporter strain SFY526 was co-transformed with the indicated pGBT-receptor and pGAD-p85 plasmid constructs, and transformants were assayed for β -galactosidase reporter gene activity. The EGFR protein tyrosine kinase domain (ER TKD, residues 647–972) and ErbB3 cytosolic domain (residues 660–1339) are shown schematically. ER/B3 is a chimaera composed of the EGFR protein tyrosine kinase domain (residues 647–918) fused to the phosphorylation domain of the ErbB3 protein (residues 938–1339) and expressed via the pGBT vector. A conserved Lys residue in the EGFR protein tyrosine kinase domain essential for protein kinase activity was replaced with Met in the ER(K/M)/B3 construct. The pGAD-p85 construct expresses the full-length p85 subunit of PI 3-kinase as a GAD fusion protein [10]. Abbreviation: U, units.

lack of phosphorylation in the yeast system, we developed an EGFR/ErbB3 chimaeric construct (ER/B3) containing a functional EGFR protein tyrosine kinase domain in place of the inactive ErbB3 protein tyrosine kinase homology domain. In this construct, the ErbB3 C-terminus was fused to the end of the EGFR protein tyrosine kinase domain in a manner that preserved the conserved amino acid sequences at the domain boundary. By itself, the EGFR kinase domain (ER TKD, residues 647–972) was incapable of association with p85 (Figure 1). However, ER/B3 strongly associated with p85, apparently in a phosphory-

lation-dependent manner, as a kinase-deficient ER/B3 construct, ER(K/M)/B3, failed to interact with p85 (Figure 1).

To ensure that ER/B3 could not activate the *lacZ* reporter gene independently of p85, ER/B3 was transfected both alone and with a pGAD vector not containing a p85 cDNA insert. No activation of the *lacZ* reporter gene was observed in either case (results not shown). Similarly, the pGAD-p85 construct did not activate the reporter gene alone or in combination with an empty pGBT vector (results not shown). Western immunoblotting revealed that the GBT-fusion proteins were expressed at similar levels (see Figure 4A), which suggested that differences in protein interactions with p85 were not due to differences in protein expression levels. Taken together, these results demonstrated a specific interaction between ErbB3 and p85 within the yeast two-hybrid system.

Interaction between ER/B3 and p85 subdomains

To determine which regions of p85 associated with ErbB3, we tested the interaction of the SH3, Bcr and dual SH2 domains (n/cSH2) of p85 with ER/B3. The n/cSH2 domain exhibited the strongest interaction of the three domains. However, this interaction was less than half of that of full-length p85 (Figure 2). The Bcr and SH3 domains of p85 seemed to associate very weakly with ER/B3. Even though the SH3 domain of p85 exhibited a minimal interaction when tested alone, we suspected that the SH3 of p85 domain might be capable of a weak interaction with a proline-rich motif in ErbB3 and contribute to the maximally observed interaction between p85 and ErbB3, which seemed to be mediated predominantly by the two SH2 domains of p85. To test this hypothesis we generated a mutant p85 protein (p85-D21N) with an Asp $\rightarrow$ Asn substitution at Asp-21, which occurs within the SH3 domain of p85. Other groups have shown that this Asp $\rightarrow$ Asn mutation in the p85 SH3 domain disrupts the specific association between the SH3 domain and proline-rich recognition sequences [24,25]. We found that

Figure 2 Interaction between ErbB3 and domains of the p85 protein

The yeast reporter strain SFY526 was co-transformed with pGBT-ER/B3 and the indicated pGAD-p85 plasmid constructs. p85-D21N has an Asp-21 $\rightarrow$ Asn substitution in the SH3 domain of p85. Distinct constructs expressing individual p85 domains as GAD fusion proteins are indicated: n/cSH2 (residues 330–720), Bcr (residues 77–353), SH3 (residues 9–87), nSH2/IS (residues 330–621), nSH2 (residues 330–434), IS/cSH2 (residues 429–720), cSH2 (residues 621–720), and IS (residues 429–621). Abbreviation: U, units.

Lysates of yeast transfected with pGBT and pGAD expression vectors were immunoblotted to detect the expression of the yeast two-hybrid fusion proteins. **(A)** Left panel: detection of the approx. 90 kDa GBT-fusion proteins with an antibody recognizing the C-terminus of ErbB3. Right panel: detection of wild-type GBT-ErbB3 (approx. 90 kDa) and ER/B3 truncation mutants (approx. 75, 65 and 50 kDa) with an antibody specific for the Gal4 DNA-binding domain. **(B)** Detection of ER/B3 Tyr → Phe site mutants. **(C)** Detection of GAD-p85 constructs with an antibody recognizing the N-terminal SH2 domain of p85.

Figure 5 Association *in vitro* of GST-p85-SH3 domain fusion proteins with ErbB3

To investigate the role of the p85 SH3 domain in ErbB3/p85 interactions, GST fusion proteins containing either the p85 SH3 domain (GST-SH3, residues 1–80), the mutant SH3-D21N domain (GST-SH3*), the SH3 domain with a small proline-rich region of the Bcr domain appended (GST-SH3-pro, residues 1–101), or the contiguous SH3 and Bcr domains (GST-SH3-bcr, residues 1–339) were generated. (A) GST-p85 fusion proteins (2.0 nmol of each) were incubated with a recombinant ErbB3 cytosolic domain protein (2.0 pmol) and samples of glutathione-agarose precipitates were subsequently resolved by SDS/PAGE. Association of the ErbB3 protein with precipitated GST or GST-p85 fusion proteins was detected by immunoblotting with an ErbB3-specific antibody. (B) GST and GST-p85 fusion proteins (0.9 nmol of each) analysed by SDS/PAGE with Coomassie Blue staining.

p85-D21N exhibited a weaker association than wild-type p85 with ER/B3. Interestingly, the p85-D21N and n/cSH2 domain associations with the ER/B3 construct were comparable. The p85-D21N and n/cSH2 domain proteins were expressed at a level similar to or higher than wild-type p85 (see Figure 4C). These results suggested that the tandem SH2 domains of p85 are predominantly responsible for the observed ErbB3/p85 interaction but also suggested that the SH3 domain might have a role in the association of p85 with ErbB3.

Association of the N-terminal and C-terminal SH2 domains of p85 with ER/B3

We next tested whether both SH2 domains were required for the binding of p85 to ErbB3. The individual N-terminal (nSH2) and

C-terminal (cSH2) SH2 domains were capable of binding to ErbB3, with or without the IS domain (Figure 2). Inclusion of the IS domain increased the interaction between either nSH2 or cSH2 and ErbB3, perhaps by altering the conformation of the SH2 domains, as the IS domain alone did not interact with ErbB3 (Figure 2). The independent SH2 domains, however, bound much more weakly to ER/B3 than the dual SH2 domain, although comparable expression of these proteins was observed (see Figure 4C). This suggested that the tandem SH2 domains might participate in the ErbB3/p85 interaction via multiple ErbB3 phosphorylation sites.

Role of the ErbB3 proline-rich sequence in the association of p85

To test whether the proline-rich sequence motif in ErbB3 interacted with the SH3 domain of p85, a mutant ER/B3 construct (ER/B3ΔPro) was generated that lacked the PPRPPRP sequence (residues 1205 to 1211). Surprisingly, ER/B3ΔPro exhibited a slightly stronger interaction than ER/B3 with p85 (Figure 3). This result indicated that any potential interaction of the p85 SH3 domain with the PPRPPRP sequence did not significantly enhance the affinity of the ErbB3/p85 interaction. It is possible that the SH3 domain interacted with a sequence of ErbB3 other than the PPRPPRP sequence deleted. Alternatively, a competing intramolecular association of the SH3 domain with proline-rich sequences within the Bcr domain of p85 might have neutralized the effect of an ErbB3/SH3 interaction (see the Discussion section).

Association of p85 with ER/B3 C-terminally truncated mutants and Tyr → Phe site-specific mutants

We also generated several ER/B3-derived constructs with truncations of the ErbB3 C-terminal domain and the corresponding loss of YXXM motifs from the ErbB3 sequence (Figure 3). One truncated mutant (ER/B3Δ1237) deleted the three most C-terminal YXXM motifs containing Tyr-1257, Tyr-1273 and Tyr-1286. ER/B3Δ1237 mediated a weaker association with p85 than was observed with ER/B3. A more pronounced effect was observed with a truncation (ER/B3Δ1204) that removed an additional YXXM sequence corresponding to the loss of Tyr-1219. A third truncation, which resulted in the loss of Tyr-1194 and left only one YXXM motif (Tyr-1051) in the C-terminal domain of ErbB3, produced ER/B3Δ1121; this associated very weakly with p85. Because the accumulated loss of YXXM motifs correlated with progressively weakened associations between ER/B3 and p85, these results suggested that multiple YXXM motifs in ER/B3 contributed to the association with p85.

To study the roles of YXXM motifs further, we generated a mutant ErbB3 protein (ER/B3-6F) that contained Tyr → Phe substitutions at all six YXXM consensus sites. ER/B3-6F was unable to interact with p85, as expected (Figure 3). Consequently we constructed several YXXM add-back mutants. Here individual Tyr residues previously mutated to Phe in ER/B3-6F were restored to Tyr. Interestingly, the ER/B3-1051Y and ER/B3-1194Y add-back mutants, which contained single YXXM consensus sites for p85 binding, were able to interact with p85, albeit extremely weakly when compared with ER/B3. Furthermore the interaction with p85 was considerably augmented when two YXXM motifs were present in ER/B3, as was observed with the double add-back mutant ER/B3-1051/1194Y. These sites were not unique in interacting with p85 because a double mutant, ER/B3-1051/1194F, which contained Phe residues substituted for Tyr at positions 1051 and 1194, but retained the four C-terminal YXXM consensus sites, was able to mediate a stronger interaction than did ER/B3-1051/1194Y. In fact, as

suggested by analysis of the deletion mutants, it seemed that the larger the number of YXXM sites present in ER/B3, the stronger the interaction was with p85, regardless of the location of the sites. It also seemed from an examination of ER/B3-6F that at least one YXXM motif was required for binding and that other Tyr residues in ErbB3, which occur in consensus binding sites for other SH2 domain-containing proteins [29], could not substitute. Additionally, it seemed that multiple YXXM motifs co-operated in p85 binding, because add-back mutants containing multiple motifs associated in a more than additive manner compared with single-site add-back mutants. The mutant proteins showed expression levels similar to that of ER/B3 (Figures 4A and 4B).

Association *in vitro* of the p85 SH3 domain with ErbB3

To investigate further the role of the p85 SH3 domain in interactions with ErbB3, we generated GST–p85 fusion proteins containing either the p85 SH3 domain (GST–SH3), an SH3 domain carrying the Asp-21 → Asn substitution (GST–SH3*), an SH3 domain with a small proline-rich sequence of the Bcr domain appended (GST–SH3-pro), or the contiguous SH3 and Bcr domains (GST–SH3-bcr) (see Figure 5). GST or GST–p85 fusion proteins were incubated with a recombinant ErbB3 cytosolic domain protein, after which the GST fusion proteins were precipitated with glutathione–agarose. ErbB3 that associated with the GST fusion proteins was detected by SDS/PAGE and Western immunoblotting. Indeed, an interaction was detected between GST–SH3 and ErbB3 (Figure 5), although this interaction was much weaker than that observed with the full-length GST–p85 protein and phosphorylated ErbB3 (results not shown). In contrast, the interaction of the mutant GST–SH3* with ErbB3 was almost undetectable. Interaction of GST–SH3-pro and GST–SH3-bcr with ErbB3 was also much weaker than that of GST–SH3, which suggested that the proline-rich sequences contained in p85 might compete in SH3 domain binding with the proline-rich sequence found in ErbB3.

DISCUSSION

The results of this study show that the association of ErbB3 with the p85 subunit of PI 3-kinase is mediated predominantly by interactions between the SH2 domains of p85 and YXXM motifs within the C-terminus of ErbB3, as previously suggested [22]. Other potential Tyr-phosphorylation motifs in the ErbB3 C-terminus do not seem to substitute for the conserved YXXM consensus p85 binding sites, in contrast with one study in which low-stringency protein–protein interactions were observed between EGFR phosphotyrosine sites and SH2 domain-containing proteins [34]. Our results also suggest that multiple Tyr residues within YXXM motifs can interact with the tandem SH2 domains of p85. Spatial relations between these Tyr residues could in principle determine optimal p85 binding [35]. Tyr-1257, Tyr-1273 and Tyr-1286 are separated by fewer than 20 residues each and could be ideally spaced for associating in concert with tandem SH2 domains [35]. Tyr-1051 and Tyr-1194, however, are separated by 142 residues and, from our results, still seemed to co-operate in binding p85. This suggests flexibility in the binding of p85 tandem SH2 domains to differentially spaced Tyr residues as hypothesized [35], in contrast with the relatively rigid spatial requirement of the tandem SH2 domains of SHP-2 [36] and ZAP-70 [37]. A tandem interaction of the SH2 domain with ErbB3 might optimize PI 3-kinase activation by ErbB3. It has been observed that bisphosphopeptides are more potent in stimulating PI 3-kinase activity when compared with mono-phosphopeptides [38–40].

Differential phosphorylation of Tyr residues in YXXM consensus sites within the ErbB3 C-terminus could ultimately determine which of these sites interact with p85. Little is currently known about the stoichiometry of phosphorylation of these various Tyr residues. Co-operation of the tandem SH2 domains of p85 in high-affinity binding to ErbB3, as we have apparently observed here (see Figures 2 and 3), would require multisite phosphorylation of ErbB3 molecules. However, we have not independently determined which ErbB3 Tyr residues were phosphorylated in the context of the yeast two-hybrid system, or whether individual ErbB3 molecules were phosphorylated on multiple Tyr residues. It is therefore possible that particular YXXM sites were predominantly phosphorylated and responsible for strong ErbB3 associations with p85, or alternatively that Tyr phosphorylation was distributed relatively equally between the YXXM sites. Our preliminary studies of COS7 cells transfected with ErbB3 mutants containing single YXXM sites have shown that p85 is capable of associating with any of the YXXM sites occurring in the ErbB3 C-terminus, which indicates that each of the YXXM sites in the ErbB3 C-terminus is potentially phosphorylated *in vivo* (N. J. Hellyer and J. G. Koland, unpublished work).

The presence of a consensus SH3 domain-binding motif in ErbB3 led us to investigate the roles of this motif and the p85 SH3 domain in ErbB3/p85 interactions. Deletion of the p85 SH3 domain or substitution of an SH3 domain residue critical for peptide recognition significantly decreased the interaction of ER/B3 and p85 (Figure 2). However, a similar effect was observed when examining the interaction of p85 with the insulin receptor cytosolic domain in the yeast system (results not shown), although the insulin receptor possesses no obvious candidate SH3 domain-binding sequences within its cytoplasmic domain. In addition, deletion of the PPRPPRP candidate SH3 domain-binding motif of the ErbB3 C-terminus did not significantly alter the ER/B3 interaction with p85 (Figure 3). Together these results imply that the potential interaction between this ErbB3 proline-rich sequence and the p85 SH3 domain does not contribute significantly to the overall affinity of the ErbB3/p85 interaction.

The decrease in the apparent affinity of the ErbB3/p85 interaction observed on deletion or mutation of the p85 SH3 domain might have been a consequence of the self-association of the p85 SH3 domain and a proline-rich region within the Bcr region of p85 [15], an interaction that could modulate the affinity of p85 SH2 domains for phosphotyrosine-containing substrates. It would also be possible that ligation of p85 SH2 domains by phosphotyrosine-containing peptides could exert a reciprocal effect on the SH3 domain, possibly reversing its intramolecular association with the Bcr domain and enhancing its accessibility to other signalling molecules. In the Ras-Gap/Rho-Gap interaction, it seems that when the tandem SH2 domains of Ras-Gap bind to phosphorylated Tyr residues of Rho-Gap, the conformation of Ras-Gap changes such that there is a 100-fold increase in the accessibility of the target-binding surface of its SH3 domain [41].

Our experiments *in vitro* provided evidence for intramolecular p85 interactions, and furthermore suggested that interactions between the p85 SH3 domain and proline-rich Bcr domain sequences could negate any positive binding contribution attributed to interactions between the p85 SH3 domain and the proline-rich sequence of the ErbB3 (see Figure 5). Hence these results leave open the possibility that ErbB3/p85 SH3 domain interactions do occur and that these interactions are of regulatory significance. For example, the disruption of an intramolecular p85 Bcr/SH3 domain interaction by the ErbB3 proline-rich sequence could modulate the function of p85, perhaps by

regulating its binding to or activation of low-molecular-mass GTP binding proteins such as Cdc42Hs [15,42] or by regulation of the associated p110 catalytic domain.

Other investigators have shown that yeast two-hybrid fusion proteins can be phosphorylated within the yeast two-hybrid system when co-expressed with either the platelet-derived growth factor receptor or Src protein tyrosine kinase domains [43,44]. In those studies, protein tyrosine kinase domains were co-expressed to phosphorylate mammalian signalling proteins that otherwise would not be significantly phosphorylated in yeast cells. This technique allowed investigators to screen for target proteins that interact with phosphorylated Tyr motifs occurring in these signalling proteins. Similarly, our strategy, in which an active protein tyrosine kinase domain was substituted for the otherwise inactive kinase domain of ErbB3, provided us with a functional chimaeric EGFR/ErbB3 protein that was used in mapping the sites of interaction between ErbB3 and the p85 subunit of PI 3-kinase. This strategy could be generally applicable to the investigation of other protein-protein interactions mediated by phosphorylation of Tyr residues. The ER/B3 construct could also be used to screen for novel proteins that interact with ErbB3 by use of the yeast two-hybrid approach.

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REFERENCES

- Alroy, I. and Yarden, Y. (1997) *FEBS Lett.* **410**, 83–86
- Burden, S. and Yarden, Y. (1997) *Neuron* **18**, 847–855
- Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. and Carraway, III, K. L. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8132–8136
- Sierke, S. L., Cheng, K., Kim, H.-H. and Koland, J. G. (1997) *Biochem. J.* **322**, 757–763
- Soltoff, S. P., Carraway, III, K. L., Prigent, S. A., Gullick, W. G. and Cantley, L. C. (1994) *Mol. Cell. Biol.* **14**, 3550–3558
- Kim, H.-H., Sierke, S. L. and Koland, J. G. (1994) *J. Biol. Chem.* **269**, 24747–24755
- Carraway, III, K. L., Soltoff, S. P., Diamonti, A. J. and Cantley, L. C. (1995) *J. Biol. Chem.* **270**, 7111–7116
- Ram, T. G. and Ethier, S. P. (1996) *Cell. Growth Differ.* **7**, 551–561
- Klippel, A., Escobedo, J. A., Hirano, M. and Williams, L. T. (1994) *Mol. Cell. Biol.* **14**, 2675–2685
- Holt, K. H., Olson, A. L., Moye-Rowley, W. S. and Pessin, J. E. (1994) *Mol. Cell. Biol.* **14**, 42–49
- Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M. and Waterfield, M. D. (1994) *EMBO J.* **13**, 511–521
- Hu, P. and Schlessinger, J. (1994) *Mol. Cell. Biol.* **14**, 2577–2583
- Klippel, A., Escobedo, J. A., Hu, Q. and Williams, L. T. (1993) *Mol. Cell. Biol.* **13**, 5560–5566
- Heisterkamp, N., Stam, K., Groffen, J., de Klein, A. and Grosveld, G. (1985) *Nature (London)* **315**, 758–61
- Kapeller, R., Prasad, K., Janssen, O., Hou, W., Schaffhausen, B. S., Rudd, C. E. and Cantley, L. C. (1994) *J. Biol. Chem.* **269**, 1927–1933
- Prasad, K., Janssen, O., Kapeller, R., Raab, M., Cantley, L. C. and Rudd, C. E. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7366–7370
- Prasad, K., Kapeller, R., Janssen, O., Repke, H., Duke, C. J., Cantley, L. C. and Rudd, C. E. (1993) *Mol. Cell. Biol.* **13**, 7708–7717
- Liu, X., Marengere, L., Koch, C. A. and Pawson, T. (1993) *Mol. Cell. Biol.* **13**, 5225–5232
- Vogel, L. B. and Fujita, D. J. (1993) *Mol. Cell. Biol.* **13**, 7408–7417
- McGlade, C. J., Ellis, C., Reedijk, M., Anderson, D., Mbamalu, G., Reith, A. D., Panayotou, G., End, P., Bernstein, A., Kazlauskas, A. et al. (1992) *Mol. Cell. Biol.* **12**, 991–997
- Piccione, E., Case, R. D., Domchek, S. M., Hu, P., Chaudhuri, M., Backer, J. M., Schlessinger, J. and Shoelson, S. E. (1993) *Biochemistry* **32**, 3197–3202
- Prigent, S. A. and Gullick, W. J. (1994) *EMBO J.* **13**, 2831–2841
- Yu, H., Rosen, M. K., Shin, T. B., Seidel, D. C., Brugge, J. S. and Schreiber, S. L. (1992) *Science* **258**, 1665–1668
- Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W. and Schreiber, S. L. (1994) *Cell* **76**, 933–945
- Weng, Z. G., Rickles, R. J., Feng, S. B., Richard, S., Shaw, A. S., Schreiber, S. L. and Brugge, J. S. (1995) *Mol. Cell. Biol.* **15**, 5627–5634
- Fields, S. and Song, O.-K. (1989) *Nature (London)* **340**, 245–247
- Lamotte, B., Buccini, D., Jami, J. and Joshi, R. L. (1995) *FEBS Lett.* **373**, 51–55
- Tartare-Deckert, S., Murdaca, J., Sawka-Verhelle, D., Holt, K. H., Pessin, J. E. and Van Obberghen, E. (1996) *Endocrinology (Baltimore)* **137**, 1019–1024
- Hellyer, N. J., Kim, H.-H., Greaves, C. H., Sierke, S. L. and Koland, J. G. (1995) *Gene* **165**, 279–284
- Clark, A. J. L., Beguinot, L., Ishii, S., Ma, D. P., Roe, B. A., Merlino, G. T. and Pastan, I. (1986) *Biochim. Biophys. Acta* **867**, 244–251
- Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N. and Rosenfeld, M. G. (1987) *Nature (London)* **328**, 820–823
- Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Koland, J. G., O'Brien, K. M. and Cerione, R. A. (1990) *Biochem. Biophys. Res. Commun.* **166**, 90–100
- Soler, C., Beguinot, L. and Carpenter, G. (1994) *J. Biol. Chem.* **269**, 12320–12324
- Ottinger, E. A., Botfield, M. C. and Shoelson, S. E. (1998) *J. Biol. Chem.* **273**, 729–735
- Eck, M. J., Pluskey, S., Trueb, T., Harrison, S. C. and Shoelson, S. E. (1996) *Nature (London)* **379**, 277–280
- Hatada, M. H., Lu, X., Laird, E. R., Green, J., Morgenstern, J. P., Lou, M., Marr, C. S., Phillips, T. B., Ram, M. K., Thieriault, K. et al. (1995) *Nature (London)* **377**, 32–38
- Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S. and Cantley, L. C. (1993) *J. Biol. Chem.* **268**, 9478–9483
- Herbst, J. J., Andrews, G., Contillo, L., Lamphere, L., Gardner, J., Lienhard, G. E. and Gibbs, E. M. (1994) *Biochemistry* **33**, 9376–9381
- Rordorf, N. T., Van, H. D., Chen, D., White, M. F. and Backer, J. M. (1995) *J. Biol. Chem.* **270**, 3662–3666
- Hu, K. Q. and Settleman, J. (1997) *EMBO J.* **16**, 473–483
- Zheng, Y., Bagrodia, S. and Cerione, R. A. (1994) *J. Biol. Chem.* **269**, 18727–18730
- Lioubin, M. N., Algate, P. A., Tsai, S., Carlberg, K., Aebersold, A. and Rohrschneider, L. R. (1996) *Genes Dev.* **10**, 1084–1095
- Keegan, K. and Cooper, J. A. (1996) *Oncogene* **12**, 1537–1544