Analysis of Grb7 Recruitment by Heregulin-activated erbB Receptors Reveals a Novel Target Selectivity for erbB3*

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This paper is available on line at http://www.jbc.org

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Received for publication, August 22, 1997

Heregulin-mediated activation of particular erbB receptor combinations was used as a model system to investigate the interaction of erbB3 and erbB4 with the adaptor protein growth factor receptor-hound (Grb7). In human breast cancer cell lines, co-immunoprecipitation of Grb7 with both receptors was detected upon heregulin stimulation. This association was direct and mediated by the Grb7 Src homology (SH)2 domain. Co-expression of erbB2 with erbB3 point mutants was used to map Grb7 binding sites. This demonstrated that tyrosine 1180 and 1243 represent the major and minor sites of Grb7 interaction, respectively. Although these recognition sequences possess an Asn residue at +2 relative to the phosphotyrosine and therefore represent potential Grb2 binding sites, phosphopeptide competition and “pull-down” experiments demonstrated that they interact preferentially with the Grb7 versus the Grb2 SH2 domain. Substitution analysis indicated that an Arg residue at +3 could act as a selectivity determinant, but the effect was context-dependent. Consequently, the Grb2 and Grb7 SH2 domains possess overlapping, but distinct, specificities. These studies therefore identify Grb7 as an in vivo target of erbB3 and erbB4 and provide an underlying mechanism for the ability of erbB3 to recruit Grb7 and not Grb2, a property unique among erbB receptors.

Recently it has become evident that a complex series of interactions governs signaling by the erbB family of receptor tyrosine kinases. This family currently contains four members, the epidermal growth factor receptor (EGFR)1 or erbB1, erbB2, erbB3, and erbB4, which differ both qualitatively and quantitatively in their signaling potential (1–3) and biological activities (4–7). A variety of ligands exhibit distinct specificities. These studies therefore identify Grb7 as an in vivo target of erbB3 and erbB4 and provide an underlying mechanism for the ability of erbB3 to recruit Grb7 and not Grb2, a property unique among erbB receptors.

This work was supported by research grants from the National Health and Medical Research Council of Australia and the New South Wales State Cancer Council and also by the Australian Government Cooperative Research Centre Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: EGFR, epidermal growth factor receptor; SH, Src homology; PI3-kinase, phosphatidylinositol 3-kinase; Grb; growth factor receptor bound; GST, glutathione-S-transferase; PAGE, polyacrylamide gel electrophoresis; CMV, cytomegalovirus.

factor-α, amphiregulin, and betacellulin, but betacellulin also binds erbB4 (8), and both erbB3 and erbB4 provide receptors for the heregulin/neuregulin 1 and neuregulin 2 families of ligands (9–16). Furthermore, ligand-induced erbB receptor heterodimerization, first detected between the EGFR and erbB2 (17, 18), diversifies the signals that can be generated by particular ligands (6, 19, 20). Importantly, this occurs in a hierarchical fashion and also with directionality (20). In particular, erbB2-containing heterodimers are preferred, and this receptor favors interaction with erbB3. Moreover, heterodimerization is critical for the activity of the erbB3 receptor because it is kinase-impaired and hence reliant on transphosphorylation by other receptors for signal generation (2, 21–23).

Dimerization of erbB receptors leads to kinase activation and phosphorylation of their cytoplasmic domains on specific tyrosine residues, thus creating binding sites for signaling molecules containing phosphotyrosine binding or Src homology (SH)2 domains (24). The latter are conserved noncatalytic regions of approximately 100 amino acids which, along with other modules such as SH3 and pleckstrin homology domains, mediate inter- and intramolecular interactions involved in tyrosine kinase signal transduction. The specificity of the SH2 domain binding is determined by both the residues flanking the phosphotyrosine, in particular the three COOH-terminal amino acids, and the residues in the SH2 domain which interact with these sites (24, 25). For example, the NH2-terminal SH2 domain of the phosphatidylinositol 3-kinase (PI3-kinase) p85 subunit preferentially binds a pYVMVX motif, and the Grb2 SH2 domain recognizes a pYXXN motif (25, 26). Consequently, the signaling specificity of a given receptor tyrosine kinase is determined largely by its repertoire of SH2 domain docking sites and the nature of the proteins that these recruit.

Proteins that contain SH2 domains can be divided into two distinct groups: those that exhibit enzymatic activity and those that contain only noncatalytic protein modules (24, 27). Those in the latter class, which includes Crk, Nck, and Grb2, act as adaptors linking separate catalytic subunits to tyrosine-phosphorylated receptors or other signaling intermediates. For example, Grb2 recruits the Ras GDP-GTP exchange factor Sos (28). A rapidly emerging adaptor subfamily has been described recently which comprises Grb7, Grb10, and Grb14 (29–31). These proteins exhibit significant sequence homology and a common overall architecture, which includes a conserved NH2-terminal proline-rich motif, a central pleckstrin homology domain, and a COOH-terminal SH2 domain. Grb7 is bound by a variety of receptor tyrosine kinases upon ligand stimulation, including erbB2 (32, 33), the platelet-derived growth factor β-receptor (34), and Ret (35), as well as the Shc proteins (32) and the protein tyrosine phosphatase SH-PTP2 (36). Interestingly, this reflects an overlap in Grb2 and Grb7 SH2 domain speci-
ficity (33). In contrast, Grb10 is recruited directly by the insulin receptor (37, 38). However, despite the intriguing structure of these adaptors and their participation in diverse signaling systems, their detailed mechanisms and functions have yet to be identified.

Both the ERBB and GRB7 gene families exhibit differential expression in certain human cancers. Amplification and/or overexpression of ERBB2 occurs in approximately 20% of human breast cancers and correlates with a poor prognosis (39); high expression of ERBB3 and ERBB4 has also been detected in human breast cancer cell lines and primary breast cancers (21, 40–42). GRB7 maps close to ERBB2 on chromosome 17q, resulting in frequent co-amplification of these two genes, and the concomitant overexpression of the two interacting gene products is likely to up-regulate a fundamental receptor tyrosine kinase signaling pathway in human breast cancer (32). However, although the erbB3 and erbB4 receptors represent heterodimerization partners for erbB2 and are commonly expressed at high levels in human breast cancer cells, their interaction with Grb7 has yet to be characterized. We therefore addressed this issue by analyzing Grb7 recruitment by heregulin-activated erbB receptors. This demonstrated that Grb7 represents an in vivo target of the erbB3 and erbB4 receptor tyrosine kinases and identified a signaling function for two previously uncharacterized erbB3 phosphorylation sites. Furthermore, because these sites bound Grb7 and not Grb2, this provides an important insight into the signaling specificity of erbB3 and the SH2 domain selectivity of these adaptor proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The sources and maintenance of the human breast cancer, HEK-293, and HER1–2 cell lines were described previously (31, 34).

**Antibodies**—The following antibodies were used: monoclonal anti-erbB2 (Novocastra, Newcastle, U. K.); polyclonal anti-erbB3 MK4, which was used for immunoprecipitation; monoclonal anti-erbB3 2F12 (44), for Western blotting; polyclonal anti-erbB3 C-17 (Santa Cruz Biotechnology, Santa Cruz, CA), for immunoprecipitation and Western blotting; polyclonal anti-erbB4 (Santa Cruz Biotechnology), for Western blotting; recombinant anti-phosphotyrosine (RC20)-horseradish peroxidase conjugate (Transduction Laboratories, Lexington, KY); polyclonal anti-Grb7 (Transduction Laboratories); polyclonal anti-Shc (Upstate Biotechnology, Lake Placid, NY); and monoclonal anti-GST (Santa Cruz Biotechnology). The monoclonal anti-erbB4 antibody used for immunoprecipitations was produced for our laboratory from a mouse immunized with recombinant erbB4 extracellular domain.

**Cell Lysis and Immunoprecipitation**—For immunoprecipitation experiments, breast cancer cells were grown to 70–80% confluence in either 10-cm tissue culture dishes or T150 flasks. After overnight starvation in serum-free medium, recombinant heregulin β2 (α-amino acids 177–237) (45) was added to a final concentration of 5 nM. After a 10-min exposure to heregulin, the cells were lysed as described previously (46). The protein concentration was estimated using Bio-Rad protein assay reagent. Antibodies (1–2 μg) were incubated with lysates for at least 2 h at 4 °C. The immunocomplexes were then collected by incubation with 20 μl of goat anti-mouse IgG-Sepharose or protein A-Sepharose beads (Zymed Laboratories Inc., South San Francisco, CA) at least 1 h at 4 °C. The immunocomplexes were then collected by centrifugation, washed three times in cold lysis buffer, and subjected to SDS-PAGE. After transfer to nitrocellulose, the samples were Western blotted with the appropriate antibody. Visualization of bound antibody was by enhanced chemiluminescence (NEL Life Science Products). Densitometric analysis of autoradiographs was performed using the IP Lab Gel analysis program (Signal Analytics Corp., Vienna, VA).

**Binding Assays Using GST Fusion Proteins**—GST fusion proteins containing the SH2 domains of Grb2 and Grb7 were described previously (33, 37) and were prepared by standard methodology (48). Approximately 5 μg of fusion protein coupled to glutathione-agarose beads was incubated with 400 μl of lysate from control or hergulin-treated ZR-75-1 cells for 2 h at 4 °C. The beads were then collected by centrifugation and washed three times in cell lysis buffer. Bound proteins were analyzed by Western blot using erbB receptor-specific antibodies.

Direct binding of the Grb7 SH2 domain was demonstrated by a “Far Western” approach. erbB receptors were immunoprecipitated from control or hergulin-treated breast cancer cells, separated by SDS-PAGE, and transferred to nitrocellulose. The filters were incubated with fusion proteins diluted to 1 μg/ml in TBS (10 mM Tris-Cl, pH 7.4, 150 mM NaCl) containing 5% bovine serum albumin and 4 mM dithiothreitol. After washing, bound fusion protein was detected using an anti-GST monoclonal antibody.

**Metabolic Labeling of Cells and Phosphoamino Acid Analysis**—Subconfluent HER1–2 and HER2–3 cells were started over-night in serum-free RPMI medium and then incubated in phosphate-free minimal essential medium (Life Technologies, Inc.) for 2 h. The medium was then replaced with phosphate-free minimal essential medium containing 0.4 μCi/ml 32P (Amersham Australia Proprietary Ltd., Sydney, NSW, Australia) and 20 μl HEPES, pH 7.5. After 3 h the medium was removed and the cells incubated with heregulin (5 nM in phosphate-free minimal essential medium) for 10 min. Control cells received growth factor diluent (0.1% bovine serum albumin in TBS) only. Cells were washed twice with phosphate-buffered saline before preparation of cell lysates.

Immunoprecipitations were performed by incubating the 32P-labeled cell lysates (1.5 mg of protein) with 5 μl of anti-Grb7 antibody for 16 h at 4 °C. Protein A-Sepharose was then added for a further 1 h. Immunoprecipitates were then collected by centrifugation, washed eight times in RIPA buffer (20 mM Tris-Cl, pH 7.6, 300 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and resolved by SDS-PAGE. Two-thirds of each sample was transferred to a polyvinylidene difluoride membrane and exposed for autoradiography while the remaining third was subjected to Western blot analysis. Phosphoamino acid analysis was performed essentially as described (49). The Grb7 band was excised, washed twice in water, and then hydrolyzed for 2 h at 110 °C in 6 M HCl. The samples were dried by vacuum desiccation and then dissolved in 6 μl of 1.9 buffer containing 2 μg of phosphoamino acid standards (Sigma). Thin layer electrophoresis was then performed using 0.1-mm plastic-backed cellulose TLC plates (Merck, Darmstadt, Germany) in a Multiphor II apparatus (Amrad Pharmacia Biotech, Melbourne, Victoria, Australia). The run conditions for the first dimension (pH 1.9) were 1,500 V for 50 min (flatbed plate temperature 16 °C) and for the second dimension (pH 3.5 buffer) 1,000 V for 30 min. After visualization of the standards by ninhydrin staining the plate was exposed for autoradiography.

**Plasmid Constructs and Site-directed Mutagenesis**—To construct a mammalian expression vector for hGrb7, the EcoRI insert containing full-length hGRB7 (hGRB7) was excised from a pUC18 clone. This fragment was then blunt ended with Klenow enzyme and cloned into HindIII-digested, blunt-ended pRCCMV (InVitrogen, Leek, The Netherlands). To generate erbB2 and erbB3 expression vectors, a full-length ERBB2 cDNA clone was subcloned into HindIII-digested pRCCMV, and a cDNA clone encoding full-length erbB3 was subcloned into Xbal/XhoI-digested pCMVneo. Tyrosine to phenylalanine mutations in erbB3 were generated using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to produce erbB3-Y1180F, erbB3-Y1243F, and erbB3-Y1180F,1243F. Correct incorporation of these mutations was verified by DNA sequencing (fpron DNA Cycle Sequencing System, Promega).

**Transient Transfections**—HEK-293 cells stably transfected with hGRB7/pRCMV (HEK/Grb7) cells were used for transient transfection studies. To generate HEK/Grb7 cells, HEK-293 cells were transfected with hGRB7/pRCMV as described previously (33). After selection with Geneticin (500 μg/ml, Life Technologies, Inc.), single clones were analyzed for Grb7 expression by Western blot. One clone expressing a high level of Grb7 was expanded and used for transient transfections. HEK/Grb7 cells were grown to 70–80% confluence in 10-cm tissue culture dishes. The erbB2 expression construct (10 μg) was co-transfected with either the wild-type or a mutant erbB3 expression construct (10 μg) as described previously (33). Control cultures received the empty vectors alone. After 24 h, the medium was replaced with serum-free medium and the cells cultured for a further 24 h. The cultures were then stimulated for 10 min with heregulin (5 nM final concentration) or vehicle alone before preparation of cell lysates in 0.4 μl of lysate buffer.

**Phosphopeptide Compounds**—“Pull-down” assay—Synthetic phosphopeptides were synthesized by Chiron Technologies Proprietary Ltd. (Melbourne, Victoria, Australia) and were as follows; Tyr-1139, PQPQpVPYQNPQ; Q1142R, PQPQpVPYQRNP; Tyr-1196, VENPEpYLT-PQG; Tyr-1180, DEEYEpYMNRR; R1185Q, DEEYEpYMNQR; Tyr-1243, DEDYEpYMNQR, where pY represents a phosphotyrosine residue. These were purified by reverse phase high performance liquid
RESULTS

Heregulin-stimulated Association of Grb7 with erbB Receptors and Shc—Although others have demonstrated a strong association of Grb7 with erbB2 in SK-BR-3 cells (32), breast cancer cell lines exhibiting ERBB2 and Grb7 gene amplification may also overexpress other erbB receptors. For example, SK-BR-3 cells overexpress erbB3 relative to normal breast epithelial cells, whereas BT-474 cells express high levels of erbB3 and erbB4 (41, 51). Because heregulin administration leads to activation and tyrosine phosphorylation of these receptors, serum-starved SK-BR-3 and BT-474 cells were stimulated with this growth factor and Grb7 immunoprecipitates analyzed for the presence of associated proteins by Western blot. Anti-phosphotyrosine immunoblots revealed heregulin-inducible association of Grb7 with tyrosine-phosphorylated proteins of 52 kDa and 180–185 kDa (p180–185, Fig. 1A). The use of specific antibodies identified the former as the p52 isoform of Shc (Fig. 1A). Association of Grb7 with tyrosine-phosphorylated Shc proteins has been observed by other workers (32, 34). The identity of the proteins comprising the p180–185 band was determined by blotting the immunoprecipitates with erbB-specific antibodies (Fig. 1B). Increased levels of erbB2 and erbB3 were found in the complexes isolated from heregulin-treated SK-BR-3 and BT-474 cells. Furthermore, analysis of anti-erbB4 immunoprecipitates from BT-474 cells detected recruitment of Grb7 by this receptor, which was increased upon heregulin administration. The association between erbB2 and Grb7 observed in the absence of ligand administration is the result of significant basal receptor tyrosine phosphorylation often observed in breast cancer cell lines (46).

Previous studies on erbB receptor heterodimerization (20) suggest that in SK-BR-3 cells, heregulin stimulation increases the formation of erbB2-erbB3 heterodimers, whereas in BT-474 cells, erbB2-erbB4 complexes may also be formed. To confirm that the co-immunoprecipitation of Grb7 with erbB3 and erbB4 did not reflect the formation of heterodimers between these receptors and erbB2 complexed with Grb7, the stability of the respective heterodimers was investigated under our lysis and immunoprecipitation conditions (Fig. 1C). Western blotting of erbB3 immunoprecipitates from heregulin-stimulated SK-BR-3 and BT-474 cells did not detect co-immunoprecipitated erbB2. Similarly, Western blotting of erbB4 immunoprecipitates from BT-474 cells also did not detect the presence of this receptor. Taken together, these results demonstrate that upon heregulin stimulation, Grb7 is targeted not only by erbB2 but also by erbB3 and erbB4.

Regulation of Grb7 Phosphorylation by Heregulin—The growth factor-induced serine phosphorylation of Grb10 and Grb14 led to the suggestion that a serine/threonine kinase may represent a conserved component of Grb7 family signaling complexes (30, 31). To investigate the qualitative and/or quantitative changes in Grb7 phosphorylation which occur upon heregulin activation of erbB receptors, Grb7 was immunoprecipitated from serum-starved and heregulin-stimulated SK-BR-3 cells that were metabolically labeled with 32P, and the samples analyzed by SDS-PAGE (Fig. 2A) and phosphoamino acid analysis (Fig. 2B). The immunoprecipitates were also Western blotted with anti-Grb7 antibodies to normalize for protein levels and with anti-Shc antibodies to confirm effective stimulation of the cells. Despite heregulin-induced association of Grb7 with Shc and hence erbB receptor activation, no increase in total Grb7 phosphorylation was detected after heregulin stimulation. Phosphoamino acid analysis of Grb7 before and after heregulin treatment revealed serine and threonine, but not tyrosine, phosphorylation. One explanation for the lack of inducible Grb7 phosphorylation observed in SK-BR-3 cells is that the serine/threonine kinase activity involved is not activated by heregulin treatment. An example of such growth factor specificity is provided by the enhanced phosphorylation of Grb14 detected in response to platelet-derived growth factor but not EGF (31). Alternatively, the overexpression of Grb7 and erbB receptors in these cells, combined with the significant basal tyrosine phosphorylation of erbB2, leads chromatography to ≥ 95% and their identity confirmed by ion spray mass spectrometry. Phosphopeptide competition experiments were performed using lysates from HER1–2 cells, essentially as described previously (33). Peptides were coupled to Affi-Gel 15 (Bio-Rad) under anhydrous conditions. In brief, 50 μg of peptide (in dimethyl sulfoxide) was incubated with 100 μl of Affi-Gel 15 (in isopro pyl alcohol) for 2 h at room temperature. The reaction was stopped by the addition of 1 ml of ethanolamine, pH 8, and the beads were washed in 50 mM HEPES, pH 7.4, and 100 mM NaCl. This prepared sufficient coupled peptide for 20 binding reactions, which were performed as follows. The matrix was incubated with 250 μl of lysis buffer containing 1 μg of GST, GST-Grb2 SH2, or GST-Grb7 SH2 fusion protein for 2 h at 4 °C and then washed three times in 1 ml of lysis buffer. The presence of fusion protein bound to the coupled peptide was analyzed by Western blot using antibodies against GST.

![Graphical Abstract](http://www.jbc.org/)

**Fig. 1.** Heregulin (HRG)-induced association of Grb7 with erbB receptors and Shc in human breast cancer cells. Panel A, association of Grb7 with tyrosine-phosphorylated Shc and p180–185. SK-BR-3 or BT-474 cells were serum starved overnight and then treated with either vehicle alone (−) or heregulin (5 nM final concentration) (+) for 10 min at 37 °C. Cell lysates were then transferred to nitrocel lulose. The membranes were then blotted with the indicated antibodies. PY, anti-phosphotyrosine. Panel B, recruitment of Grb7 by specific erbB receptors. The experimental protocol was as in panel A except that anti-Grb7 or anti-erbB4 immunoprecipitations were performed and the complexes blotted with the indicated antibodies. Panel C, erbB receptors in heregulin-treated SK-BR-3 and BT-474 cells are not co-immunoprecipitated because of heterodimer formation. The experimental protocols were as in panel A except that erbB receptor immunoprecipitations were performed and the complexes Western blotted with the indicated antibodies. The positive control for the erbB4 immunoprecipitate is included in the right section of panel B. Lys, cell lysate.
to saturation of the pathway regulating Grb7 phosphorylation even in the absence of heregulin stimulation. Finally, inducible phosphorylation of the adaptor itself may not be essential for signaling, or the requirement may be member-specific.

Specific Binding of Grb7 to erbB3 and erbB4 Is Direct and Mediated by the SH2 Domain—Phosphotyrosines of growth factor-activated receptor tyrosine kinases provide targets for proteins containing SH2 domains (24, 52). To verify that the association of Grb7 with erbB3 and erbB4 receptors was mediated by the SH2 domain, solution binding studies were performed using a GST-Grb7 SH2 domain fusion protein coupled to glutathione-agarose beads and control or heregulin-stimulated lysates from ZR-75-1 cells. This cell line overexpresses erbB2, erbB3, and erbB4 in a heregulin-dependent association of the Grb7 SH2 domain with erbB4 also occurred.

To confirm that the apparent binding of the Grb7 SH2 domain to erbB3 and erbB4 in ZR-75-1 cell lysates was not caused by an interaction with erbB2 and erbB3 from lysates of control cells, presumably because of the basal phosphorylation of these erbB receptors observed in ZR-75-1 cells (45), but this interaction was increased significantly when lysates from heregulin-stimulated cells were used (Fig. 3A). Strong heregulin-dependent association of the Grb7 SH2 domain with erbB4 also occurred.

The latter were blotted with either anti-Grb7 antibodies or to nitrocellulose filters (for Western blotting). The left section shows an autoradiograph of the former (4-day exposure at −70 °C). The latter were blotted with either anti-Grb7 or anti-Shc antibodies to control for protein levels and stimulation, respectively (center and right sections). Panel B, phosphoamino acid analysis of Grb7. After detection of polyvinylidene difluoride-imobilized phosphorylated Grb7 the band was excised and subjected to phosphoamino acid analysis as described under “Experimental Procedures.” The mobilities of the phosphoamino acid standards phosphotyrosine (Tyr), phosphothreonine (Thr), and phosphoserine (Ser) were as indicated. The exposure time was 7 days at −70 °C.

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with erbB2 in HEK/Grb7 cells, HEK-293 cells stably transfected with a hGrb7 expression vector. In this system, heregulin administration leads to increased formation of erbB2-erbB3 heterodimers and erbB2-mediated transphosphorylation of erbB3 (53). Consequently, this provides a powerful system to investigate the signaling potential of the latter receptor.

Western blot analysis of cell lysates from control and transfected cells revealed low levels of endogenous erbB2 and erbB3 expression which were enhanced markedly upon introduction of the corresponding expression vectors (Fig. 4A). Furthermore, anti-phosphotyrosine immunoblots of these cell lysates revealed levels of heregulin-induced receptor tyrosine phosphorylation which paralleled the erbB receptor expression levels, indicating that introducing the point mutations did not alter total erbB3 transphosphorylation significantly. This was confirmed by Western blot analysis of anti-erbB3 immunoprecipitates with anti-phosphotyrosine antibodies (Fig. 4A). To confirm that the interpretation of these experiments would not be complicated by the presence of erbB2-erbB3 heterodimers during the immunoprecipitations, the stability of these complexes from the transfected cells was investigated by Western blotting erbB3 immunoprecipitates with anti-erbB2 antibodies (Fig. 4B). In accordance with our previous control experiments (Figs. 1C and 3C), the two receptors were not co-immunoprecipitated from heregulin-stimulated cells.

To assess the effect of these mutations on Grb7 binding, anti-Grb7 immunoprecipitates were Western blotted with anti-erbB3 antibodies. Grb7 associated strongly with wild-type erbB3 (53) in vivo. These results demonstrate that Tyr-1180 of erbB3 represents the major binding site for Grb7 in vivo, and a lesser contribution is made by Tyr-1180, Tyr-1243, and Tyr-1243. In contrast, the addition of Tyr-1180 and Tyr-1243 reduced binding by approximately 90% compared with untreated controls (Fig. 6). Tyr-1180 and Tyr-1243 also competed binding, but less efficiently (by approximately 70%). However, when the Grb2 SH2 domain binding to the tyrosine-phosphorylated intracellular region of erbB2 (33). A phosphopeptide corresponding to Tyr-1196 of erbB2, which does not bind either SH2 domain, served as a negative control.
After dilution with 200

bound in the absence of added phosphopeptide.

bound by each fusion protein was expressed as a percentage of that

competition. After densitometric analysis, the amount of receptor

detected by Western blot analysis.

and further incubation, the beads were washed and bound receptor detected by Western blot analysis. Panel B, quantitative analysis of competition. After densitometric analysis, the amount of receptor bound by each fusion protein was expressed as a percentage of that bound in the absence of added phosphopeptide.

and Tyr-1243 represent binding sites predominantly for the Grb7 SH2 domain, and Tyr-1180 interacts with a higher affinity.

Comparison of Grb2 and Grb7 SH2 Domain Specificity by Phosphopeptide Pull-down Analysis—Binding the Grb2 and Grb7 SH2 domains to Tyr-1180 and Tyr-1243 was also investigated by pull-down experiments. Synthetic phosphopeptides corresponding to these sites were coupled to Affi-Gel beads and investigated for their ability to bind GST-Grb2 SH2 and GST-Grb7 SH2 fusion proteins (Fig. 7). It should be noted that since the particular phosphopeptides may couple with different efficiencies to the Affi-Gel beads, these experiments provide only an insight into the relative interaction of Grb2 and Grb7 SH2 domains with a particular phosphopeptide and cannot be used reliably to compare binding affinities for different ligands.

As in Fig. 6, Tyr-1139 and Tyr-1196 of erbB2 acted as positive and negative controls, respectively. Both the Tyr-1180 and Tyr-1243 phosphopeptides bound the Grb7 SH2 domain, whereas binding of the Grb2 SH2 domain was not detected. These results confirmed that Tyr-1180 and Tyr-1243 represent Grb7, but not Grb2, binding sites.

An interesting feature of the Tyr-1180 and Tyr-1243 phosphorylation sites is the presence of an Arg residue at the +3 position relative to phosphotyrosine (Fig. 7). Because the consensus sequence for the Grb2 SH2 domain binding is pYØ/QNØ/Q, where Ø represents a hydrophobic residue (26), the presence of an Arg residue at +3 may be detrimental to binding. We therefore replaced the latter residue in the Tyr-1180 phosphopeptide with Gln and investigated binding to the Grb2 and Grb7 SH2 domains (Fig. 7, R1183Q peptide). This phosphopeptide bound strongly to both the Grb2 and Grb7 SH2 domains. This effect was investigated further by introducing Arg at +3 into the Tyr-1139 phosphopeptide, which represents an optimal Grb2 binding site. Interestingly, this phosphopeptide bound the Grb2 SH2 domain more strongly than the Grb7 SH2 (Fig. 7, Q1142R peptide). The relative interaction of this phosphopeptide with the respective SH2 domains is therefore the inverse of the results obtained with the erbB3-derived ligands. These results indicate that an Arg residue at +3 can modulate the relative binding of the Grb2 and Grb7 SH2 domains, but the effect depends on the sequence context. Consequently, the Grb2 and Grb7 SH2 domains possess overlapping but distinct selectivities.

DISCUSSION

The signaling pathways triggered by specific erbB receptors and receptor combinations remain incompletely characterized. This is particularly true for the more recently identified family members erbB3 and erbB4. Initial studies investigating the signaling potential of erbB3 used an EGFR-erbB3 chimera expressed in NIH 3T3 cells. This revealed that this receptor was particularly potent in activating PI3-kinase but did not couple to phospholipase C-γ or Ras-GAP (Ras-GTPase-activating protein). The strong association with PI3-kinase was because of the presence of multiple phosphorylation sites with the consensus pYXXM sequence for binding the p85 subunit of this enzyme (1, 55). This system also identified Tyr-1309 as the binding site for the phosphotyrosine binding domain of Shc (55, 56). Importantly, these recruitment events also occurred upon transphosphorylation of native erbB3 by other erbB receptors; that is, EGF stimulated binding of PI3-kinase to erbB3 in cells expressing high levels of the EGFR and erbB3 (2, 44), and heregulin induced the association of both PI3-kinase and Shc with this receptor in cells expressing erbB2 and erbB3 (53). More recently it has become evident that erbB3 and erbB4 exhibit overlapping substrate specificities in that both receptors target Shc and PI3-kinase, but erbB4 also binds Grb2 and Ras-GAP (45, 57–59).

The adaptor protein Grb7 is co-expressed with erbB3 and erbB4 not only in a subgroup of human breast cancers but also in a restricted range of normal tissues, such as kidney (41, 60, 61), and represents a potential tissue-specific target for these receptors. Therefore, we investigated the interaction of Grb7 with erbB3 and erbB4, initially using heregulin stimulation of human breast cancer cell lines co-expressing these proteins as a model system. In SK-BR-3 cells, based on the receptor expression profile and the hierarchical nature of erbB receptor interactions (20), adding heregulin leads predominantly to increased formation of erbB2-erbB3 heterodimers with subse-
quent tyrosine phosphorylation of erbB2 (via autophosphorylation) and erbB3 (via transphosphorylation by erbB2). Although these cells also express the EGFR, the formation of erbB2-erbB3 heterodimers in response to heregulin stimulation is favored strongly over erbB1-erbB3 heterodimers. Western blotting of Grb7 immunoprecipitates with receptor-specific antibodies revealed an association of Grb7 with both erbB2 and erbB3 after treatment with heregulin (Fig. 1). Similar results were obtained with a more defined model system based on transient expression of these receptors in HEK-293 cells (Fig. 5). In BT-474 human breast cancer cells, heregulin stimulation also leads to erbB4 tyrosine phosphorylation, predominantly at the terminal domain of erbB4 contains five YN motifs (11), but Tyr-1180 and Tyr-1243 can be phosphorylated, but they represent targets for the relative contribution of these potential sites to Grb2 and Grb7 binding remains to be determined. Consequently, both Tyr-1180 and Tyr-1243 were initially suggested as Grb2 binding sites because of the presence of an Asn residue at +2 relative to the phosphotyrosine (19, 26). However, Prigent and Gullick (55) were unable to detect binding of a GST-Grb2 fusion protein to an EGFR-erbB3 chimera in vitro, and we could not immunoprecipitate Grb2 with erbB3 in heregulin-stimulated ZR-75-1 cells, despite clear association of Shc with this receptor and of Grb2 with erbB2 and erbB4 (45). This suggested that these sites were not phosphorylated in vivo, did not bind Grb2, or were tightly bound by a competing protein. In addition, this indicated that erbB3, like erbB2 (54), does not recruit Grb2 indirectly via Shc (45). Site-directed mutagenesis revealed that both Tyr-1180 and Tyr-1243 are required for efficient binding of Grb7 to erbB3 in vivo, although the former residue makes the largest contribution (Fig. 5). Phosphopeptide competition experiments (Fig. 6) demonstrated that this is because of a higher affinity of Tyr-1180 for the Grb7 SH2 domain, but differential phosphorylation of the two sites in vivo may also be a contributing factor. Consequently, both Tyr-1180 and Tyr-1243 can be phosphorylated, but they represent targets for the Grb7, and not the Grb2, SH2 domain. Interestingly, the COOH-terminal domain of erbB4 contains five YNX motifs (11), but the relative contribution of these potential sites to Grb2 and Grb7 binding remains to be determined.

Therefore, erbB3 is unique among erbB receptors in binding Grb7 directly and not Grb2. The underlying mechanism appears to be the atypical nature of the candidate binding sites, which exhibit Arg residues at either the +3 to +5 position (Tyr-1180) or the +3 and +5 (Tyr-1243) relative to the phosphotyrosine (Fig. 7). However, although the consensus sequence for Grb2 SH2 binding exhibits either a hydrophobic or Gln residue at +3 (26), substitution analysis using synthetic phosphopeptides revealed that the effect of an Arg residue at +3 on Grb2 SH2 binding is context-dependent. In particular, although R1183Q and not Tyr-1180 bound the Grb2 SH2, introduction of an Arg residue at +3 into the Tyr-1139 phosphopeptide did not eliminate binding (Fig. 7, Q1142R). These experiments also highlighted the overlapping but distinct specificity of the Grb7 SH2 domain, which bound strongly to the erbB3-derived peptides; however, when compared with the Grb2 SH2, it bound less well to Q1142R. Distinct specificities for these adaptors were described originally by Koegam and Cooper (36), who demonstrated that Tyr-542 of SH-PTP2 (PYTN) bound the Grb2, but not the Grb7, SH2 domain.

In the crystal structure of the Grb7 SH2 domain complexed with a high affinity phosphopeptide, Trp-121 in the EF loop forces the ligand to adopt a novel β-turn conformation (62), and we have speculated that because of the presence of an insertion in the EF loop relative to Src, the Grb7 SH2 binds phosphopeptides in a similar mode (33). In the Grb2 structure, the β-turn is stabilized by an interaction between the +2 Asn of the phosphopeptide and the βD6 Lys of the SH2 domain. In the Grb7 SH2 domain the βD6 residue also plays a key role in SH2 selectivity (33) but is a hydrophobic amino acid (Leu), not basic. It is therefore possible that the differential selectivity of the Grb7 and Grb2 domains toward phosphopeptides with a high proportion of charged, basic amino acids at +3 to +5 (e.g. Tyr-1180 and Tyr-1243), is determined by the nature of the βD6 residue. This hypothesis will be analyzed further by modeling and structural studies.

In summary, Grb7 represents an in vivo target of at least three of the four identified erbB receptors. Because the tissue distribution of Grb7 is limited (61), the normal physiological role of this protein must be relatively tissue-specific. However, in breast cancer cells exhibiting concomitant erbB receptor and Grb7 overexpression, the recruitment of Grb7 not only to multiple erbB receptors but also to Shc (32) must represent a major component of the erbB receptor signaling cascade. The demonstration that Grb7 represents a downstream target of erbB3 is of particular note in this regard because co-expression of erbB2 and erbB3 commonly occurs in human breast cancer cells, presumably resulting from the enhanced signaling potential of this heterodimer compared with the respective receptor homodimers (53, 63). The future characterization of the signaling mechanism and function of this protein will therefore be of fundamental significance to our understanding of erbB receptor signaling in normal and cancerous cells.

Acknowledgments—We thank Dr. M. H. Kraus and Dr. J. G. Koland for providing anti-erbB3 antibodies and Dr. Tadashi Yamamoto for the ERBB2 cDNA clone.

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J. Biol. Chem. 1998, 273:7717-7724.  
doi: 10.1074/jbc.273.13.7717

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