Chemistry & Biology
Article

System-wide Investigation of ErbB4 Reveals 19 Sites of Tyr Phosphorylation that Are Unusually Selective in Their Recruitment Properties

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DOI 10.1016/j.chembiol.2008.07.006

SUMMARY

The first three members of the ErbB family of receptor tyrosine kinases activate a wide variety of signaling pathways and are frequently misregulated in cancer. Much less is known about ErbB4. Here we use tandem mass spectrometry to identify 19 sites of tyrosine phosphorylation on ErbB4, and protein microarrays to quantify biophysical interactions between these sites and virtually every SH2 and PTB domain encoded in the human genome. Our unbiased approach highlighted several previously unrecognized interactions and led to the finding that ErbB4 can recruit and activate STAT1. At a systems level, we found that ErbB4 is much more selective than the other ErbB receptors. This suggests that ErbB4 may enable ErbB2 and ErbB3 to signal independently of EGFR under normal conditions, and provides a possible explanation for the protective properties of ErbB4 in cancer.

INTRODUCTION

The ErbB family of receptor tyrosine kinases (RTKs) comprises four single-pass transmembrane proteins which are expressed in a wide variety of cell types and are responsible for mediating a diverse range of cellular outcomes, including proliferation, migration, differentiation, survival, and apoptosis (Porter and Vaillancourt, 1998; Schlessinger and Lemmon, 2003; Yarden and Sliwkowski, 2001). Ligand binding to the extracellular domain of a receptor promotes either homo- or heterotypic receptor dimerization and activation of the intracellular tyrosine kinase domain. Activated receptors then phosphorylate each other on a number of tyrosine residues, which serve as docking sites for downstream enzymes or adaptor proteins. The proteins that are recruited directly to the phosphorylated receptors often contain Src homology 2 (SH2) domains or phosphotyrosine binding (PTB) domains, which recognize these phosphotyrosine (pTyr) sites in a sequence-specific fashion. Thus, one way to learn about the signaling pathways that are activated by a receptor is to uncover interactions between its sites of tyrosine phosphorylation and SH2 or PTB domains (Jones et al., 2006; Kavanaugh and Williams, 1994; Songyang et al., 1993, 1994).

The ErbB family of RTKs is unique in that two of its members are partially defective: ErbB3 does not have a functional kinase domain (Kim et al., 1998; Yarden and Sliwkowski, 2001) and ErbB2 does not recognize an extracellular ligand (Marmor et al., 2004; Yarden and Sliwkowski, 2001). Although much has been learned about the intracellular signaling events initiated by EGFR, ErbB2, and ErbB3, much less is known about ErbB4. Like EGFR, ErbB4 has a functional kinase domain and several extracellular ligands that induce either homo- or heterodimerization (Carpenter, 2003). Unlike the other ErbB receptors, however, ErbB4 can signal in two ways: it can dimerize with another ErbB and recruit proteins to the plasma membrane (Cohen et al., 1996; Jones et al., 1999), or it can undergo proteolytic cleavage in a γ-secretase-dependent manner, translocate to the nucleus, and act as a nuclear chaperone (Ni et al., 2001; Williams et al., 2004).

Although most disease-related studies of ErbB4 have focused on its role in neuronal signaling and schizophrenia (Norton et al., 2006; Silberberg et al., 2006), recent reports suggest that ErbB4 also plays a role in cancer, but in a different way than the other ErbB receptors. In the case of bladder cancer, ErbB4 and one of its ligands, Heregulin-4, appear to be protective: their expression correlates with prolonged patient survival, and this effect is strongest when either EGFR and ErbB3, or ErbB2 and ErbB3, are also expressed (Memon et al., 2004, 2006a, 2006b). A similar pattern is observed in mammary carcinomas. It is well established that EGFR and ErbB2 are overexpressed in many breast cancers, and their overexpression correlates with poor prognosis (Klijn et al., 1992; Slamon et al., 1987). In contrast, ErbB4 expression correlates with lower tumor grade and more favorable prognosis (Barnes et al., 2005; Tovey et al., 2004). Interestingly, coexpression of ErbB4 with ErbB2 in medulloblastomas and ependymomas—where EGFR and ErbB3 are absent—leads to an increased proliferative index of the tumor and decreased patient survival (Gilbertson et al., 1997, 2002).

We asked whether a broad and unbiased investigation of the signaling capabilities of ErbB4 could shed light on its biological role in signaling, as well as on how its overall properties differ from those of the other ErbB receptors in cancer. We previously reported high-throughput methods to uncover binding

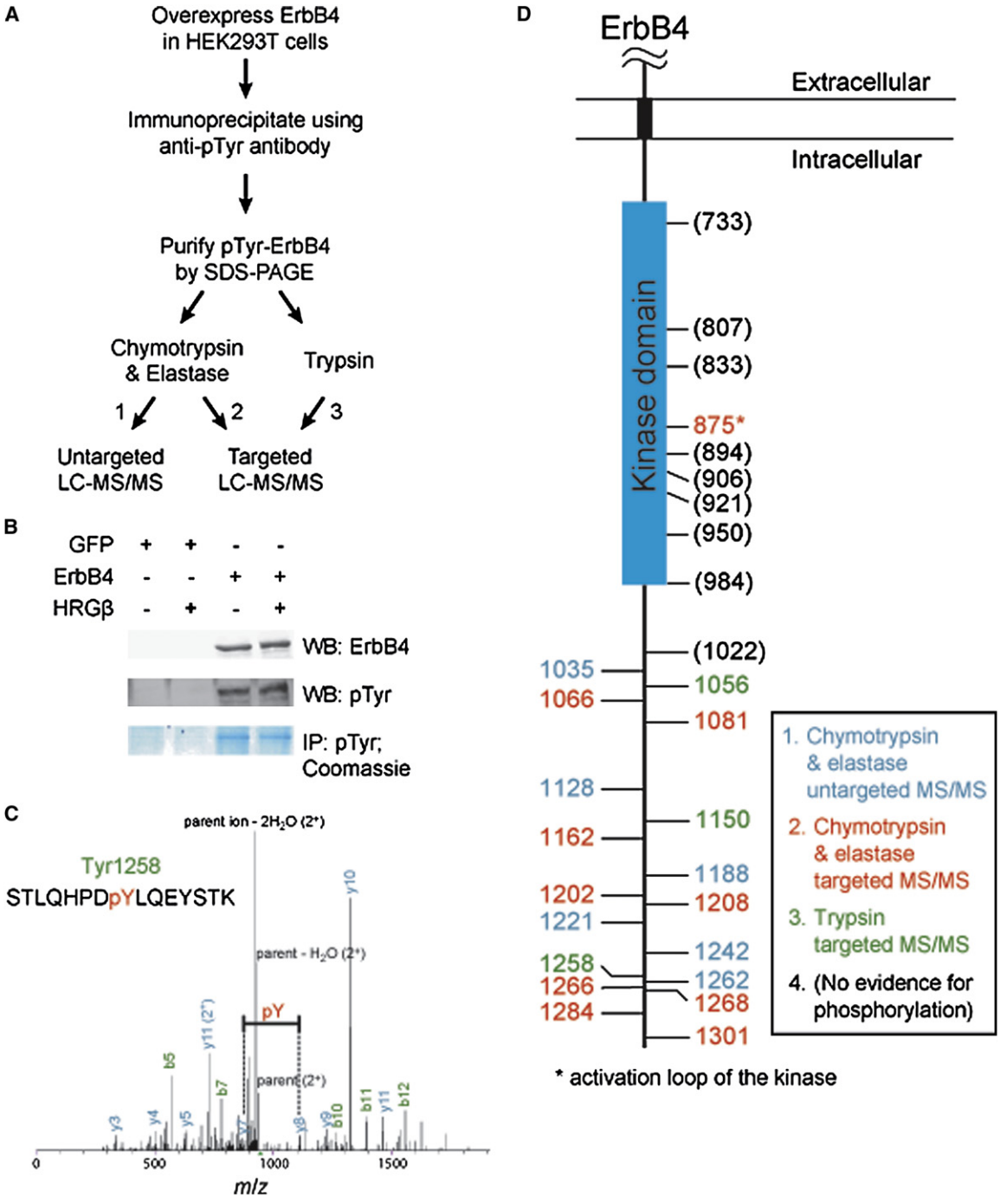

Figure 1. Identifying Sites of Tyrosine Phosphorylation on ErbB4

(A) Experimental procedure for identifying pTyr sites. ErbB4 is overexpressed in HEK293T cells and phosphorylated receptor is isolated by pTyr immunoprecipitation (IP) and gel electrophoresis. pTyr sites are identified by μHPLC-MS/MS, using both untargeted and targeted methods.

(B) ErbB4 is detected by western blotting (WB) in HEK293T cells transfected with ErbB4, but not in cells transfected with GFP. Western blots with an anti-pTyr antibody show that ErbB4 is phosphorylated independently of stimulation with Heregulinβ1 (HRGβ1). ErbB4, recovered by pTyr IP from ErbB4-transfected cells, is obtained at sufficiently high levels to visualize by Coomassie staining.

(C) Representative MS/MS spectrum, showing the identification of Tyr1258 as a site of phosphorylation.

(D) Schematic representation of ErbB4, showing which of the intracellular tyrosine residues is phosphorylated.

interactions between phosphopeptides, representing sites of tyrosine phosphorylation on RTKs, and recombinant SH2 or PTB domains, representing full-length signaling proteins (Gordus and MacBeath, 2006; Jones et al., 2006). By probing protein microarrays comprising most of the SH2 and PTB domains encoded in the human genome with eight concentrations of each fluorescently labeled phosphopeptide, we were able to obtain equilibrium dissociation constants (K_Ds) for all possible domain-peptide interactions with $K_D \leq 2$ μM. Because EGFR, ErbB2, and ErbB3 have been studied extensively, many—if not all—of their pTyr sites are known. As such, we were able to construct near-complete quantitative interaction maps for these receptors (Jones et al., 2006).

To our knowledge, however, there are no published reports identifying sites of tyrosine phosphorylation on ErbB4, although peptide competition experiments suggest that tyrosines 1056, 1188, and 1242 are phosphorylated (Cohen et al., 1996). Here we use tandem mass spectrometry (MS/MS) to identify 19 sites of tyrosine phosphorylation on ErbB4 and protein microarrays to construct a quantitative signaling map for this receptor. This broad and unbiased approach revealed several new interactions, including specific interactions between two pTyr sites on ErbB4 and the core domain of signal transducer and activator of transcription 1 (STAT1). Consistent with these biophysical data, we find that STAT1 copurifies with ErbB4, is phosphorylated on Tyr701, and forms a STAT1/STAT1 homodimer in ErbB4-transfected cells. On a broader level, our microarray experiments show that ErbB4 is very different from the other ErbB receptors: it is much more selective and does not interact with any proteins that are not also recruited by at least one of the other ErbBs. Based on these findings, we submit that ErbB4 may act under normal conditions to furnish the missing functions of ErbB2 and ErbB3, and may play a protective role in cancer by buffering the oncogenic effects of heterodimers formed between the other three ErbB receptors. Importantly, the methods described here are not specific to ErbB4 and so can be applied in a systematic fashion to the study of other RTKs where little is known about their signaling properties.

RESULTS AND DISCUSSION

Identifying Sites of Tyrosine Phosphorylation on ErbB4

To identify sites of tyrosine phosphorylation on ErbB4, we followed the strategy outlined in Figure 1A. The full-length coding region of ErbB4 was transfected into human embryonic kidney (HEK) 293T cells, which normally do not express any of the ErbB receptors at levels detectable by immunoblotting. After serum starvation, the transfected cells were stimulated for 15 min with Heregulinβ1 (HRGβ1), a potent ligand of ErbB4 that promotes the formation of ErbB4 homodimers. Immunoblotting revealed that ErbB4 was expressed at high levels and that it was phosphorylated on tyrosine residues independently of treatment with HRGβ1 (Figure 1B). Autoactivation of ErbB4 is not surprising, because other RTKs have also been observed to autoactivate when transfected into HEK293T cells (Hinsby et al., 2003) and ErbB2 is well known to autoactivate when overexpressed (Yarden and Sliwkowski, 2001).

To enrich for tyrosine-phosphorylated ErbB4, we performed an immunoprecipitation (IP) using a mixture of anti-pTyr antibodies. We then separated phospho-ErbB4 from other pTyr-containing proteins by one-dimensional SDS-polyacrylamide gel electrophoresis (Figures 1A and 1B). Immunoblotting confirmed that ErbB4 and pTyr were detected at the same molecular weight in ErbB4-transfected cells; no corresponding band was observed in cells transfected with the coding region for green fluorescent protein (GFP). Moreover, enough phospho-ErbB4 was recovered to visualize its band by Coomassie staining (Figure 1B), thereby enabling near-complete coverage of the receptor in our subsequent MS/MS experiments. We chose to perform the IP using a mixture of anti-pTyr antibodies rather than an anti-ErbB4 antibody because we wanted to separate phosphorylated receptor from nonphosphorylated receptor. Phosphorylated peptides are often more difficult to detect by mass spectrometry than nonphosphorylated peptides, so minimizing the presence of nonphosphorylated peptides increases sensitivity.

To identify pTyr sites, the Coomassie-stained band was subjected to in-gel proteolytic digestion and analyzed by micro-high-performance liquid chromatography (μHPLC) coupled with nanospray MS/MS. To maximize coverage, we split the sample in half, digested each portion with either chymotrypsin or elastase, and performed separate untargeted analyses, subjecting the six most abundant ions in each survey scan to MS/MS. This process revealed 7 pTyr sites (Figure 1D). We then performed targeted analyses of the same samples, selecting ions of the expected *m/z* during the appropriate retention-time window of the μHPLC run. These targeted analyses enabled us to select ions that were previously ignored in the untargeted runs, revealing an additional 9 sites (Figure 1D). Finally, we performed targeted runs on trypsin-digested phospho-ErbB4, revealing an additional 3 sites (Figure 1D). Overall, we observed peptides spanning >95% of ErbB4, including 25 of the 28 intracellular tyrosine residues (see Figure S1 available online) and found evidence for 19 sites of tyrosine phosphorylation (Figure 1D).

No evidence was found for phosphorylation of tyrosine residues in the extracellular portion of the receptor or in the kinase domain, with the exception of Tyr875, which lies in the activation loop of the kinase and was therefore expected to be phosphorylated. These negative observations strongly suggest that ErbB4 is correctly folded and appropriately inserted in the plasma membrane. As further support, when ErbB4 was expressed with an epitope tag placed N-terminal to its signal peptide, the receptor was produced but not tyrosine phosphorylated (data not shown). This shows that when ErbB4 is inappropriately localized, it is not subject to aberrant phosphorylation.

Interestingly, all but one of the tyrosine residues that are located outside the kinase domain were found to be phosphorylated. Although our data do not provide information on the stoichiometry of phosphorylation, they show that these tyrosines are able to serve as substrates of the ErbB4 kinase, or of other intracellular tyrosine kinases, within a cellular context. Although we found more sites than have previously been observed on any other RTK, we note that ErbB4 has more intracellular tyrosine residues than most other receptors. It is not unusual to find evidence for phosphorylation on nearly every tyrosine in the C-terminal tail of an RTK. For example, evidence has been found for the phosphorylation of every tyrosine in the C-terminal tail of EGFR and all but one tyrosine in the C-terminal tails of FGFR1 and IGF1R (for a summary of these investigations, see

Table 1. Phosphopeptides Derived from Sites of Tyrosine Phosphorylation on ErbB4

pTyr Site	Peptide Sequence
ErbB4-875	dRLLEGDEKEpYNADGGKMD
ErbB4-1035	dQAFNIPPPIpYTSRARIDD
ErbB4-1056	dEIGHSPPPApYTPMSGNQD
ErbB4-1066	dTPMSGNQFVpYRDGGFAAD
ErbB4-1081	dAAEQGVSVPpYRAPTSTID
ErbB4-1128	dVQEDSSTQRpYSADPTVFD
ErbB4-1150	dPRGELDEEGpYMTPMRDKD
ErbB4-1162	dPMRDKPKQEpYLNPVEEND
ErbB4-1188	dDLQALDNPEpYHNASNGPD
ErbB4-1202	dNGPPKAEDEpYVNEPLYLD
ErbB4-1208	dEDEYVNEPLpYLNTFANTD
ErbB4-1221	dFANTLGKAEpYLKNNILSD
ErbB4-1242	dAKKAFDNPDpYWNHSLPPD
ErbB4-1258	dPRSTLQHPDpYLQEYSTKD
ErbB4-1262[a]	dLQHPDYLQEpYSTKYFYKD
ErbB4-1266[a]	dDYLQEYSTKpYFYKQNGRD
ErbB4-1268	dLQEYSTKYFpYKQNGRIRD
ErbB4-1284[b]	dRPIVAENPEpYLSEFSLKD
ErbB4-1301	dPGTVLPPPPpYRHRNTVVD

All peptides were labeled on their N terminus with 5(6)-TAMRA (denoted "d") and contained a C-terminal Asp residue. pY, phosphotyrosine.
[a] No product of the correct molecular weight was obtained for these peptides.
[b] This peptide exhibited high levels of background binding on the protein microarrays, precluding an analysis of its interactions.

Kaushansky et al., 2008). We therefore set out to conduct a broad and unbiased study of pTyr-dependent protein recruitment to ErbB4 by testing and quantifying biophysical interactions between these newly found sites of tyrosine phosphorylation and a large collection of human SH2 and PTB domains.

Defining a Quantitative Protein Interaction Map for ErbB4

To study the interaction of SH2 and PTB domains with individual sites of tyrosine phosphorylation on ErbB4, we synthesized pTyr-containing phosphopeptides as surrogates for the full-length receptor. Structural studies have shown that recognition can occur as far upstream as the −7 position of the peptide for some PTB domains (Eck et al., 1996; Zhou et al., 1995, 1996) and as far downstream as the +5 position of the peptide for some SH2 domains (Case et al., 1994; Lee et al., 1994; Yaffe et al., 1997). To ensure that we included all relevant residues, we synthesized 18 residue phosphopeptides that featured 9 residues upstream of the pTyr and 7 residues downstream (Table 1). An Asp residue was appended to the C terminus of each peptide to promote solubility and a fluorescent dye was appended to the N terminus to visualize binding. After purifying the peptides by reverse-phase HPLC, we probed protein microarrays comprising 96 SH2 domains and 37 PTB domains with eight concentrations of each peptide ranging from 10 nM to 5 μM. The resulting data were fit to an equation that describes saturation binding (Jones et al., 2006), yielding equilibrium dissociation constants (K_Ds) for each domain-peptide interaction (Figure 2; Table S1). As positive controls, we also synthesized peptides corresponding to every known site of tyrosine phosphorylation on EGFR, ErbB2, and ErbB3 and analyzed these peptides along with the ErbB4-derived peptides. These data, which recapitulate our previous results with few exceptions (Jones et al., 2006), are also provided in Table S1. Any discrepancies between our previous results and these new data arise either from minor differences in K_D values that cause interactions to pass the 2 μM threshold in one data set but not the other, or from improvements in the quality and concentration of the spotted domains in the current version of our microarrays.

The interaction map of Figure 2 provides an unbiased view of ErbB4, showing biophysical interactions between phosphopeptides and SH2 or PTB domains derived from signaling proteins. Which proteins are actually recruited in a given cell will depend on many additional factors, including the concentrations of the proteins in the vicinity of ErbB4 and how they interact with each other. As such, this diagram should be viewed as a quantitative map of the receptor, rather than as a depiction of protein recruitment in any particular cell type or cell state. In addition, the map of Figure 2 is based on our identification of pTyr sites on ErbB4. It is possible that some of these sites are only phosphorylated in response to certain ligands, or by nonreceptor tyrosine kinases that are only expressed in certain cells. In addition, it is possible that some of the pTyr sites that we identified are phosphorylated only when the receptor is autoactivated by overexpression. Thus, if anything, this map overrepresents the signaling properties of ErbB4, rather than underrepresents them.

The observed biophysical interactions with ErbB4-derived peptides are consistent with previous studies of ErbB4 signaling. For example, ErbB4 has been shown to activate the mitogen-activated protein kinase (MAPK) signaling cascade (Kainulainen et al., 2000), and peptide competition studies have implicated Tyr1188 and Tyr1242 as direct recruitment sites for Shc1 (Cohen et al., 1996). Consistent with this finding, we observed strong interactions (K_D < 1 μM) between the PTB domain of Shc1 and phosphopeptides derived from pTyr1188 and pTyr1242 (Figure 2). Likewise, the Cyt1 isoform of ErbB4, but not the Cyt2 isoform, has previously been shown to activate Akt, which lies downstream of phosphoinositide 3 kinase (PI3K) (Kainulainen et al., 2000). In addition, peptide competition studies have implicated Tyr1056 as a recruitment site for PI3K (Cohen et al., 1996). Consistent with these findings, we observed strong interactions between the SH2 domains of all three isoforms of PI3K's regulatory subunit and pTyr1056 of ErbB4. This tyrosine is present in the Cyt1 isoform of ErbB4, but not in the Cyt2 isoform.

ErbB4 Activates STAT1 in HEK293T Cells

The protein microarrays also revealed interactions that, to our knowledge, have not previously been reported. Interactions were observed between ErbB4-derived phosphopeptides and the SH2 domains of Abl2, Src, Syk, Crk, CrkL, Ras-GAP, Vav2, PLCγ2, Cbl, and STAT1 (Figure 2A). We asked whether the information uncovered by these microarray experiments could be used to discover previously unrecognized signaling events mediated by ErbB4. To address this question, we focused on the observed interactions between ErbB4 phosphopeptides and

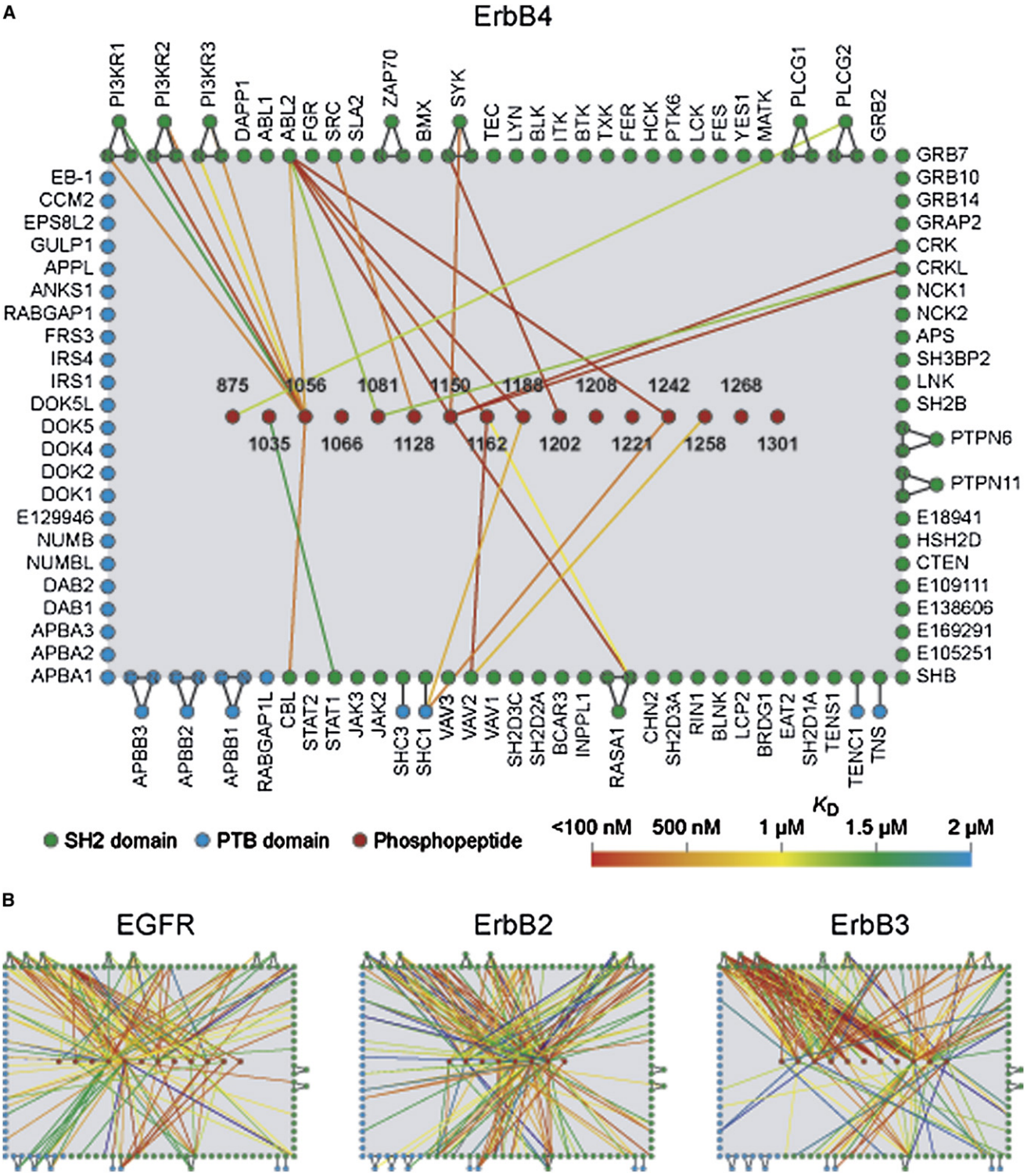

Figure 2. Quantitative Protein Interaction Maps for the ErbB Receptors

(A) Interaction map for ErbB4.

(B) Interaction maps for EGFR, ErbB2, and ErbB3.

Red circles represent phosphopeptides, green circles represent SH2 domains, and blue circles represent PTB domains. Lines connecting peptides to domains indicate observed interactions, colored according to the affinity of the interaction (see legend). The circles that lie outside the rectangle of individual domains, and are connected by black lines to two other circles, represent tandem domains. They comprise the two domains to which they are connected. The circles that lie outside the rectangle but are connected to only one other circle represent proteins that contain both an SH2 domain and a PTB domain.

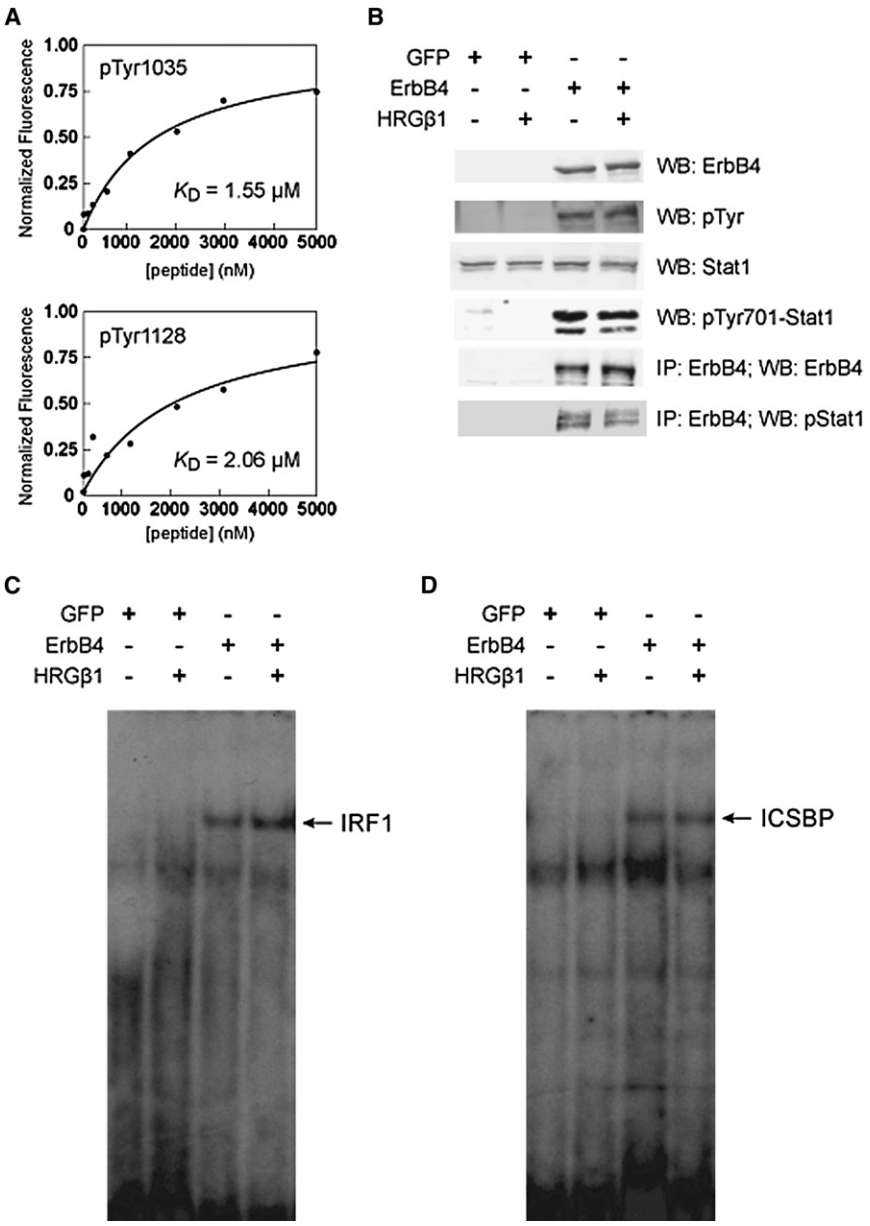

Figure 3. ErbB4 Recruits and Activates STAT1 in HEK293T Cells

(A) Two ErbB4-derived phosphopeptides representing pTyr1035 and pTyr1128 bind the core domain of STAT1 on protein microarrays, with K_Ds of 1.55 μM and 2.06 μM, respectively.

(B) When HEK293T cells are transfected with ErbB4, STAT1 is phosphorylated on Tyr701 and copurifies with ErbB4.

(C and D) Electromobility shift assays show that DNA sequences derived from the promoter regions of IRF1 and ICSBP are shifted upon the addition of lysates derived from ErbB4-transfected HEK293T cells. Both DNA sequences are established targets of STAT1/STAT1 homodimers.

STAT1. Our microarrays showed that the core domain of STAT1, which includes its SH2 domain, binds ErbB4 pTyr1035 with a K_D of 1.55 μM (Figure 3A). In addition, STAT1 binds several other ErbB4 phosphopeptides, but with affinities that fall just below our K_D cutoff of 2 μM. For example, STAT1 binds pTyr1128 with a K_D of 2.06 μM (Figure 3A).

STAT1 is a DNA-binding protein that is produced as a mixture of two isoforms: α (91 kDa) and β (87 kDa). Although it has been described to induce different outcomes in different cellular environments, it is generally considered to be the most pro-apoptotic of the STAT proteins (Battle and Frank, 2002; Calo et al., 2003; Kim and Lee, 2007; Schindler, 1998; Stephanou and Latchman, 2003). To test whether ErbB4 activates STAT1 in a cellular context, we transfected HEK293T cells with ErbB4 and measured the phosphorylation status of endogenous STAT1 using a phosphospecific antibody. We found that STAT1 was phosphorylated on Tyr701 in ErbB4-transfected cells, but not in cells transfected with a control protein (GFP; Figure 3B). In addition, we found that endogenous phospho-STAT1 physically associates with ErbB4: it copurifies with ErbB4 when the receptor is immunoprecipitated from ErbB4-transfected cells (Figure 3B). Because STAT1 is activated in an HRGβ1-independent fashion, we asked whether the two recruitment sites for STAT1 are phosphorylated under these conditions. We therefore repeated our targeted mass spectrometry experiments using ErbB4 that had been immunoprecipitated from unstimulated cells and found that both Tyr1035 and Tyr1128 were indeed phosphorylated. It has previously been shown that, upon γ-secretase-dependent cleavage, ErbB4 can physically associate with STAT5A and translocate to the nucleus (Williams et al., 2004). We do not observe cleavage of ErbB4 in HEK293T cells and submit that recruitment and activation of STAT1 may provide a way for ErbB4 to activate STAT signaling when it is expressed as its noncleavable isoform (Carpenter, 2003) or when it is expressed in cells where proteolytic processing does not occur.

When STAT1 is activated in the cytosol, it translocates to the nucleus where it can function as a transcription factor. STAT1 is known to function either as a STAT1/STAT1 homodimer or as a member of the ISGF3 ternary complex which comprises STAT1, STAT2, and IRF9. Both complexes have been observed in HEK293T cells (Parisien et al., 2001). Because STAT2 did not recognize any of the ErbB4-derived phosphopeptides (Figure 2A)—but was clearly active because it bound phosphopeptides derived from EGFR, ErbB2, and ErbB3—our microarray results suggest that STAT1 forms a homodimer in response to ErbB4 activation, rather than the ISGF3 complex. To test this prediction, we transfected HEK293T cells with ErbB4 and prepared cellular lysates. We then added ^{32}P-labeled DNA to the lysates and used an electromobility shift assay to determine which

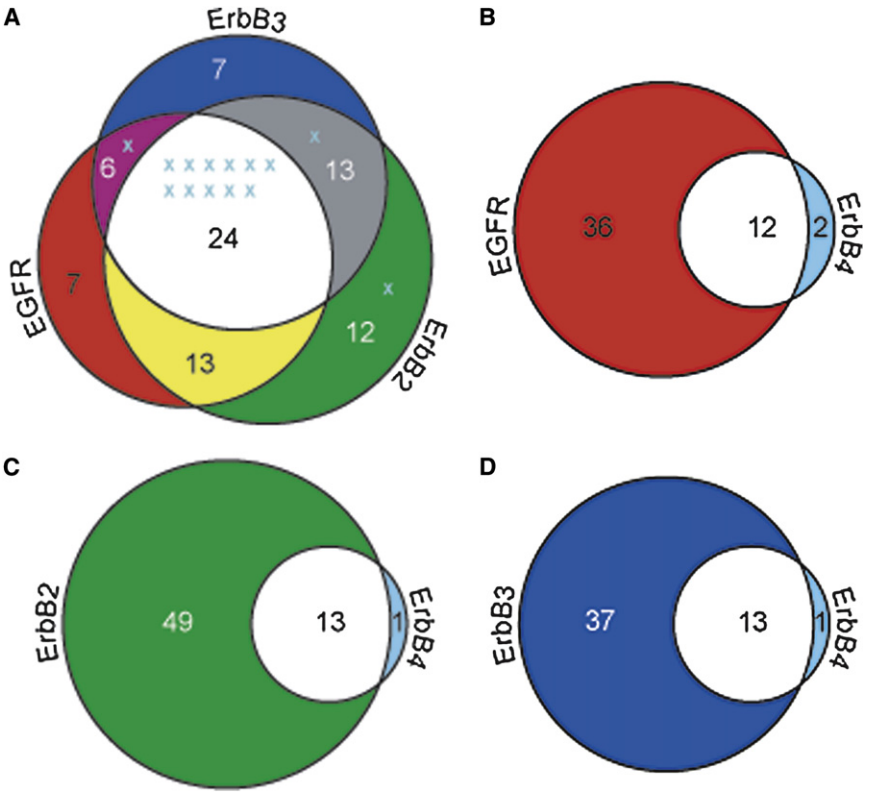

Figure 4. Venn Diagrams Illustrating which Signaling Proteins Can Be Recruited by Each of the ErbB Receptors

Proteins are considered able to be recruited by a receptor if they contain at least one SH2 or PTB domain that binds at least one phosphopeptide derived from that receptor with a $K_D \leq 2\ \mu M$. (A) Venn diagram for EGFR, ErbB2, and ErbB3. The 14 proteins that can be recruited by ErbB4 are indicated by light blue Xs.

(B–D) Venn diagrams for (B) EGFR and ErbB4; (C) ErbB2 and ErbB4; and (D) ErbB3 and ErbB4.

STAT1-responsive sequences were recognized by components of the lysate. We found that DNA sequences derived from the promoter regions of IRF1 and ICSBP, which are well-established binding sites for the STAT1/STAT1 homodimer (Schindler et al., 2007), were shifted in their electromobility (Figures 3C and 3D). In contrast, sequences derived from the ISG15 promoter region, which is a well-established binding site for the ISGF3 complex (Tang et al., 2007; Wagner et al., 2002), were not shifted (data not shown). These results demonstrate that noninteractions observed on the microarrays, as well as positive interactions, can inform biological investigations of RTK signaling.

System-Level Properties of ErbB4

In addition to providing a list of previously unrecognized biophysical interactions, our arrays also offer an unbiased, system-level view of ErbB4 that was previously unavailable. Most noticeably, the interaction map in Figure 2A shows that ErbB4 is recognized by very few signaling proteins. On average, each phosphopeptide is recognized by 1.5 SH2 or PTB domain-containing proteins. It is possible we have overrepresented pTyr sites on ErbB4 due to overexpression of the receptor. However, even if we eliminate the five phosphopeptides that are not recognized by any SH2 or PTB domains, each phosphopeptide is still only recognized by, on average, 2.2 proteins. This contrasts sharply with what we observed for phosphopeptides derived from EGFR, ErbB2, and ErbB3, which are recognized by, on average, 9.8, 18.4, and 11.4 proteins, respectively (Figure 2B). Thus, although we observed very little selectivity at the level of ErbB4 phosphorylation, there is striking selectivity at the level of protein recruitment.

When we consider the identity of the proteins that recognize pTyr sites on EGFR, ErbB2, and ErbB3, we note that each receptor is able to recruit proteins that the other receptors cannot (Figure 4A). This suggests that heterodimeric complexes involving EGFR, ErbB2, and ErbB3 should all induce a broader range of signals than any of the individual homodimers. Consistent with this observation, heterodimers involving EGFR, ErbB2, and ErbB3 have been shown to be more transforming than any of the homodimers (Alimandi et al., 1995; Wallasch et al., 1995) and ErbB2-ErbB3, EGFR-ErbB3, and EGFR-ErbB2 are all more mitogenic than EGFR-EGFR and ErbB2-ErbB2 when transfected into murine hematopoietic cells (Pinkas-Kramarski et al., 1996). In contrast, all of the signaling proteins that recognize phosphopeptides derived from ErbB4 also recognize phosphopeptides derived from at least one of the other three ErbB receptors, and most of these proteins (11 of 14) recognize peptides derived from all three (Figure 4).

That the recruitment profile of ErbB4 constitutes a subset of the recruitment profiles of EGFR, ErbB2, and ErbB3 suggests a unique biological role for ErbB4. Because ErbB4 recognizes an array of extracellular ligands and has an active kinase domain, it can function on its own as a highly selective homodimer. In addition, it can heterodimerize with ErbB2, which has no known ligand, or with ErbB3, which lacks an active kinase, to enable these two receptors to signal independently from EGFR. ErbB4 thus has the potential to complement the defective properties of ErbB2 and ErbB3 without adding substantially to the range of signaling pathways they induce. In effect, it can enable ErbB2 and ErbB3 to signal as though they were active homodimers.

The protein microarray data also provide a potential explanation for the observed protective role of ErbB4 in cancer. Recent epidemiological studies of bladder and breast cancers have shown that expression of ErbB4 correlates with improved prognosis when at least two other ErbBs are also expressed (Barnes et al., 2005; Memon et al., 2004, 2006a, 2006b; Tovey et al., 2004). In the presence of an appropriate ligand, ErbB4 can interact with each of the other ErbB receptors. We submit that ErbB4 acts like a buffer, decreasing levels of the more oncogenic heterodimers in favor of more benign complexes with itself.

Interestingly, in cancers of the brain where ErbB2 is expressed but EGFR and ErbB3 are not, ErbB4 expression correlates with poor prognosis (Gilbertson et al., 1997, 2002). In this case, we submit that ErbB4 heterodimerizes with ErbB2 and thus promotes, rather than dampens, ErbB2 signaling.

In summary, we have combined the use of tandem mass spectrometry with quantitative protein microarray technology to gain insight into the signaling properties of ErbB4, a receptor that has received considerably less attention to date than the other three ErbB family members. Through this broad and unbiased approach, we were able to identify a novel pathway activated by ErbB4, as well as to propose a model that explains the protective effects of ErbB4 expression in cancer. Because our core strategy did not rely on the use of ErbB4-specific reagents, it is relatively straightforward to apply this same approach systematically to the investigation of other RTKs about which little is known.

SIGNIFICANCE

The first three members of the ErbB family of receptor tyrosine kinases have been studied extensively and have been shown to mediate a wide variety of cellular processes by activating a diverse range of signaling proteins. They are potent oncogenes and are frequently misregulated in cancer. In contrast, ErbB4 has received much less attention, is not a potent oncogene, and even appears to play a protective role in some cancers. In order to gain insight into the signaling properties of ErbB4 in an unbiased fashion, we used tandem mass spectrometry to identify sites of tyrosine phosphorylation. We then used protein microarrays comprising most of the SH2 and PTB domains encoded in the human genome to identify and quantify biophysical interactions between these pTyr sites and the signaling proteins they recruit. In addition to confirming reported interactions, we uncovered several previously unrecognized interactions, including specific interactions with STAT1. We found that STAT1 physically associates with ErbB4 in HEK293T cells, is phosphorylated on Tyr701, and forms a STAT1/STAT1 homodimer that binds STAT1-responsive DNA sequences. This shows that the information revealed by our in vitro microarray experiments can be used to discover previously unrecognized signaling events. On a systems level, we found that ErbB4 is substantially more selective than the other ErbB receptors: the proteins it recruits constitute a small subset of the proteins recruited by each of the other receptors. This suggests that ErbB4 may enable ErbB2 and ErbB3 to signal independently of EGFR, because both ErbB2 and ErbB3 are normally inactive as homodimers. The highly selective nature of ErbB4 also provides a plausible explanation for its protective role in cancer. ErbB4 may act like a buffer, decreasing levels of the more oncogenic ErbB heterodimers in favor of more benign complexes with itself.

EXPERIMENTAL PROCEDURES

Subcloning of ErbB4

The wild-type sequence of ErbB4 was obtained from the Harvard Institute of Proteomics, cloned in the vector pDNR-Dual (Clontech, Mountain View, CA, USA). A Myc tag (5′-GCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTG-3′) was introduced between nucleotides 90 and 91 using PCR, and the product of this reaction was inserted into pDONR-221 using the BP cloning procedure (Invitrogen, Carlsbad, CA, USA). This vector served as the template for a λ-recombinase-mediated directional transfer into pDEST-40 (Invitrogen) to create pDEST-40-ErbB4. Although this construct contains a Myc tag at the N terminus of ErbB4, the tag was not detected by immunoblotting or by mass spectrometry and so was presumably removed proteolytically in the HEK293T cells.

Overexpression of ErbB4

HEK293T cells were grown to 50% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM (L)-Glu and transfected with either pDEST-40-ErbB4 or a control vector expressing GFP. All transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Following transfection, cells were allowed to recover for 36 hr, starved of serum for 18–20 hr, and stimulated for 15 min with 10 nM HRGβ1 (R&D Systems, Minneapolis, MN, USA). Cells were washed with PBS and lysed on ice for 30 min with NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40 [v/v], 5 mM EDTA, 5 mM EGTA [pH 8.0]), supplemented with 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 100 μl phosphatase inhibitor cocktail 2 (Sigma Aldrich, St. Louis, MO, USA), and 1 tablet of complete mini protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) per 10 ml lysis buffer. Lysate was centrifuged at 14,000 × *g* for 20 min to remove cellular debris.

Immunoprecipitations and Immunoblotting

All immunoprecipitations (IPs) were performed according to standard procedures using 2 μg of primary antibody per mg of lysate. ErbB4 IPs were performed without preclearing using antibody sc-283 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Prior to the pTyr IPs, lysates were precleared using 4 μg of normal mouse or rabbit serum (Santa Cruz Biotechnology) per mg of lysate. Anti-pTyr IPs were performed using an equimolar mix of p-100 and p-102 antibodies (Cell Signaling Technology, Danvers, MA, USA). Immunoblots were performed according to standard procedures using the following primary antibodies: sc-283 (Santa Cruz Biotechnology) or c-erbb-4/HER-4 Ab-2 (Lab Vision Corporation, Fremont, CA, USA) for ErbB4; p-100 (Cell Signaling Technology) for phosphotyrosine; STAT1 antibody 9172 (Cell Signaling Technology) for STAT1; and phospho-STAT1 (Tyr701) antibody 9171 (Cell Signaling Technology) for pTyr701-STAT1. Immunoblots were developed using either an Alexa-680-conjugated anti-rabbit antibody or an Alexa-800-conjugated anti-mouse antibody (LI-COR Biosciences, Lincoln, NE, USA). Membranes were visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

Mass Spectrometry

Immunoprecipitated proteins were separated on an 8% SDS-polyacrylamide gel at 100 V for 1 hr and stained with colloidal Coomassie (Invitrogen). The band corresponding to phospho-ErbB4 was excised from the gel and washed three times with acetonitrile/water (1:1, v/v). For the untargeted run, the gel band was split equally and digested with either chymotrypsin or elastase. Peptide sequence analysis of each digestion mixture was performed by microcapillary reverse-phase high-performance liquid chromatography coupled with nanoelectrospray tandem mass spectrometry (μLC-MS/MS) on an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The Orbitrap repetitively surveyed an *m/z* range from 395 to 1600, whereas data-dependent MS/MS spectra on the six most abundant ions in each survey scan were acquired in the linear ion trap. MS/MS spectra were acquired with relative collision energy of 30%, 2.5 Da isolation width, and recurring ions dynamically excluded for 60 s. Preliminary sequencing of peptides was facilitated with the SEQUEST algorithm and the Uniprot/Swissprot database (uniprot_sprot.fasta), taking into account the following: appropriate restriction to chymotrypsin specificity; static modification of cysteine to carboxyamidomethylcysteine; and differential modification of methionine to its sulfoxide. Data sets for both digest results were combined in silico, culled of minor contaminating keratin or autolytic peptide spectra, and re-searched with SEQUEST against the ErbB4 sequence without taking into account enzyme selectivity. Differential modifications of phosphorylated tyrosine, serine, and threonine residues were included. The discovery of phosphopeptides and subsequent manual confirmation of their MS/MS spectra were facilitated by the in-house programs MuQuest, GraphMod, and FuzzyIons (Proteomics Browser

Suite, Thermo Fisher Scientific). For the targeted runs, peptides of interest were continuously subjected to MS/MS analysis within a 10 min window centered on their expected retention times.

Peptide Synthesis

Peptides were synthesized at 2 μmol scale in a 96-well plate with an Intavis MultiPep synthesizer (Koeln, Germany) using standard Fmoc chemistry. Preloaded NovaSyn TGT resin was from Novabiochem (San Diego, CA, USA). Fmoc-protected amino acids were activated in situ with HBTU/*N*-methylmorpholine and coupled at 5-fold molar excess over peptide. Each coupling cycle was followed by capping with acetic anhydride. At the end of the synthesis, peptides were labeled on the resin with 5- (and 6)-carboxytetramethylrhodamine (5[6]-TAMRA) from Anaspec (San Jose, CA, USA). 5(6)-TAMRA was coupled using HATU/diisopropylethylamine at a 7-fold molar excess over peptide. After labeling, the peptides were deprotected and cleaved from the resin with trifluoroacetic acid/triisopropylsilane/water (38:1:1, v/v/v) and subsequently precipitated by addition of cold ether.

Crude peptides were purified by reverse-phase HPLC using a Kromasil 100-5-C18 semipreparative column (Peeke Scientific, Sunnyvale, CA). Fractions containing the correct product were identified by MALDI-TOF mass spectrometry using a Voyager DE Pro (Applied Biosystems, Foster City, CA).

Fabrication and Processing of Protein Microarrays

Purified recombinant domains were spotted at a concentration of 40–200 μM onto 112.5 mm × 74.5 mm × 1 mm glass substrates, chemically modified to display aldehyde functionalities (Erie Scientific Company, Portsmouth, NH, USA), using a NanoPrint microarrayer (TeleChem International, Sunnyvale, CA). Ninety-six microarrays (two different sets of 48 identical arrays) were fabricated in an 8 × 12 pattern on the glass, with a pitch of 9 mm. Each array consisted of a 16 × 17 pattern of spots, with a center-to-center spacing of 250 μm. All proteins were spotted in quadruplicate. Following a 1 hr incubation, the glass was attached to the bottom of a bottomless 96-well microtiter plate (Greiner Bio-One, Kremsmuenster, Austria) using an intervening silicone gasket (Grace Bio-Labs, Bend, OR). The arrays were stored at −80°C. Immediately before use, the plates were quenched with buffer A (20 mM HEPES, 100 mM KCl, 0.1% Tween 20 [pH 7.8]) containing 1% BSA (w/v) for 30 min at room temperature, followed by several rinses with buffer A. Arrays were probed with eight different concentrations of 5(6)-TAMRA-labeled peptides, dissolved in buffer A: 5 μM, 3 μM, 2 μM, 1 μM, 500 nM, 200 nM, 100 nM, and 10 nM. Following a 1 hr incubation at room temperature, the peptide solution was removed and the arrays were washed with 150 μl buffer A for 10 s. The arrays were rinsed briefly with ddH_2O and spun upside down in a centrifuge for 1 min to remove residual water.

Electromobility Shift Assays

DNA oligonucleotides corresponding to the interferon-stimulated response elements of IRF1, ICSBP, and ISG15 were synthesized with the following sequences: IRF1: 5′-GATCGATTTCCCCGAAATG-3′; ICSBP: 5′-AGTGATTTCTCGGAAAGAGAG-3′; ISG15a: 5′-GCTTCAGTTTCGGTTTCCCTTTCCCGAG-3′; and ISG15b: 5′-GGGAAAGGGAAACCGAAACTGAA-3′. Oligonucleotides were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) according to standard procedures. Five micrograms of lysate in a 15 μl volume was incubated for 30 min with 1 μg dIdC (to block nonspecific binding). Labeled oligonucleotide was then added and the reaction was incubated for an additional 20 min. Samples were loaded on a 6% TBE-polyacrylamide gel and run for 2.5 hr. The gel was dried and exposed to X-ray film overnight.

SUPPLEMENTAL DATA

Supplemental Data include one figure and one table and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/8/808/DC1/.

ACKNOWLEDGMENTS

We thank Sze-Ling Ng for assistance with electromobility shift assays; Tom Maniatis for helpful discussions; Kermit Carraway III for sharing unpublished data; Renee A. Robinson for assistance with mass spectrometry; Jeffrey Knott, Susan Rogers, and Colleen Hunter for help with peptide synthesis; and the Harvard FAS Center for Systems Biology for support with instrumentation. This work was supported by awards from the W. M. Keck Foundation, the Arnold and Mabel Beckman Foundation, and the Camille and Henry Dreyfus Foundation, and by a grant from the National Institutes of Health (1 R33 CA128726-01). A.K. was supported in part by an NIH Molecular, Cellular, and Chemical Biology Training Grant (5 T32 GM07598-25) and A.G. is the recipient of an NSF Graduate Research Fellowship. J.R. is an employee of and stockholder in Cell Signaling Technologies, Inc., and G.MacB. is an advisor for and stockholder in Merrimack Pharmaceuticals, Inc. and Makoto Life Sciences, Inc.

Received: May 1, 2008
Revised: June 25, 2008
Accepted: July 1, 2008
Published: August 22, 2008

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