Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins*

Received for publication, July 5, 2001, and in revised form, August 9, 2001
Published, JBC Papers in Press, August 10, 2001, DOI 10.1074/jbc.M106239200

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Amplification of the type I receptor tyrosine kinase ErbB-2 (HER2/Neu) is observed in 20–30% of human mammary carcinomas, correlating with a poor clinical prognosis. We have previously demonstrated that four (Tyr1144, Tyr1201, Tyr1226/1227, or Tyr1253) of the five known Neu/ErbB-2 autophosphorylation sites can independently mediate transforming signals. The transforming potential of at least two of these autophosphorylation sites (Tyr1144 and Tyr1226/1227) has been further correlated with their ability to associate with Grb2 and Shc adapter proteins, respectively. To confirm the specificity of these interactions, we have created a series of second site mutants in these phosphorylation sites. The results showed that Grb2 recruitment to site 1144 is absolutely required for transforming signal from this autophosphorylation site, whereas association of Shc-mediated transformation is dependent on conservation of the NPXY motif spanning Tyr1227. A stretch of amino acid identity around tyrosines 1201 (ENPEYLTP) and 1253 (ENPEYLDL) exists, and mutation of key residues within this motif reveals distinct requirements for an intact protein tyrosine-binding protein (NPXY). We show that Dok-R, a protein tyrosine-binding site-containing protein implicated in Ras signaling, interacts with Neu/ErbB-2 at Tyr1253 as do two unidentified proteins, p150 and p34, the latter correlate with transformation. Together these data argue that ErbB-2/Neu is capable of mediating transformation through distinct effector pathways.

Neu/ErbB-2, along with the epidermal growth factor receptor, ErbB-3, and ErbB-4, are transmembrane receptor tyrosine kinases (RTKs) that comprise the class I or ErbB RTK family (reviewed in Ref. 1). Although elevated expression of the ErbB family members is implicated in the etiology of human ovarian and breast cancers, ErbB-2 most clearly plays a causal role in this process. ErbB-2 amplification and elevated expression correlates with a poor clinical prognosis in breast cancer patients (2–4). Although these data are consistent with a role in mammary tumorigenesis, transgenic mice expressing either ErbB-2 or Neu (the rat ErbB-2 homolog) were generated to directly assess ErbB-2 oncogenic causality. Expression of wild type Neu or constitutively active Neu or ErbB-2 alleles in the mammary epithelia of transgenic mice results in the induction of metastatic mammary tumors (reviewed in Ref. 5). Taken together, these data suggest that activation of ErbB-2/Neu plays a causal role in mammary tumorigenesis.

Despite the importance of ErbB-2/Neu in human malignancies, the molecular basis for the potent transforming properties of this oncogene remains to be elucidated. Activation of the ErbB-2 receptor via receptor dimerization results in the phosphorylation of a number of tyrosine residues within the carboxyl-terminal regulatory region of the receptor. These phosphorylated tyrosines provide binding sites for a variety of Src homology 2 (SH2) and/or protein tyrosine binding (PTB)-containing proteins involved in transducing proliferative, transforming, or differentiating signals to the nucleus. For example, ErbB-2 associates with intracellular signaling molecules such as Crk, Grb7, PLCγ1, and c-Src, with the latter two proteins becoming activated by ErbB-2 (reviewed in Refs. 5 and 6). Another class of adapter proteins associate with ErbB-2 to modulate Ras activity by either promoting the active Ras-GTP complex formation through Sos GDP/GTP exchange proteins (Shc, Grb2, and Nck) or by accelerating the hydrolysis of Ras-GTP to its inactive Ras-GDP state (Ras-GAP) (7–10). Whereas these studies have provided compelling evidence that ErbB-2 can associate with a number of downstream mediators, the relative contribution of these signaling pathways remains to be elucidated.

To address the role of the various signaling pathways in ErbB-2-mediated transformation, we have generated a series of ErbB-2 mutants that harbor mutations in the tyrosine auto-phosphorylation sites (9). These studies revealed that a constitutively active ErbB-2 allele lacking all of the major phosphorylation sites within the regulatory region (NYPD mutant) was severely impaired in transformation. However, restoration of single phosphorylation sites to NYDP or to its inactive Ras-GDP state (NYPD mutant) (7–10). Whereas these studies have provided compelling evidence that ErbB-2 can associate with a number of downstream mediators, the relative contribution of these signaling pathways remains to be elucidated.

To address the role of the various signaling pathways in ErbB-2-mediated transformation, we have generated a series of ErbB-2 mutants that harbor mutations in the tyrosine auto-phosphorylation sites (9). These studies revealed that a constitutively active ErbB-2 allele lacking all of the major phosphorylation sites within the regulatory region (NYPD mutant) was severely impaired in transformation. However, restoration of single phosphorylation sites to NYDP, creating a series of add-back mutants, demonstrated that four of five phosphorylation sites (sites B–E) can independently mediate transforming signals, whereas tyrosine 1028 functions to repress transforming signals from the receptor (9). Further biochemical analyses demonstrated that Grb2 associates directly with Tyr1144 (site B) and indirectly through tyrosine-phosphorylated Shc proteins at Tyr1226/1227 (site D) (9), whereas Crk binds through tyrosine residue 1201 (site C) (10).

Here we have demonstrated that mutations in the Grb2 and Shc consensus binding sites revealed that transformation from these add-back mutants strictly correlates with their ability to bind Grb2 or Shc adapters, suggesting that these are the prin-
and effector molecules of these sites. Furthermore, we show the PTB-containing protein, DOK-R, interacts with Neu/ErbB-2 at Tyr1253 (site E) as do two unidentified proteins, p34 and p150. Interestingly, although p150 and DOK-R associate with transformation deficient NPX mutants at site E, p34 associates with only wild type site E in a transformation-dependent manner. Taken together, these results suggest that Neu/ErbB-2 induces cellular transformation through multiple ostensibly redundant tyrosine phosphorylation sites through the recruitment of at least four different signaling molecules.

**EXPERIMENTAL PROCEDURES**

**Autophosphorylation Mutants, Cell Lines, and Transformation Assays**—The generation of rat ErbB-2 NYPD and add-back mutant plas-
mids and Rat1-derived plasmids has been described previously (9).
Secondary mutations around sites B, D, and E were generated by
polymerase chain reaction, and all cloned polymerase chain reaction
amplified regions were sequenced in their entirety. In all cases ErbB-2
was expressed in a Moloney murine leukemia virus long terminal
repeat expression vector (pMlAI) (11). Details of plasmid constructions
are available on request.
Rat1 fibroblasts were maintained in Dulbecco's modified Eagle's
medium supplemented with 10% fetal bovine serum, penicillin, strep-
tomycin, gentamycin, and fungizone. DNA was introduced into Rat1
fibroblasts via electroporation for the focus formation assays of Figs. 2,
4, and 5 as described (9). Briefly, 10^7 Rat1 fibroblasts along with 50 μg
of each mutant plasmid DNA and 2 μg of PGK-puro were electroporated
and were immediately seeded at 10^5 cells/100-mm tissue culture dish.
Plates were maintained in supplemented Dulbecco's modified Eagle's
medium for 14 days with the medium being changed every third day.
Following the manufacturer's instructions, the plates were stained with
Giemsa, and the foci were counted from six plates/construct. Relative
transformation potential was normalized to that obtained with acti-
vated Neu (NT) in each experiment and expressed as a percentage. Soft
agar assays were as described using clonal Rat fibroblasts engineered to
express the various Neu autophosphorylation mutants (9).
Affinity Purification, Immunoprecipitation, and Immunoblotting—
The cells were washed twice in 1× phosphate-buffered saline at 4°C, and
the lysates were made in PLC lysis buffer (50 mM HEPES, pH 7.5,
150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl_2, 10 mM NaF,
50 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 μg/ml aprotinin,
10 μg/ml leupeptin) as described (9). Grb2 and Shc co-immunoprecipitates
were as described in Refs. 9 and 10. DOK-R
immunoprecipitated with DOK-R-specific antisera (12) from 1 to
1 mg of cell lysate and were washed five to seven times in PLC lysis
buffer prior to immunoblot analyses. Neu was immunoprecipitated with
Neu immunoprecipitates from the stable cell lines expressing various Neu
phosphorylation mutants were probed with
32P-labeled GST fusion proteins containing the Shc PTB (A) or SH2 domain (B).
These immunoprecipitates were also probed with anti-phosphotyrosine specific
antisera (C). Note the specific binding of the Shc PTB domain to site D (lane 6). IP,
immunoprecipitation; IB, immunoblot; Ab, antibody.

RESULTS
To directly assess the role of the ErbB-2/Neu tyrosine phospho-
rylation sites in mediating transforming signals, we have generated a Neu tyrosine phosphorylation-deficient mutant (NT-NYPD) through single point mutations at each of the defined tyrosine phosphorylation sites (Tyr^{1028},
Tyr^{1144},
Tyr^{1201},
Tyr^{1226/1227},
Tyr^{1253}) (Fig. 1). Activated ErbB-2/
Neu NYPD alleles are severely impaired in transforming activities in culture and when expressed in the mammary epithelium of transgenic mice (9, 10). To determine whether particular tyrosines were sufficient in mediating a trans-
forming signal, individual tyrosine phosphorylation sites were reconstituted to NT-NYPD, creating a series of add-
back mutants each possessing only one of these sites. For the
purpose of clarity, activated Neu mutants harboring individual
 tyrosine phosphorylation sites are termed NT-YA (tyro-
sine at 1028), NT-YB (tyrosine 1144), NT-YC (tyrosine 1201),
NT-YD (tyrosines 1226 and 1227), and NT-YE (tyrosine 1253)
To assess transformation potential, we performed soft agar colony formation assays. Activated ErbB-2 (NT) readily conferred anchorage-independent growth to expressing Rat1 fibroblasts, a process requiring the major phosphorylation sites, because NYPD failed to form colonies in these assays (Fig. 1B). As we had observed in focus formation assays (9), NT-YA was transformation defective. Restoration of any one of four tyrosine autophosphorylation sites (NT-YB, NT-YC, NT-YD, or NT-YE) results in anchorage-independent growth activities comparable with those observed in the parental NT in Rat1 fibroblasts (Fig. 1B). Similar results were observed in multiple Rat1-derived cell lines expressing the various add-back mutants (data not shown). We consistently observed smaller colonies formed in stable lines expressing NT-YB compared with those from the other transforming mutants. The ability to grow in an anchorage-independent manner was not acquired through the cloning and passaging of these lines, because pooled colonies from electroporated Rat1 cells behave in a similar manner (data not shown). Notably, pooled Rat1 cells expressing NT-YB appeared to form colonies of various sizes, suggesting that the small colonies formed from stable lines reflect the particular clones used in these assays. Taken together, these data demonstrate that four of five phosphorylation sites are sufficient to mediate transforming signals from activated Neu.

Association of Grb2 with Site B and Shc with Site D Correlates with Transformation—We have previously used Far Western (direct blot) and co-immunoprecipitation analyses to demonstrate that Grb2 binds to Tyr1144 (site B) (9). Although the Grb2 binds to NT-YB, it is conceivable that an additional protein interacts with this phosphorylation site to mediate transformation. Site B conforms to a consensus Grb2 SH2 domain-binding site as defined with degenerate peptide pools (Y\[X\]N\[X\]) (15). Four alterations of the conserved asparagine (Asn1146 in the \[H\]11001\(\)2 position; Fig. 2A) were engineered in NT-YB to evaluate whether loss of the asparagine (and presumably Grb2 association) correlated with a transformation defect. To ensure that any phenotype observed was due to the loss of the asparagine and not due to the acquisition of a new binding site for a different SH2-containing molecule, several amino acids were substituted. Alteration of this residue to histidine, leucine, valine, or proline had little effect on the phosphorylation status of the receptor (Fig. 2B), although these mutants had dramatically decreased transformation potentials (Fig. 2A) to levels observed for YB lacking Tyr1144 (i.e. NT-NYPD). This decrease in transformation potential was correlated with a loss of Grb2 binding to the NT-YB-derived mutants (Fig. 2B). Together, these data suggest that Grb2 binding is required for efficient NT-YB-mediated transformation.

Our previous biochemical analyses demonstrated that Grb2 also associates with site D, albeit indirectly, and that Shc proteins likely mediate the indirect interaction of Grb2 (9, 15).
The three Shc isoforms each contain a PTB and a carboxy-terminal SH2 domain separated by a collagen homology region that harbors two tyrosine phosphorylation sites capable of binding Grb2 (16, 17). To define the domains of Shc that bound Neu, bacterial GST fusions that included a protein kinase A recognition site and either the SH2 or PTB domain were purified and radiolabeled to high specific activity and used in Far Western/direct blot analyses. Although equivalent amounts of tyrosine-phosphorylated Neu were detected in each immune-precipitate (Fig. 3C), the Shc PTB (Fig. 3A) but not the SH2 (Fig. 3B) domain bound to NT and site D (Tyr1226/1227) demonstrating the Shc PTB domain was sufficient in mediating this interaction. This same SH2 fusion protein has been shown to interact with the epidermal growth factor receptor (13).

The observation that Shc-Neu interactions are mediated by the Shc PTB domain implies that the residues of Neu required for Shc binding are different from those which SH2 domains bind. Specifically, SH2 domains recognize sequences to the carboxyl-side of a phosphorylated tyrosine residue, whereas PTB domains recognize sequences predominantly to the amino side of the phosphotyrosine (reviewed in Ref. 18). The consen-sus binding sequence determined for the Shc PTB domain is XφNXXY, where φ is a large hydrophobic residue (19). Comparing the sequences surrounding site D (1226-NLYY1227) to those found in the Shc binding site of MT (312-NPSY315), both Asn1224 and Tyr1227 appear to create a partial PTB binding site for Shc (NXXY). Because both Tyr1226 and Tyr1227 were mutated to create site D mutants, we individually reconstituted these residues to tyrosines in the context of NT-NYPD to create YD-NLFY and YD-NLYF (Fig. 4A). Additionally, Asn1224 was changed to alanine or aspartic acid in the context of NT-YD creating YD-ALYY and YD-DLYY, respectively. Consistent with the notion that Shc interacts through its PTB domain with Neu, mutation of either Tyr1227 or Asn1224 (YD-DLYY) dramatically reduced transformation potential (Fig. 4A), whereas mutation of Tyr1226 had little effect. Additionally, alteration of Asn1224 to alanine (YD-ALYY) resulted in an impairment of transformation intermediate that of NT-NYPD and NT-YD. The levels of Shc bound to these Neu mutants appeared to directly correlate with their transformation potential. Specifically, although low Shc levels are found associated with NT-NYPD, elevated Shc levels were detected in Neu immune-precipitates from NT- and NT-YD-expressing cells (Fig. 4C). Mutation of Asn-1144 to aspartic acid reduced Shc binding to levels observed for NT-NYPD, whereas an alanine in this position reduced to levels intermediate that of NT and NT-NYPD (lanes 5–7). Reduced Shc binding was not a consequence of reduced receptor or Shc protein expression (Fig. 4, B and E) and was further coupled to a loss of detectable Grb2-Neu complex formation that cannot be ascribed to Grb2-NTPD and NT-YD.

**Fig. 5.** Transformation from tyrosine 1253 (site E) occurs in a sequence specific manner and does not correlate with DOK-R association. A, generation of specific point mutations around the NPXY motif of site E involving mutation of an asparagine residue (YE-APEY and YE-DPEY) or a proline residue (YE-NAEY and YE-NDEY). The specific transforming activity of each of these mutants is also shown. Note that YE-APEY, YE-DPEY, and YE-NDEY mutants are transformation defective. B, DOK-R was immunoprecipitated from 1.0 mg of protein lysates derived from the indicated Neu point mutant-expressing cell lines. Upper panel, the immunoprecipitates were analyzed by anti-Neu immunoblot analysis. Lower panel, Neu was immunoprecipitated from 500 µg of the same lysates, and the immunoprecipitates were subjected to anti-phosphotyrosine immunoblot analyses. C, upper panel, DOK-R immunoprecipitates from the indicated cell lines were subjected to anti-Neu immunoblot analysis as in B. Pooled lines were used for YE-NAEY, YE-NDEY, YE-APEY, and YE-DPEY mutants. Lower panel, the phosphotyrosine content of Neu was assessed by anti-phosphotyrosine immunoblot analyses as in B. Indicated is the migration of Neu. IP, immunoprecipitation; IB, immunoblot.
cells (Fig. 4, D and F). Together, these data demonstrate that Shc binding tightly correlates with transformation from site D.

Requirement for Intact NXXY Motif at Site E—A short stretch of amino acid identity around tyrosines 1201 and 1253 exist (site C, ENPEYLTG, and site E, ENPEYLDL, respectively). The presence of a β-turn-forming NPXY motif in the core Shc and IRS-1 PTB domain-binding sites suggests that one or more PTB-containing proteins mediates transformation from sites C and E. Mutations were made to codons encoding the conserved asparagine (Asn1250) or proline (Pro1251) to those encoding either alanine or aspartic acid. These mutations were made in the context of NT-YE creating YE-NAEY, YE-NDEY, encoding either alanine or aspartic acid. These mutations were aspartic acid, but not asparagine 1250 for efficient site E-mediated transformation, whereas alteration of proline 1199 in NT-YC to aspartic acid or alanine lead to a reduction in transformation potential (data not shown). The presence of a N1250D mutation (DPEpY).

Further analyses suggest that DOK-R interacts with Tie-2/Tek RTK in a yeast two-hybrid screen (12). Sequence analysis revealed homology to p62DOK and is termed DOK-R for DOK-related protein. DOK is a 62-kDa Ras-GAP-associated protein that is tyrosine-phosphorylated in response to transforming and mitogenic PTK activation (21). The transforming potential of tyrosine 1253 is correlated with transformation from site E, a tyrosine-tyrosine 1253 (site E) affinity purified several cellular proteins. Proteins from [35S]methionine-labeled Rat1 (lanes 1–3) and Jurkat T-cell (lanes 4–6) extracts were affinity purified on non-phosphorylated (NPEY) and phosphorylated (DPEpY) wild type peptides as well as to a phosphorylated peptide containing a N1250D mutation (DPEpY). Black arrowheads indicate the migration of p34 and the mobility of an ~150-kDa phosphotyrosine-dependent associated protein. The left and right panels represent long and short exposure of the same experiments.

The Transforming Potential of Tyrosine 1253 Is Correlated with Binding of a 34-kDa Protein—Given that DOK-R-Neu interactions do not correlate with transformation, we have undertaken a search for site E-interacting proteins. To this end, chemically synthesized phosphorylated and nonphosphorylated peptides spanning site E from the −11 to the +7 position relative to the phosphotyrosine residue (wild type NPEY peptide: FEGTPTAPENPEYLGLDVPV) were used in vitro association assays. The amino terminus of each peptide was covalently linked to biotin, providing a means of immobilizing the peptides via streptavidin-conjugated agarose beads. To these immobilized peptides, equivalent amounts of Rat1 [35S]methionine-labeled extracts were incubated, the beads were extensively washed, and specifically associated proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 6). Consistently, two proteins of ~150 and 34 kDa associate with the phosphorylated but not the nonphosphorylated peptide in both Rat1 (Fig. 6, lanes 1 and 2) and NIH-3T3 fibroblasts (not shown). The lack of binding to the unphosphorylated peptide is not a result of decreased peptide concentration because dephosphorylation of the wild type peptide decreased the amount of the 34- and 150-kDa proteins detected (not shown). To determine whether p34 or p150 binding correlated with transformation from site E, a tyrosine-
phosphorylated peptide was synthesized to contain the N1250D mutation (DPEY peptide: FEGTPTAEDPEpYLGLD-VPV), which renders this site transformation incompetent (Fig. 5A). The 34-kDa protein, but not the 150-kDa protein, failed to interact with this DPEpY peptide when isolated from Rat1 and NIH-3T3 fibroblasts (not shown) as well as in Jurkat T cells (Fig. 6). The 36-kDa protein associated with the phosphorylated DPEY peptide was not consistently observed, nor was it detected in all cell types such as Jurkat cells (Fig. 6, lanes 5 and 6). These data suggest that a number of proteins associate with site E through the phosphorylation of tyrosine 1253, but the in vitro association of a single 34-kDa protein correlates with transforming potential from this site.

**DISCUSSION**

The ability of activated RTKs to signal cellular proliferation and differentiation is dependent on their capacity to couple to a variety of cytoplasmic signaling molecules through a mechanism involving the binding of either SH2 or PTB domain-containing proteins with specific phosphotyrosine residues. These sites are required for efficient transformation by ErbB-2 because mutation of the known tyrosine phosphorylation sites (NT-NYPD) renders the RTK transformation deficient despite being efficiently expressed and harboring similar catalytic activities (9). Reconstitution of individual tyrosine phosphorylation sites to NT-NYPD reveals that individually sites B–E are sufficient for restoration of wild type transforming abilities. Biochemically, it is clear that site B (Tyr1144) binds directly to Grb2 and that this ErbB-2/Grb2 complex is required for transformation (Fig. 1), likely by recruiting mSos proteins to activate Ras (9, 10). In a similar fashion Shc proteins interact with site D, tethering Grb2 to ErbB-2 and thereby providing a cogent explanation explaining the Ras requirement and Erk activation (10). In either case mutation of the residues required for Grb2 or Shc binding in NT-YB or NT-YD, respectively, ablates transforming activities.

In this study we provide additional evidence that integrity of Grb2- or Shc-binding sites are required for efficient ErbB-2 mediated transformation from either the NT-YB or NT-YD add-back mutants. Indeed replacement of a conserved asparagine residue within the Grb2- or Shc-binding sites can dramatically impair transforming activities of these add-back mutants (Figs. 2 and 4). Significantly this impaired transforming activity correlated with a corresponding loss of the ability of these mutant ErbB-2 receptors to associate with either Grb2 or Shc. These data argue that the YB and YD sites are highly specific binding sites for Grb2 and Shc, respectively, that can independently mediate a transforming signal. Although these observations suggest that Shc and Grb2 binding to ErbB-2 result in functionally redundant transforming signals, there is an increasing body of evidence that recruitment of either of these adapter proteins to ErbB-2 results in the generation of distinct signals. For example, microinjection of a Grb2 dominant negative inhibitor can suppress DNA synthesis emanating from NT-YB mutant but has little impact on the NT-YD mutant (10). Further evidence supporting this view stems from transgenic mice expressing the YB and YD ErbB-2 mutants in mammary epithelium. In these studies, although mammary epithelial expression of either ErbB-2 mutant was capable of inducing mammary tumors, only the YB-derived strains developed efficient metastatic disease (10). Taken together, these observations suggest that the ability of ErbB-2 to couple to either Grb2 or Shc may have distinct biological outcomes.

In addition to the adapter proteins coupled to the B and D sites, we have presented evidence that transforming signal from the YE site is dependent on the conservation of the NPXY motif. Indeed, mutations that alter either the conserved asparagine or proline residues within the context of the YE add-back mutant exhibit an impaired transforming activity (Fig. 5). Given the importance of the PTB-binding motif, we further tested the possibility that a PTB-containing protein known as DOK-R is capable of interacting with ErbB-2. Somewhat surprisingly, DOK-R appears to specifically interact with site E but not C, even though the surrounding sequences are quite similar (site C: -GGAVENPEYLV-3 and site E: -TPTAEN-...
PEYLGL, although DOKR-ErbB-2 complexes demonstrated specificity for site E, this interaction did not correlate with transformation from site E. Specifically, NPY motif mutants severely impaired in their transformation potential remain capable of DOK-R binding (Figs. 5). Taken together these data represent the first demonstration that DOK family members interact with ErbB-2 and furthermore suggest that they are not positive mediators of signal transduction RTKs. Indeed, forced expression of DOK-R (also known as DOK-2) suppresses proliferation of cultured myeloid leukemia cells in response to several to a variety of growth factors (granulocyte-colony-stimulating factor, granulocyte macrophage colony-stimulating factor, interleukin-3, and stem cell factor) (22). Moreover, mice made deficient for p62DOK expression demonstrate that the Ras-GAP-associated p62DOK is a negative regulator of signaling through the B cell receptor. Derived p62DOK-deficient B cells demonstrate hyper-responsiveness to antigen stimulation resulting in increased Erk activation, presumably a reflection of decreased localized Ras-GAP activity (23). Transient overexpression of DOK-2 in 32D cells leads to a decrease in interleukin-2-dependent Erk activation (24). Taken together, these data suggest that although NT-YE is transforming, signaling from this phosphorylation site is negatively regulated by DOK-R recruitment.

We have demonstrated that at least two additional proteins (p150 and p34) are capable of interacting with site E in vitro. Although both proteins interact in a phosphotyrosine-dependent manner, only p34 associates specifically with the wild type but not the mutant DPEpY peptide. Additionally, p34 does not but not the mutant DPEpY peptide. Additionally, p34 does not associate with a similar peptide to site C (data not shown). It is intriguing to note that p150 is similar in size to PLCγ, which is known to interact with a collinear site in the epidermal growth factor receptor (25) and that a DOK-R-related protein interacts with PLCγ (26). We have ruled out both Crk and Nck proteins as being p34 by immunoblot analyses. Indeed, in similar peptide association assays, both proteins associate with site C (data not shown and Ref. 10). The in vitro association of p34 correlates with transformation from site E, suggesting that p34 is an excellent candidate molecule mediating signaling from site E. Clearly future identification of this protein will provide important insight into the mechanism of Ras activation and the biological basis of ErbB-2 transformation from this site.

Tyrosine phosphorylation mutants have proved to be a tool for the study of specific protein interactions. Using single phosphorylation point mutations we have previously demonstrated that no tyrosine phosphorylation site is absolutely required for ErbB-2-mediated transformation. As the removal of all the known tyrosine phosphorylation sites (NYP) abrogates transformation, tyrosine reconstitution to generate a series of add-back mutants allows a coupled study cleanly correlate protein association with biological transformation. These data taken together suggest there are at least four distinct proteins that mediate transformation from activated ErbB-2 (Fig. 7).

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