Oligomers of ERBB3 Have Two Distinct Interfaces That Differ in Their Sensitivity to Disruption by Heregulin*

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ErbB receptors associate in a ligand-dependent or -independent manner, and overexpression of epidermal growth factor receptor (ErbB1) or ErbB2 results in ligand-independent activation. Ligand-independent activation is poorly understood, and dimerization alone is not sufficient for activation. ErbB receptors also form higher order oligomers, but the mechanism of oligomer formation and their contribution to signaling are not known. The kinase-deficient ErbB3 as well as its extracellular domains are particularly prone to ligand-independent oligomerization, and oligomers are destabilized by binding of the ligand heregulin. In contrast, ligand binding facilitates heterodimerization with ErbB2 and is expected to stabilize an extended conformation of the ErbB3 extracellular domain (ECD) in which the dimerization interface is exposed. In the absence of ligand, ErbB3 can adopt a closed conformation that is held together by an intramolecular tether. We used a constitutively extended form of the ErbB3-ECD to analyze the conformation of the ECD in oligomers and the mechanism of oligomer disruption by heregulin. The extended conformation of the ECD forms oligomers more readily, suggesting the crystallographically defined dimer interface is one of the interfaces involved in oligomerization. Heregulin destabilizes oligomeric complexes but not dimers, which are neither stabilized nor disrupted by ligand binding, indicating a distinct second interface in oligomers of ErbB3. Cross-linking and activation studies on membrane-embedded ErbB3/ErbB2 chimeras confirm this dual effect of heregulin. Most of the ErbB3-ECD on the cell surface is apparently kept in an open conformation through oligomerization, and the resulting oligomers adopt a conformation representing a state of reduced activity.

The ErbB or EGFR† family of receptors in humans includes four members, EGFR (ErbB1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4), that are involved in a wide range of differentiation and growth control events. Overexpression, especially of EGFR or ErbB2, has been observed in a variety of tumors (1–5). Controlled activation of these receptors requires binding of a ligand of the EGF or heregulin family of growth and differentiation factors, resulting in cross-phosphorylation of the dimerized receptors at specific tyrosines in the cytoplasmic portion. However, under conditions of overexpression, tyrosine phosphorylation occurs constitutively, and cells expressing elevated levels of ErbB2 show a more aggressive growth behavior (5).

Of the different pairs of receptor dimers that can form, the combination of ErbB2 and ErbB3 shows the strongest potency in terms of stimulating cell proliferation (6). Many tumors that show overexpression of ErbB2, especially those that are more prone to become resistant to conventional treatment, also show elevated levels of ErbB3 (7). The ErbB2/ErbB3 dimer is unique in that ErbB2 has a potent cytoplasmic kinase domain, but its extracellular domains (ECD) fail to bind any known ligand directly (8, 9). On the other hand, the ECD of ErbB3 binds a variety of isoforms of heregulin, but its cytoplasmic kinase domain is catalytically deficient (10). Another distinct feature of ErbB3 is its enhanced propensity to self-associate. Ligand-independent oligomerization, which is the formation of complexes of more than two receptors, can be observed for full-length ErbB3 as well as for the ErbB3-ECD. Oligomerization is not driven by ligand binding. Instead, oligomers are destabilized by the binding of heregulin, thereby favoring the formation of heterodimers with ErbB2 in the context of signaling (11).

The activation of ErbB receptors has traditionally been attributed to ligand-induced dimerization of the extracellular portions of the receptors. The recent crystal structures of EGFR, ErbB2, and ErbB3 (12–14), in some cases with ligand and in a dimerized state, have provided significant insights into the nature of receptor dimers in the presence of ligand. These structures demonstrated that dimerization involves loops on domains two and four of each receptor ECD that interacts with their respective counterparts on a second receptor molecule. We refer to this interface below as the crystallographically defined dimer interface. The receptor interaction is modulated but not physically mediated by the ligand, which binds in a binding pocket on the outer faces of the symmetric dimer between domain one and three of each receptor (reviewed in Ref. 15). A crystallographically observed closed conformation of ErbB3-ECD, in which domains two and four are kept in an open conformation through oligomerization, and the resulting oligomers adopt a conformation representing a state of reduced activity.

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† The abbreviations used are: EGFR, epidermal growth factor receptor; ECD, extracellular domains; EGF, epidermal growth factor; GEMMA, gas-phase electrophoretic mobility molecular analysis; trx, thioredoxin; hrg, heregulin; PVDF, polyvinylidene difluoro; HRP, horseradish peroxidase; WT, wild type; PBS, phosphate-buffered saline; GST, glutathione S-transferase; BSA, BSA (sulfosucinimidyl) suberate; ESI, electrospray ionization; CHO, Chinese hamster ovary.

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teract instead within a single receptor monomer, was initially thought to represent a locked conformation, critical for preventing dimerization in the absence of ligand. The fact that ErbB2 failed to show such a locked conformation reconciled well with the observed enhanced autophosphorylation of overexpressed ErbB2 (16). However, although the closed ECD conformation is indeed incompatible with the receptor dimers observed for the open form, its prevalence and significance in suppressing basal activation have since been questioned (17). 

Constitutively open mutants of EGFR show more high affinity binding sites for EGF but no significant increase in basal activation (17, 18).

Although ligand-bound dimers are at the core of ErbB activation, several lines of data suggest a more complex mechanism in which receptors can be associated prior to ligand binding with parts of the cytoplasmic portion and the transmembrane segment contributing to the interaction. Specifically, the presence of two GXXXG consensus motifs in the transmembrane span of ErbB receptors has been shown to provide intrinsic dimerization potential. ErbB3 is unique in its absence of a good GXXXG consensus, and its transmembrane span consequently shows the weakest tendency to self-associate (19). In contrast, the ErbB2 transmembrane span dimerizes strongly, and ErbB2 shows enhanced constitutive activation if most of the extracellular domain is absent (20, 21), suggesting that the ECD, in the absence of ligand, acts to suppress the activity of intrinsically associated receptors. In addition, ErbB2 receptors can form large clusters of receptors on the cell surface. ErbB2 has been shown to form such clusters independent of its ligand-dependent association with ErbB3 or EGFR, especially in cells that overexpress ErbB2 (22). However, in contrast to ErbB3, the contributions of specific portions of the ECD of ErbB2 to these ligand-independent interactions are too weak to be biochemically studied in solution, and the role of such clusters in signaling is not clear.

ErbB3 provides a good model system for the evaluation of higher order oligomers of ErbB receptors. In contrast to ErbB2 or EGFR, the oligomerization of ECDs can be studied in solution. The putative oligomerization interfaces in ErbB3 are likely to differ from those found in other ErbBs in the affinity of their respective interactions but are likely to be qualitatively conserved within this family of structurally highly homologous receptors. However, several aspects of ErbB3 self-association have not been conclusively answered, most importantly, the role of ligand binding in ErbB3 self-association. By using different approaches, ErbB3 has been reported to self-associate upon ligand binding (23), not to homodimerize upon binding of ligand (24), and we have previously demonstrated that oligomers or ErbB3 are disrupted by the binding of ligand (11).

Because the crystal structure of the ECD of ErbB3 shows a closed conformation in which the high affinity ligand-binding pocket between domains one and three is disrupted (Fig. 1), the binding of ligand is expected to stabilize the extended form of the receptor over the closed conformation. For ErbB3, the presence of a closed and extended conformation and different association states means that the receptor exists in at least three different forms: oligomers, dimers, and monomers in either the closed or extended conformation. Based on the current models for the dimerization of ErbB receptors, the unlocking of the tether is expected to enhance dimerization. In the case of EGFR, unlocking of the tether provides a larger population of receptors that can transition to a high affinity form (18), but for ErbB3 no significant stabilization has been observed upon binding of heregulin to homodimers of ErbB3/EGFR chimera carrying an ErbB3 ECD (24). In addition, the formation of symmetrical dimers does not readily explain the formation of larger order oligomers, which would require at least one additional interface. If the extended and ligand-binding form of the receptor takes part in both dimerization and oligomerization, an increase in oligomerization could be expected following ligand binding. However, this appears to be in contrast to our previous observation that the binding of ligand reduces oligomerization. The work presented here addresses the question of the conformation of ErbB3 and the nature of interfaces in the oligomeric state. Specifically, we evaluate the impact of a constitutively open conformation of the ECD on oligomerization.

Our current findings indicate that oligomers are assembled from the extended form of the receptor ECD, and oligomerization involves two different types of interfaces. The extended form of ErbB3-ECDs can dimerize constitutively, and this dimerization interface is neither disrupted nor stabilized by ligand binding. A second interface is responsible for the subsequent oligomerization of ECD dimers. This second interface is subject to destabilization by heregulin. We confirmed this dual impact of heregulin on interactions of the ErbB3-ECD in a cellular context. Chimera of the ErbB3-ECD and the transmembrane and cytoplasmic portions of ErbB2 provide tyrosine phosphorylation as a readout for the formation of dimers in an activated conformation. Although the addition of heregulin greatly reduces cross-linking of this chimera, which is consistent with the destabilization of receptor clusters by heregulin, the remaining complexes in the presence of heregulin show greatly enhanced tyrosine phosphorylation, confirming that heregulin does not stabilize ErbB3-ECD-mediated dimers but is fully consistent with the constitutively formed dimers assuming an activated conformation.

EXPERIMENTAL PROCEDURES

Plasmids and Protein Purification—ErbB3-ECD cDNA was cloned into the pMT/BI/P/V5-His expression vector (Invitrogen) as described previously (11). The H565F point mutation and the C-terminal (S tag) epitope were generated by using a two-step PCR and were subsequently confirmed by sequencing. Drosophila S2 cells were cotransfected with the desired construct and the pCBHYTRO vector (Invitrogen) using Effectene reagent (Invitrogen). Stable cell lines were obtained after 3 weeks of selection with 500 μg/ml hygromycin B (Invitrogen). Cells were grown in serum-free media (HyClone, Logan, UT) and induced with 500 μM CuSO4. The ECDs were purified by nickel-nitrilotriacetic acid column chromatography as described previously (11), dialyzed in phosphate-buffered saline (PBS, 150 mM NaCl, 2.5 mM KCl, 81 mM Na2HPO4, 14.7 mM KH2PO4), and judged >95% pure by SDS-PAGE.

The GST-S tag and a hexahistidine tag, with a C-terminal S tag (GST-S tag) was generated in the pGEX-3x expression vector (Invitrogen) by PCR, expressed in Escherichia coli BL21 (DE3), and purified as described above. Human hergulin-β1 was generated as a fusion protein to the C terminus of thioredoxin (trx-hrg) in the pET-32a vector (Novagen, Madison, WI) and expressed and purified from E. coli BL21 (DE3) as described above (25). The trx-hrg fusion protein has binding properties to the ECD that are equivalent to the EGF-like domain of hrg and carries both an S tag and a hexahistidine tag, facilitating purification and detection. For experiments that utilized S-tagged ECDs, recombinant EGF-like domains of hrg-β1 (R & D Systems) were used in binding studies instead of trx-hrg. Full-length recombinant heregulin-β1 was obtained from R & D Systems. Chimera of ErbB3 and ErbB2 were generated by PCR amplification of the extracellular region of ErbB3 and the transmembrane and cytoplasmic region of ErbB2, using the previously described full-length constructs (11) as templates. The chimera and full-length ErbB2 were initially cloned into the pIND expression vector (Invitrogen), and the upstream edcsyne control region in this vector was subsequently exchanged for a tetracycline control region (pTRE2, BD Biosciences) to obtain higher expression levels. The corresponding constructs are referred to below as pIND-TRE based vectors.

ErbB3-ECD Pull Down—S-protein-agarose (Novagen) was blocked with bovine serum albumin (1 mg/ml, New England Biolabs) for 15 min and washed with PBS. S tag ECDs were precipitated in PBS for 15 min at room temperature at a final concentration of 0.50 μg/ml. The total of 1 × 10^10 mol was subsequently immobilized on the bovine serum albumin-blocked S-protein-agarose resin (equivalent of 50 μl of initial S-protein...
Erbb3 Pull Down with Pretreated Immobilized ECDs—Pull-down experiments were carried out as described above. For the pretreatment with urea, the immobilization step was carried out overnight in the presence of 8 M urea in order to elute the even distribution of (monomeric) ECDs. For pretreatment with heregulin, the Erbb3-ECD constructs containing the S tag epitope were coincubated for 1 h at room temperature with a 5-fold molar excess of recombinant human herge-
lin-β1 peptide (R&D Systems, Inc., Minneapolis, MN). The urea and free heregulin were subsequently removed together with the nonimmo-
bilized ECDs by centrifugation and three PBS washes, respectively.

Heregulin Sensitivity of Interfaces in Oligomers of ErbB3

In order to evaluate the contribution of the extended form of ErbB3-ECD to oligomerization, we generated a constitutively extended form of the ECD by mutating a key residue in its intramolecular tether. The crystal structure of the closed form of the ErbB3-ECD identifies several key residues that are critical for the stabilization of the intramolecular tether between domains two and four. Mutation of any of these key residues will break critical hydrogen bonds that stabilize the tether, and mutations in the analogous positions in EGFR have been used to generate a constitutively open conformation (17, 18). We chose to mutate histidine 565 of ErbB3 to phenylala-
nine. Histidine 565 is located in domain four and engages in a hydrogen bond with glutamine 252 of domain two as part of the intramolecular tether (Fig. 1). Phenylalanine is the residue corresponding to His-565 in ErbB2. ErbB2-ECD is structurally highly homologous to ErbB3-ECD but does not form the intramolecular tether (30), based on the crystal structure of the ErbB2-ECD. An equivalent mutation that unlocks the intramo-
lar tether in EGFR has been evaluated recently and shown not to disturb receptor stability and surface presentation (17, 18). ErbB3-ECD (H565F) expresses well in S2 insect cells at levels comparable with the wild-type ECD, and we will now refer to this extended mutant form of the receptor ECD as H565F.

Next, we addressed whether the destabilization of the intramo-
lar tether results in enhanced self-association of purified ECDs. Compared with the ligand-stabilized dimers of full-length and membrane-embedded receptors, the in vitro interactions of the ECDs in solution are significantly less stable, and oligomers tend to easily dissociate during analysis. In order to reproducibly and specifically detect the transient interactions of oligomeric ECDs, we adopted a pull-down assay with a series of rapid and controlled 100-fold dilutions. This approach provided more reproducible results, especially for weak interactions with lower signal-to-noise ratio. By using immobilized and S tag-labeled ECDs as the bait, we measured the V5 epitope-tagged ECDs (V5-ECDs) that bind to the immobilized ECD under various conditions. As a control for the extent of unspecific binding, we used immobilized S-tagged GST.

Under the stringent wash conditions employed in this assay, the H565F-ECD shows significantly enhanced binding to im-
mobilized wild-type ECD (Fig. 2). Most surprisingly, in this initial implementation of the assay, this finding is reversed when S tag-H565F is immobilized on the resin. We speculated that this apparent reversal of the relative strength of the inter-
action of H565F versus wild-type ECD with different immobilized ECDs is because of the fact that, without the modifications applied in subsequent assays (Figs. 3 and 4), both the
in the closed conformation. Domain I (amino acids 1–190) is colored green; domain II (amino acids 191–305) is colored lavender; domain III (amino acids 306–500) is colored cyan; and domain IV (amino acids 501–611) is colored blue. Bottom, close-up of the intramolecular tether between domain two and four. Histidine 565 and glutamine 252 form a key hydrogen bond that stabilizes the tether interaction and maintains ErbB3 in the closed conformation.

The extended form of the ECD self-associates more strongly. Top row, pull-down assay of V5-tagged ECDs (WT, wild type; M, H565F mutant), probed with anti-V5-HRP antibody conjugate. Samples represent the input (load) or the V5-ECDs recovered by a pull down with the immobilized S tag ECD species indicated at the bottom. Immobilized ECDs were pretreated with urea or heregulin prior to the pull down as indicated below. The amount of immobilized S-tagged ECD was evaluated by reprobing with S-protein-HRG conjugate (bottom row).

We subjected the immobilized ECD on the resin either to a preincubation with heregulin or a wash with 2 M urea. Whereas both urea and excess heregulin were removed prior to the addition of V5-tagged ECDs, the treatment with urea was more aggressive and unspecific, resulting not only in the disruption of oligomers of the S-tagged ECD but also the overall loss of some immobilized S-tagged ECD. In addition, monomers of immobilized wild-type ECDs are likely to adopt at least in part a closed conformation after the removal of urea. In contrast, heregulin should stabilize immobilized wild-type ECDs as open monomers as long as the ligand remains bound. Although interactions with the urea-treated S tag ECDs are overall significantly lower than those observed after pretreatment with heregulin, the relative intensities of H565F interactions compared with wild-type ECDs are now consistent. H565F shows stronger interactions with both wild-type ECD and its own immobilized species.

**Oligomers Have Two Distinct Interfaces with Respect to Sensitivity to Heregulin**—Although the pretreatment of the immobilized ECDs now consistently indicates stronger interactions by H565F, it also raises new questions. The fact that immobilized H565F not only requires heregulin pretreatment for efficient binding of additional incoming ECDs but shows the strongest binding after pretreatment may be explained by two different models. By itself, the data could suggest that heregulin is in fact needed for monomers of H565F to interact with other ECDs. However this model is inconsistent with the observed oligomerization of ErbB3-ECD, the disruption of oligomers by heregulin, and the reported inability of heregulin to facilitate ErbB3 dimerization (24). The second model would require that prior to heregulin binding more H565F exists in preformed oligomers than wild-type ECD, and that those oligomers have an upper limit of stoichiometry. From this second model it follows that there are two types of interactions between ECDs, one that can be disrupted by heregulin, allowing the dissociation of preformed S tag ECD oligomers, and a
second interaction that allows for the interactions with incoming ECDs despite the saturation of the immobilized S tag ECDs with heregulin. We carried out additional experiments to evaluate specifically the assumptions for the second model.

In order to confirm that the pretreatment indeed did not induce interactions of monomeric ECDs but did instead strip the resin of pre-oligomerized ECDs, we evaluated the fraction of immobilized ECDs that were bound to the resin in a manner that can be disrupted by ligand. We considered indirectly bound ECDs to be ECDs that are associated with the resin via their interactions with other ECDs in a heregulin-sensitive manner rather than through the binding of their S tags to S-protein (Fig. 4). For this analysis we evaluated only the immobilization of S-tagged ECDs, and we measured the fraction of ECDs that could be eluted from the resin with heregulin and subsequently eluted the remainder with SDS. The total amount of immobilized ECDs was determined in a parallel experiment by direct SDS elution.

In this analysis, most of the H565F-ECD was eluted with heregulin, suggesting an indirect binding mode or the immobilization of large oligomers of the ECD through few direct S tag interactions. Some wild-type ECDs could also be eluted with heregulin, but the ratio of heregulin-eluted ECDs to ECDs that are subsequently eluted with SDS (Fig. 4, HRG, SDS) is much smaller, suggesting more direct immobilization events that involve smaller oligomeric clusters. The large proportion of “indirectly” bound H565F ECDs is therefore consistent with the assumption that the failure to bind additional H565F-ECD without pretreatment (Fig. 2) was likely due to extensive oligomerization of the immobilized species and suggests an upper limit of oligomerization. This assumption is also consistent with a previously observed upper limit of oligomerization of wild-type ECDs in solution under saturating conditions (11).

As a control for unchallenged dissociation, samples were incubated with PBS. As an additional control, we evaluated the sensitivity of the oligomers to EGF. Wild-type ECDs showed much less indirect association than H565F, and no response to EGF. In contrast, H565F showed a response to EGF that is weak compared with heregulin but is clearly above the background defined by unchallenged dissociation in PBS. This sensitivity to EGF appears to be linked to the presence of larger oligomeric clusters of the ECD and may indicate a weak binding event for EGF to the ECDs at the high concentrations of EGF used in this assay (1 μM).

The pull-down assays with heregulin-treated ECDs suggest the presence of two distinct protein interfaces within the oligomers. The first ECD interface is apparently subject to disruption by heregulin during cross-linking. The addition of heregulin during cross-linking produces a comparable amount of ligand cross-linked to the monomeric receptor for both ECDs, indicating that both species are equally capable of binding ligand. However, the addition of heregulin results in an almost complete loss of higher order cross-linking products for both the wild-type and H565F, as well as much of the band corresponding to 2 eq of the H565F-ECD. Combined with the previous finding that heregulin disrupts larger oligomeric complexes and the disappearance of higher order cross-linked products in this experiments, this suggests that the ligand-bound EGFR. Furthermore, this suggests that a considerable amount of the wild-type ECD is already in an extended conformation and forms transient dimers that are captured by cross-linking, even without the addition of ligand. Those dimers do not appear to receive any additional stabilization by the ligand but do represent a mode of interaction of the ECD that is resistant to disruption by heregulin.

Overall, the significantly enhanced formation of higher order products by H565F is in agreement with the assumption that the open conformation of the ECD forms oligomers more readily. This difference between wild-type ECD and H565F is almost completely abolished by the addition of ligand. Furthermore, the resulting cross-linked products in the presence of ligand, especially at 100 nM ECD, are almost identical for both forms of the ECD. These observations are consistent with our earlier finding (Fig. 3) that the binding of ligand results in the same population of ECD complexes for WT and H565F and eliminates the more pronounced formation of ligand-sensitive H565F oligomers.

A comparison of cross-linking products at 10 and 100 nM ECD provides some insight into the relative strength of the interactions that hold oligomers of the ECD together. The total amount of cross-linked material cannot be compared between the experiments carried out with 100 versus 10 nM ECD. In fact the experiment carried out with 10 nM ECD (Fig. 5) has more
In contrast to the results at 100 nM ECD, the dimer band for disappearance of higher order cross-linked products. However, in contrast to the results at 100 nM ECD, the dimer band for H565F is also reduced drastically. This finding may reflect a tendency of H565F to exist in clusters of larger sizes that are primarily held together through the avidity effects of a large number of weak interactions. In contrast, wild-type ECDs form oligomers that have a larger proportion of constitutive dimer interfaces that are resistant to heregulin and are apparently strong enough to be captured by cross-linking at 10 nM ECD. This assumption is also consistent with the observation of a significantly larger proportion of indirectly immobilized H565F ECDs in Fig. 4. The difference in the relative sensitivity of wild-type ECD and H565F may also partially be due to the expected higher affinity of the open form of the ECD for the ligand due to an improved on-rate. This assumption is based on the reported increase in affinity for the open form of EGFR-ECD toward EGF (31).

The cross-linking studies support the model of a heregulin-resistant dimer interface for ErbB3 ECDs. However, the data provide no direct information on the nature of the cross-linked species in the absence of ligand or the composition of oligomeric clusters at saturating ECD concentrations. In order to evaluate if the oligomers formed by the constitutively extended H565F-ECD are qualitatively different from those formed by the wild-type ECD, we measured the size distribution of oligomers at saturating concentrations of ECDs by GEMMA (Fig. 6a). GEMMA separates gas-phase, singly charged particles based on their differential electrophoretic mobility in air. ESI with mass spectrometry can measure noncovalently bound protein complexes (32, 33). With GEMMA, ESI generates gas-phase ions representing the intact protein complexes from solution, and the ions are separated based on their electrophoretic mobilities and counted by a condensation particle counter (see “Experimental Procedures”). Based on their measured mobilities, the electrophoretic mobility diameter and a molecular weight are calculated (27, 29). Wild-type ECD and H565F ECD were compared at 800 nM to initially examine the stoichiometry of the complexes. A direct comparison of wild-type ECD and H565F ECD at 800 nM shows a very comparable distribution of complexes, indicating that, under saturating conditions, both ECD species form comparable oligomeric assemblies.

Most interestingly, the only significant difference in the normalized distribution for wild-type ECD and H565F-ECD is the broadness of the monomer peak. For H565F, this peak is much sharper, whereas wild-type ECD shows a significant peak broadening toward a smaller electrophoretic mobility diameter. This broadening of the distribution for wild-type ECD possibly reflects the equilibrium of the monomeric species between the extended form, coinciding with the position of H565F, and the closed and more compact conformation.

The Dimer Interface Is neither Stabilized nor Disrupted by Heregulin — The cross-linking experiments (Fig. 5) suggest that the dimer interface of the ErbB3-ECD is neither stabilized nor disrupted by heregulin. Under the conditions of the GEMMA analysis at 100 nM ECD, the predominant peaks are monomeric ECD and a small component of ECD dimer (Fig. 6d). This relative abundance of monomer and dimer over higher order products provides an opportunity to confirm the impact of the binding of heregulin on the dimer interface. Under the condition in which the monomer is the dominant species, the formation of the intramolecular tether is more likely to occur and modify ECD interactions. To eliminate this effect from the analysis, we carried out the experiment with H565F-ECD. At 100 nM, H565F primarily displays a peak that is consistent with the monomer of the ECD (geometric mean = 7.35 nm) but shows some dimer (geometric mean = 9.56 nm) and a slight trailing shoulder toward higher molecular weight species. Consistent with our earlier observation, the addition of ligand neither disrupts the dimer nor does it cause a significant increase in dimerization. The main effect of ligand binding is a broadening of the peak for the monomeric species and a modest shift of this peak toward a higher molecular weight (geometric mean = 7.78 nm), confirming the formation of a monomer-
ligand complex. The small increase in the relative height of the peak representing the dimer coincides with the disappearance of the shoulder toward larger size complexes, observed in the absence of ligand.

**Dual Effects of Heregulin on Cellular ErbB3**—We previously used direct cross-linking and coimmunoprecipitation of differentially tagged ErbB3 receptors to demonstrate that the majority of interactions of full-length ErbB3 on the cell surface can be disrupted by the addition of heregulin (11). However, coimmunoprecipitation by its nature cannot distinguish dimerization from higher order associations. Cross-linking under conditions that do not result in excessive unspecific cross-links primarily produces dimers and hence also provides no insight into the oligomerization context in which receptors were cross-linked. In order to evaluate our model that binding of heregulin is compatible with dimers but not oligomers, we contrasted the overall association state, as determined by cross-linking, with the activation state of an ErbB3/ErbB2 chimera. The receptor chimera consisted of the extracellular domains of ErbB3 and the transmembrane and cytoplasmic portions of ErbB2.

A similar chimera containing the ECD and transmembrane region of ErbB3 and the cytoplasmic region of EGFR was previously used to demonstrate that the binding of heregulin does not stabilize homodimers of ErbB3 (24). This chimera showed only low levels of constitutive phosphorylation, indicating either a failure to self-associate in the absence of ligand or a state of reduced activation in the absence of ligand. The reported failure to respond to heregulin in this system is consistent with our finding that heregulin disrupts oligomers and does not stabilize the dimer interface of ErbB3 homodimers. In order to generate phosphorylation as a readout for functional dimers, we used the transmembrane region of ErbB2 instead of ErbB3. In contrast to the transmembrane helix of ErbB3, the transmembrane region of ErbB2 shows intrinsic dimerization potential (19).

As shown in Fig. 7A, the chimeras with wild-type ErbB3 ECD show a strong response to heregulin. Constitutive phosphorylation in serum-starved cells is at a relatively low level and is similar to that observed for an ErbB2 control at comparable expression levels. A comparison of a chimera based on the wild-type (WT) ECD of ErbB3 with that of the H565F mutant (M) (Fig. 7B, WT and M) shows elevated basal phosphorylation for H565F, but the difference is small compared with the overall stimulation observed with heregulin. To evaluate if the lack of robust tyrosine phosphorylation in the absence heregulin is because of a failure of the chimeric receptors to self-associate or the presence of an inhibited conformation, we combined the analysis of tyrosine phosphorylation with cross-linking (Fig. 7C).

Cross-linking with the membrane-impermeable cross-linker BS3 reveals significant cross-linking in the absence of heregulin. The yield of cross-linked chimera is substantially reduced in the presence of heregulin, consistent with our previous observation of disruption of most of the interactions of full-length ErbB3 in a cellular setting (11). As seen in these earlier experiments with ErbB3, a residual amount of cross-linking occurs in the presence of ligand. In the case of the ErbB3/ErbB2 chimera, this cross-linked species shows strong tyrosine phosphorylation.

**DISCUSSION**

Several lines of evidence suggest that the traditional view, *i.e.* ErbB receptors are activated by ligand-induced dimerization of monomeric receptors, is likely not to describe the full nature of the control of activation within the ErbB family. Ligand-independent self-association has been primarily studied for EGFR, and several groups have reported dimers or oligomers in the absence of ligand. However, the resulting complexes have a suppressed kinase activity (18, 34–37), and the contribution of the extracellular domains of EGFR alone appears to be insufficient to stabilize the complexes, especially in the course of procedures such as coimmunoprecipitation (34, 37). Dimerization itself appears to be controlled by various competing interactions that involve multiple parts of the receptor, including the transmembrane spans and cytoplasmic portions in addition to the ECDs. The ligand-induced or ligand-stabilized interactions of the ECDs within dimers of the receptor apparently serve to modulate the activity of, at least in part, preexisting dimers or complexes. For some receptor complexes, such as mixed dimers of ErbB2 and ErbB3, this clearly includes an overall stabilization of the dimeric assembly as is evident by ligand-dependent coimmunoprecipitation of receptor complexes. Compared with ErbB dimerization, very little is known about the impact of oligomerization on ligand-induced or ligand-independent signaling or the nature of receptor interactions within oligomers.

A better understanding of the nature of interactions within oligomers of ErbB receptors may provide insight into the source of constitutive activity that occurs at conditions of overexpression, as found in several cancer cells. Under those conditions, the ability of the ECDs to suppress the inherent kinase activity apparently breaks down, at least in part. A key question is whether the extent of oligomerization and the nature of additional interactions within oligomers are fully or in part responsible for this partial breakdown of suppression of constitutive activity. Alternatively, the observed activation could merely reflect the percentage at which a given dimer exists in an active state, enhanced by the larger number of dimers in overexpressing cells. Although ErbB3 lacks kinase activity, the ability to observe transient oligomers of its ECD in solution makes it a good model system for the analysis of ECD interactions within oligomers of ErbB receptors. The enhanced tend-
Heregulin Sensitivity of Interfaces in Oligomers of ErbB3

The evaluation of resin-immobilized ECDs of wild-type ErbB3 as well as H565F shows that a large portion of the ECD can be released from the resin by incubation with heregulin, indicating that it was immobilized through heregulin-sensitive interactions with other ECDs rather than direct immobilization via its S tag (Fig. 4). Consistent with our overall observation of stronger oligomerization for the extended form of the ECD, this effect is much more pronounced for H565F. Without pretreatment with heregulin, resin-immobilized H565F shows little interaction with incoming V5-tagged H565F (Fig. 2). However, treatment of the immobilized H565F with heregulin prior to the binding experiment disrupts the pre-existing oligomers of immobilized ECDs and facilitates the binding of new incoming H565F. This ability to bind incoming ECDs also suggests the presence of a second type of interaction that is not subject to disruption by heregulin. By itself, the recruitment of oligomers to the previously heregulin-saturated and -immobilized ECDs could be due to partial dissociation of heregulin from the immobilized species prior to interaction with incoming ECDs. However, the presence of two types of interactions was also confirmed in other experiments. The presence of an interaction that is subject to disruption by heregulin is evident from the fact that the cross-linking of higher order products was efficiently suppressed by heregulin (Fig. 5). The disruption of interactions by heregulin was also confirmed by the elution of half of the wild-type and most of the H565F V5-ECD from immobilized S tag ECD (Fig. 4).

Cross-linking data suggest that the second heregulin-insensitive interaction can be found in the formation of dimers of the ECD. For H565F at 100 nM, treatment with excess heregulin resulted in a residual dimer band that was equivalent in intensity to the dimer band observed for wild-type ECD. This constitutive and heregulin-resistant dimer band probably represents cross-linking of the dimer, as defined by the ligand-bound structure of the homologous EGFR-ECD. The significant drop in intensity of this band for H565F compared with wild-type ECD suggests that, consistent with the higher degree of oligomerization of H565F, most of the cross-linking of H565F occurs between neighboring ECDs in the oligomer but not through the established dimer interface. Consequently, most of this cross-linking is eliminated as oligomers dissociate upon addition of heregulin. Although cross-linking cannot distinguish the nature of the interfaces that gave rise to cross-linked dimers, the assumption that the heregulin-resistant cross-linked dimer is derived from the crystallographically defined interface. This biochemical characterization of the involved oligomerization interfaces among homologous ErbB receptors as well as the analysis of the contribution of oligomerization to the modulating of receptor activity.

Oligomers Are Formed by ErbB3-ECD in the Open Conformation—The oligomerization of the ECD necessitates the presence of at least two interfaces, which may or may not be symmetrically arranged. We previously showed that the formation of higher order oligomers of ErbB3 is not only independent of ligand binding but that oligomers of the ECD are in fact destabilized by the binding of ligand. Based on the crystallographic evidence, the closed conformation of the ErbB receptor ECDs is expected to be incapable of high affinity ligand binding because of a disruption of the ligand binding pocket between domains one and three of the ECD. Hence, the binding of the ligand is expected to stabilize the extended conformation. A model of oligomers of ErbB3-ECD in the closed conformation could therefore easily be reconciled with the observed disruption of oligomers by the ligand. However, because the closed conformation buries the structurally conserved dimerization interface, this model would require two additional protein interfaces for oligomerization. Based on this model of oligomers composed of closed forms of the ECD, we also would have anticipated that the weakened intermolecular tether of H565F should have resulted in a reduction of oligomerization. However, we find the opposite to be true, indicating that the open form of the receptor drives oligomerization (Figs. 2 and 3). Hence, the crystallographically and biochemically characterized dimer interface is likely to provide one of the required interfaces for oligomerization.

An additional implication of this finding is that the wild-type ErbB3-ECD must exist, at least to a significant extent, in the extended conformation in the absence of ligand in order to form oligomers. In the equilibrium between extended and closed conformations of the ECD, the extended conformation may be trapped in oligomers that are stabilized through avidity effects of multiple weak interactions. A simplified model of the association states of the ErbB3-ECD is shown in Fig. 8.

If membrane localization favors receptor associations, and oligomers stabilize the open form of the ErbB3 ECD, one would expect the H565F mutant to have relatively little impact on self-association for receptors on the cell surface. Our results obtained for the ErbB3/ErbB2 chimera are consistent with this assumption. With respect to constitutive activation and cross-linking, and compared with the pronounced differences seen between the wild-type and mutant ECDs in solution, H565F shows only relatively modest enhancements compared with chimeras based on wild-type ErbB3-ECDs. This would argue that the closed conformation only plays a minor role on the surface of cells. Similar findings have been reported for mutants of EGFR where equivalent mutations of the tether forming residues in domain four have little impact on basal activation or EGFR response (17, 18).

ErbB3 Oligomers Possess Two Classes of Interfaces, Ligand-destabilized and Ligand-insensitive—Based on our findings, we propose that the ErbB3-ECD can interact with other ErbB3 receptors via two different types of interactions. One interaction for the open conformation of the receptor is not disrupted by ligand binding. The second interaction results in the formation of oligomers and is destabilized by the binding of ligand. Oligomers are stabilized by the avidity effect of multiple weak interactions and may use dimers of the ECD as building blocks. The two different classes of interactions are evident from both the pull-down data and the cross-linking data.
dimer interface is consistent with the known ECD structures. For the structure of the homologous ECD of EGFR with bound ligand, the binding pocket between domains one and three and the dimer interface between domains two and four are located on opposite faces of the ECD (13, 14).

The Dimer Interface of ErbB3-ECD Is neither Disrupted nor Stabilized by Ligand Binding—Our cross-linking data suggested that the dimer interface is resistant to disruption by ligand binding, at least at elevated concentrations of the ECD, but is also not stabilized by the binding of ligand. No increase in a cross-linked dimer band was observed in cross-linking studies, neither for the ECDs in solution nor for the chimeric receptor on the cell surface. We confirmed the neutral impact of heregulin binding on dimer stability using GEMMA analysis (Fig. 6b). Under conditions in which H565F shows primarily a monomer peak with a small component of dimer, addition of heregulin only shifts the position of peaks marginally, consistent with the mass and size increase from the ligand binding event itself. No significant increase in the fraction of dimers was observed. This indicates that under conditions in which the stabilization of the extended over the closed conformation is not a parameter that influences dimerization, heregulin binding contributes little or nothing to the stability of the dimer interface but also does not disrupt the small amount of constitutive dimers. This lack of stabilization of dimers of ErbB3-ECDs by heregulin is consistent with the recent report (24) that heregulin binding does not result in dimerization of ErbB3 receptor.

Estimates of the Contribution of Different Interfaces to Oligomer Stability—Our initial analysis of the oligomerization behavior of wild-type ECD had suggested an apparent $K_d$ between 15 and 50 nm depending on the method used for the analysis (11). These estimates are limited in their accuracy as our current data suggest that they represent the contributions from different types of interfaces within oligomers and additive effects from multiple interactions. An accurate measurement of the contribution of each interface will require mutations that selectively disable the individual interfaces. However, our cross-linking data (Fig. 5) suggest that the affinity of the interface that is subject to disruption by heregulin is stronger than 10 nm in the absence of ligand, resulting in efficient oligomerization at 100 and 10 nm ECD. Although this presence of oligomers at 10 nm suggests that oligomers are held together by interactions that have an apparent $K_d$ that is lower than 10 nm, this may be the result of avidity effects, and individual interactions may be significantly weaker. The saturation of oligomers with heregulin reduces the affinity of this heregulin-sensitive interaction enough for oligomers to dissociate, even at concentrations as high as 100 nm. However, we have observed that at concentrations of several hundred nanomolar, even a 5-fold molar excess of heregulin is often insufficient to achieve complete dissociation of oligomers of the ECD (data not shown), thus placing the $K_d$ for the oligomerization interface of heregulin-saturated ECDs between $10^{-7}$ and $10^{-6}$ M.

The heregulin-resistant dimer interface could be detected by GEMMA analysis at 100 nm ECD (Fig. 6b) and remained largely intact in cross-linking studies in the presence of heregulin at 100 nm ECD. However, at lower concentrations of the ECD the proportion of cross-linked dimers drops, especially for H565F. Once the additional stabilization through avidity effects within oligomers is removed, the remaining dimer interaction is apparently too weak to generate significant dimeric complexes at 10 nm. This would indicate a $K_d$ for the constitutive dimerization of the ECD that is closer to 100 nm than to 10 nm.

Partial Disruption by Heregulin May Cause “Reshuffling” of Oligomers—By using various methods, we have demonstrated an enhanced tendency for the constitutively extended form of the ErbB3-ECD to undergo oligomerization. This raises the question whether under the saturating conditions more likely to reflect the environment on the cell surface, oligomers formed by a homogeneous species of extended ECDs are qualitatively the same as oligomers formed by wild-type ECDs. We evaluated this question by GEMMA analysis at 800 nm ECDs. At these elevated concentrations, the distribution of oligomers that were detectable after separation showed a comparable distribution of oligomeric species for the wild-type ECD and H565F (Fig. 6c). The only observed difference was a more narrow size distribution for the monomer peak of H565F compared with wild-type ECD, which possibly reflects the fact that monomeric H565F does not alternate between a closed and open conformation.

The effect of heregulin on oligomers under saturating concentrations of receptors, which may be more comparable with overexpressing cells, is more difficult to evaluate. We reported previously (11) that the disruption of oligomers by ligand at high concentrations of ECD is only partial. Under conditions where the concentration of the ECDs exceed the $K_d$ value of the weakened oligomer interface, even a saturation of the receptors with heregulin may be insufficient to completely disrupt the assembly. We therefore propose that the result of heregulin binding under such conditions may be to facilitate the reshuffling of clusters as opposed to their complete disruption. This reshuffling and exchange model may also explain previous reports that heregulin at high ECD concentrations can appear to stimulate ECD interactions between immobilized and soluble species of the ECD. The evaluation of such a model in a cellular setting is currently limited by the lack of techniques that sufficiently discriminate between the different types of interactions that underlie clustering versus dimerization or the potential exchange of cell surface receptors between clusters. The identification and targeted disruption of the specific interfaces will be essential to untangle the contribution of dimers and clusters to signaling.

Dual Effect of Heregulin on Membrane-anchored ErbB3-ECDs, Implications for Signaling—A comparison of cross-linking and activation data for chimeras of ErbB3-ECDs and the transmembrane and cytoplasmic portions of ErbB2 are consistent with our model that the ErbB3-ECD favors the formation of receptor clusters and that binding of heregulin destabilizes such clusters but not receptor dimers. When additional interactions in the transmembrane span aid in the stabilization of the dimers, heregulin triggers strong tyrosine phosphorylation but reduces overall cross-linking of receptors. In the presence of heregulin, the interaction of the ECDs alone is apparently not sufficient to stabilize dimers of the receptors as chimeras with the poorly dimerizing transmembrane span of ErbB3 fail to show tyrosine phosphorylation upon addition of heregulin (24). This is also consistent with similar reports, pointing out that transmembrane and cytoplasmic contributions are needed for preformed dimers or oligomers of EGFR to be stable (34, 37).

The absence of strong constitutive phosphorylation under conditions that give enhanced cross-linking (Fig. 7) suggests that constitutive activation in clusters of ErbB3s is largely suppressed in the absence of ligand. We observed a comparable constitutive activation of ErbB3/ErbB2 chimera and ErbB2. Whether this constitutive activation is dependent on cluster formation or reflects a small pool of dimers that stochastically exists in the active state will require additional studies on mutant receptors carrying specific mutations in the two distinct self-association interfaces.

Conclusion—Recent data on the activation of ErbB receptors have provided a new model for activation in which ligand...
binding results in the formation of activated dimers and may stabilize dimers, depending on the type of receptor complex, but receptor association can precede ligand binding. Ligand-independent self-association can be stabilized by interactions from different parts of the receptors and the relative contributions differ between ErbBs. ECD interactions are strong for ErbB3 and weak for ErbB2, whereas the opposite is true for transmembrane contributions. The state of the extracellular domain in the absence of ligand appears to suppress a constitutively active mode, regardless of the association state. Although the contribution of the ECD to self-association may be weak for ErbB2 compared with ErbB3, the residual interaction may be significant in the context of a membrane-embedded and overexpressed receptor, and we found comparable constitutive activation for recombinant ErbB2 and ErbB3/ErbB2 chimera when expressed alone in CHO cells.

Our data for ErbB3 suggest that the established dimer interface for ErbBs is one of the two interfaces needed for oligomerization. Its interaction is neither stabilized nor disrupted by heregulin, therefore favoring the formation of more stable heterodimers with ErbB2 in the presence of heregulin. The second interface, which we termed oligomerization interface, is destabilized by heregulin. To what extent this additional interface is conserved among ErbB receptors and whether it contributes to the constitutive activation of ErbB receptors with kinase activity remain to be seen as the contribution of clustering to constitutive activation is not certain at this point. Our data indicate that the level of activation in such clusters, as judged by tyrosine phosphorylation, is at least significantly lower compared with activated dimers. However, the outcome of activation in a cellular setting may also differ for activated dimers and modestly activated clusters. The model system provided by ErbB3-ECD allows a detailed evaluation of different modes of ECD interactions that are difficult to evaluate in dimers and modestly activated clusters. The state of the extracellular domain in the absence of ligand appears to suppress a constitutively active mode, regardless of the association state. Although the contribution of the ECD to self-association may be weak for ErbB2 compared with ErbB3, the residual interaction may be significant in the context of a membrane-embedded and overexpressed receptor, and we found comparable constitutive activation for recombinant ErbB2 and ErbB3/ErbB2 chimera when expressed alone in CHO cells.

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