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Disulfide-Linked and Noncovalent Dimers of p185^{HER-2} in Human Breast Carcinoma Cells

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Abstract Enhanced levels of disulfide-linked dimers of the *neu* oncogene product have been suggested to be associated with the transformed state [Weiner DB, Liu J, Cohen JA, Williams WV, Greene MI: *Nature* 338:230–231, (1989)]. We, therefore, investigated the properties of the dimeric forms of p185^{HER-2}/*neu* from the human breast carcinoma cell line, SK-BR-3. We found disulfide-linked dimers as well as noncovalently associated dimers that were detected by cross-linking with bis(sulfosuccinimidyl) suberate (BS^3). However, the disulfide-linked dimers did not exist in intact cells, since they were eliminated when the cells were lysed in the presence of the alkylating agent, sodium iodoacetate. Moreover, the disulfide-linked dimeric molecules were not the activated form of p185^{HER-2} since they incorporated about the same level of phosphate in an in vitro kinase reaction as the monomeric molecules. In contrast, the noncovalent dimers appeared to be present on the surface of intact cells and were phosphorylated at levels at least tenfold higher than monomers in an in vitro kinase reaction.

Key words: Her-2/*neu*, proto-oncogene, dimers, breast carcinoma

Growth factor receptor tyrosine kinases transcend the plasma membrane once with their ligand binding domain extending outside the cell and their tyrosine kinase domain located in the cytoplasm. Receptor activation leads to tyrosine phosphorylation of intracellular proteins and to a mitogenic response [Yarden, 1988]. One model to explain how binding of ligand to the extracellular domain activates the catalytic domain proposes a role for receptor dimerization [Yarden and Schlessinger, 1987; Yarden, 1988; Schlessinger, 1988; Ullrich and Schlessinger, 1990]. In this mechanism, ligand binding would stabilize receptor dimers, which are the activated form. The effects of dimerization on kinase activation have been assessed but only with dimers formed in vitro with partially purified receptors. While kinase activation has been strongly correlated with PDGF receptor dimerization [Heldin et al., 1989], the in vitro autophosphorylation activity of EGF receptor dimers has been reported to be mildly enhanced [Boni-Schnetzler and Pilch, 1987] or at a similar level as monomers [Yarden and Schlessinger, 1987].

Receptor dimers are linked through weak, noncovalent bonds or through disulfide linkages. Ligand-induced disulfide dimers of the colony stimulating factor (CSF-1) have been found to be selectively internalized and they appear to be associated with kinase inactivation [Li and Stanley, 1991]. The platelet-derived growth factor (PDGF) receptor has also been found to form disulfide-linked dimers in intact cells in response to ligand binding [Li and Schlessinger, 1991]. In contrast, it appears that only noncovalent dimers of the EGF receptor are formed in response to EGF [Yarden and Schlessinger, 1987; Boni-Schnetzler and Pilch, 1987; Li and Schlessinger, 1991].

The disulfide-linked dimers of the HER-2/*neu* proto-oncogene product, p185^{HER-2}, which is a member of the EGF receptor family, are of particular interest. The HER-2/*neu* product has been reported to form elevated levels of disulfide-bonded dimers at the cell surface as the result of

Abbreviations used: BS^3, bis(sulfosuccinimidyl) suberate; D-MEM, Dulbecco's modified Eagle's medium; EDC, [1-ethyl-3-(3-dimethyl-aminopropyl)]carbodiimide; EGF, epidermal growth factor; FCS, fetal calf serum; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid; PAGE, polyacrylamide gel electrophoreses; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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an amino acid substitution in its transmembrane domain [Weiner et al., 1989]. This point mutation causes kinase activation and oncogenic activation of c-*neu* which is the rat equivalent of the human HER-2 gene [Bargmann and Weinberg, 1988; Stern et al., 1988]. The observation that enhanced dimerization occurs for the oncogenic form of the HER-2/*neu* product is of great importance because it suggests that alterations in receptor aggregation may lead to the transformed state.

In the work described here, we demonstrate that disulfide-linked dimers of p185^{HER-2} are formed after the cells are lysed and do not appear to exist on the surface of intact cells. Further we demonstrate, for the first time, that noncovalent dimers of p185^{HER-2} from intact breast carcinoma cells have a greatly enhanced autophosphorylation capacity compared to the monomeric or disulfide-linked dimeric p185^{HER-2} molecules.

MATERIALS AND METHODS

Materials

Cell culture medium and fetal calf serum (FCS) were supplied by GIBCO BRL, (Gaithersburg, MD). Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoreses (PAGE) were obtained from Bio-Rad (Richmond, CA). Other chemicals were purchased from Sigma (St. Louis, MO) unless specified. Anti-peptide antibody against the C-terminus of p185^{HER-2} was prepared as described [Lin et al., 1990]. Antiserum to phosphotyrosine was generated in rabbits injected with hemocyanin coupled to phosphotyrosine as described [Kamps and Sefton, 1988]. Production of specific antibodies to phosphotryosine was screened by Western blotting and immunoprecipitation of autophosphorylated EGF receptor from A431 cell membrane vesicles prepared as previously described [Lin et al., 1990]. SK-BR-3 cells (human breast carcinoma) were obtained from American Type Culture Collection (A.T.C.C., Rockville, MA).

Radiolabelling, Cross-Linking, and Immunoprecipitation

Cells, cultured in D-MEM supplemented with 10% FCS, were plated at a density of 3×10^6 cells per 60 mm culture dish for 24 h. Cells were starved in methionine/cysteine-free medium without serum for 90 min and then 200 μCi/ml of ^{35}S-methionine/cysteine (EXPRE^{35}S^{35}S) (NEN, Boston, MA) was added and cells were incubated for 16–18 h. The cross-linking reagent, BS^3, (Pierce, Rockford, IL), was added to the medium at a final concentration of 2 mM at room temperature for 30 min. Then 10 mM Tris, pH 7.4, was added to quench the cross-linking reaction. The labelled cells were solubilized in TG buffer (50 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol, 1% Aprotinin) and immunoprecipitated as previously described [Lin et al., 1990]. The proteins in the immune complex were analyzed by SDS-PAGE containing 4.5% acrylamide. For autoradiography of ^{35}S-labelled proteins, gels were soaked in water for 30 min and then in Fluoro-Hance Enhancer (RPI, Corp., Mt. Prospect, IL) for 30 min. Gels were dried and visualized by autoradiography at −70°C using Kodak X-Omat K film. The in vitro phosphorylation assay was conducted by suspending the immune complex in a kinase reaction mixture containing [γ-^{32}P]ATP under previously described conditions [Lin et al., 1990]. The phosphorylated immune complex was washed three times with M-RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.15 M NaCl, 1 mM PMSF, 10 mM sodium pyrophosphate, 10 mM NaF, 4 mM EDTA, 2 mM sodium vanadate) [Akiyama et al., 1988] and analyzed by SDS-PAGE.

RESULTS

Disulfide-Linked p185^{HER-2} Dimers From Human Breast Carcinoma Cells

We investigated the human breast carcinoma cell line, SK-BR-3, for the presence of disulfide-linked dimers of p185^{HER-2}. We chose these cells since they have enhanced expression of HER-2 [Kraus et al., 1987; Lin and Clinton, 1991] which is believed to contribute to their malignant growth. The cells were labelled with ^{35}S-methionine/cysteine and the HER-2 gene product was immunoprecipitated from the cell lysate with anti-peptide antibodies against a sequence near the C-terminus, (residues 1242–1255) [Lin et al., 1990]. A high molecular weight protein of about 360 kDa was observed in the absence of the reducing agent (Fig. 1A, lane 2), but disappeared when β-mercaptoethanol was added to the sample prior to electrophoresis (Fig. 1A, lane 1). Under nonreducing conditions the monomeric form of p185^{HER-2} migrated faster indicating the presence of intra-subunit disulfide bonds [Lin and Clinton, 1991]. The distortion in the migration of proteins near the edge of the lanes

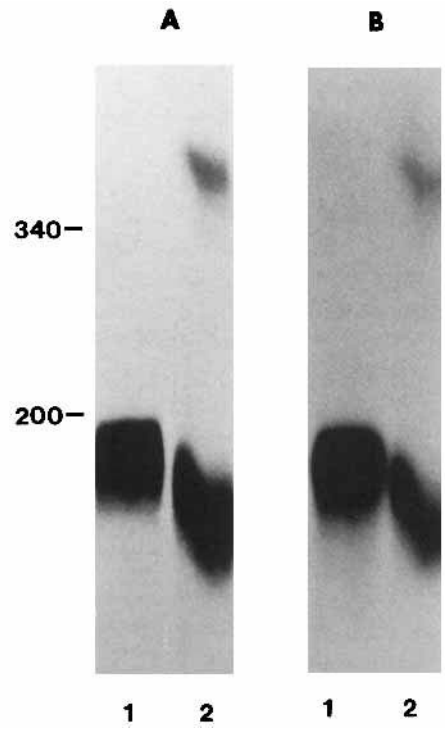

Fig. 1. Disulfide-linked p185^{HER-2} dimers. ^{35}S-labelled SK-BR-3 cells were extracted and immunoprecipitated as described in Methods (**A**). Unlabelled cell extracts were immunoprecipitated and incubated in an immune complex kinase assay with [γ-^{32}P]ATP (**B**). Samples in lanes 2 of A and B were electrophoresed in the absence of β-mercaptoethanol.

was caused by diffusion of reducing agent from the neighboring lane. The high molecular weight protein of 360 kDa appeared to be a dimer of p185^{HER-2} because it was about twice the relative molecular mass and because disruption of the dimer with β-mercaptoethanol resulted in a corresponding increase in the monomeric HER-2 product. In four experiments the disulfide-linked dimer represented from 18–25% of the total amount of p185^{HER-2}. This approximated the 20–30% of the c-*neu* product which was previously observed in a disulfide-linked dimer in *neu* transfected 3T3 cells [Weiner et al., 1989].

In Vitro Phosphorylation of Disulfide-Linked Dimers and Monomers of p185^{HER-2}

Because autophosphorylation of tyrosine kinase receptors reflects their state of activation [Yarden, 1988; Ullrich and Schlessinger, 1990], we examined phosphorylation in an immune complex kinase reaction which is commonly used to assess autophosphorylation levels. The SK-BR-3 cell extracts were reacted with anti-p185^{HER-2}, and the washed immune complex was incubated in a kinase reaction mixture containing [γ-^{32}P]ATP. The phosphorylated proteins were resolved by electrophoresis under reducing and nonreducing conditions (Fig. 1B). Excision of the ^{32}P-labelled proteins of about 360 kDa and 185 kDa from the gels and quantitation by Cerenkov counting indicated that about 25% of the ^{32}P was incorporated into the higher molecular weight protein in the nonreduced sample. This proportion did not change following treatment of gels with 1 M KOH at 55°C for 1 h (data not shown), conditions that are known to preferentially preserve phosphotyrosine while hydrolyzing phosphoserine and phosphothreonine residues [Cooper et al., 1983]. In replicate experiments, the percentage of ^{32}P incorporated into dimers ranged from 20% to 30%. Comparison with the proportion of the ^{35}S label that was in dimers (Fig. 1A) suggests that ^{32}P incorporation, per molecule of p185^{HER-2}, did not differ significantly between monomers and dimers.

Effects of the Alkylating Agent, Iodoacetate, on Formation of Disulfide-Linked Dimers

To determine whether the disulfide-linked dimers existed in intact cells or were formed after the cells were lysed, 25 μM of freshly prepared sodium iodoacetate was added to cell extracts to alkylate cysteine residues thus blocking their availability for disulfide bonding. The cell extracts were then immunoprecipitated and the immune complex was incubated in a kinase reaction system exactly as described in Figure 1B. Figure 2 lane 2 shows that no phosphorylated dimers were detected when cell extracts were treated with alkylating agent indicating that the disulfide-linked dimers were not present in the intact cell but were formed after the cells were lysed. Similarly, when ^{35}S-labelled cell extracts were treated with sodium iodoacetate, ^{35}S-labelled dimers were not detected (data not shown).

In Vitro Phosphorylation of Chemically Cross-Linked p185^{HER-2}

Because dimers of the HER-2 gene product did not survive treatment with alkylating agent, we tested for the presence of dimers at the cell surface using chemical cross-linking. The homobifunctional cross-linking reagent, BS^3, which does not penetrate the plasma membrane [Staros, 1982], was added to SK-BR-3 cells. Immunoprecipitation of the HER-2 protein product from ^{35}S-labelled cells revealed small amounts of a

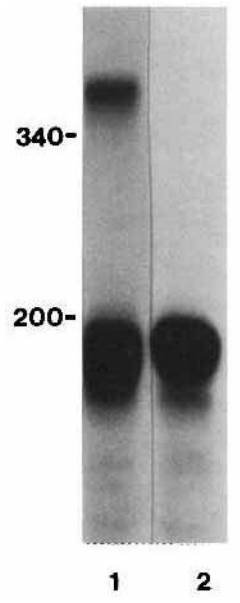

Fig. 2. Effect of sodium iodoacetate on formation of disulfide linked dimers. Cell extracts with (lane 2) or without (lane 1) 25 μM sodium iodoacetate were immunoprecipitated with anti-p185HER-2 and incubated in an immune complex kinase reaction containing [γ-32P]ATP.

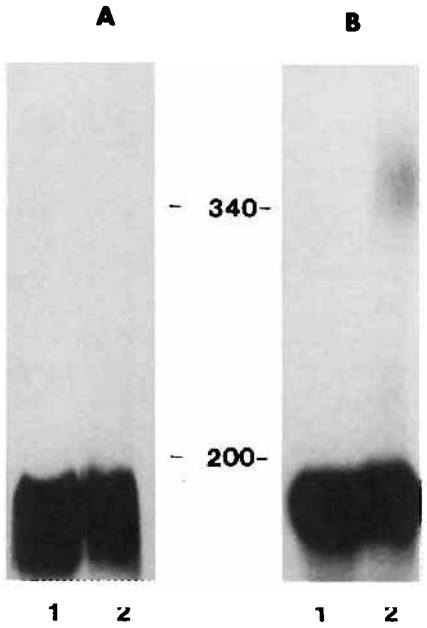

Fig. 3. Chemical cross-linking of p185HER-2 in intact cells. 35S-labelled SK-BR-3 cells were incubated in the presence of 2 mM BS3 for 30 min (**A** and **B**, lanes 2) or in the absence of BS3 (lanes 1). An aliquot of 35S-labelled cell extract was immunoprecipitated (A) or immunoprecipitated and then phosphorylated in an in vitro kinase assay (B). Samples were resolved by SDS-PAGE.

high molecular weight (about 350–370 kDa) protein from the BS3 treated cells when the gel was autoradiographed for longer times (data not shown). P185HER-2, which migrated as a monomer, was detected whether or not cells were treated with BS3 (Fig. 3A).

To assess the autophosphorylation capacity of noncovalent dimers, we immunoprecipitated aliquots of the 35S-labelled cell extracts and incubated the immune complex with [γ-32P]ATP in a kinase reaction system. The reason we used 35S-labelled proteins for the autophosphorylation assay was so that the amount of the protein and the level of autophosphorylation could be determined in the same sample. This would serve as a control for the possibility of differences in extent of dimerization from experiment to experiment which would confound determinations of specific autophosphorylation capacity. A higher molecular weight protein of about 360 kDa as well as p185HER-2 were phosphorylated (Fig. 3B, lane 2). The 360 kDa protein was about twice the relative molecular mass of p185HER-2 and was only observed in the presence of cross-linker. The partial proteolytic digests of the phosphopeptides of the 360 kDa protein matched those of the monomeric p185HER-2 indicating identity between the two (data not shown). Thus the phosphorylated 360 kDa protein appeared to be noncovalent dimers of p185HER-2. Because there were large amounts of 35S-labelled monomeric p185HER-2 in the immune complex, the exposed film gave the impression of a higher proportion of monomers (Fig. 3B). To accurately determine 32P levels without interference from 35S, the proteins were excised from the gel and Cerenkov counted. About 30% of the total 32P incorporated into immunoprecipitated protein was in the 360 kDa protein. In replicate experiments, the proportion varied from 24% to 32%. In contrast, less than 3% of the 35S-labelled p185HER-2 was in the higher molecular weight protein. Therefore, the 360 kDa protein incorporated at least tenfold higher levels of 32P compared to the monomeric form of the HER-2 protein product.

Immunoprecipitation of Disulfide-Linked and Noncovalent Dimers With Anti-Phosphotyrosine Antibodies

Phosphorylation of dimers in the immunecomplex kinase reaction likely occurred at tyrosine residues since antiserum specific for p185HER-2 tyrosine kinase was used for immunoprecipitation. To further analyze the phosphorylated

dimers, the ^{32}P-labelled proteins were released from the immunecomplex by incubation at 100 °C for 1 min with RIPA containing 8 M urea and 2 M sodium thiocyanate. This procedure caused release of greater than 90% of the ^{32}P-labelled proteins and denatured the anti-peptide antibodies against $p185^{HER-2}$. To test for tyrosine phosphorylation, the proteins were dialyzed, immunoprecipitated with anti-phosphotyrosine antibodies, and then analyzed by gel electrophoresis and autoradiography. As expected, the autophosphorylated $p185^{HER-2}$ monomers were immunoprecipitated with the anti-phosphotyrosine antiserum (Fig. 4b). In addition the ^{32}P-labelled dimers, whether they were cross-linked or nonreduced, also reacted with the anti-phosphotyrosine antiserum (Fig. 4b). Reactivity was specific for tyrosine phosphorylated dimers since immunoprecipitation was blocked by preincubation of the antiserum with excess phosphotyrosine (Fig. 4d). These results strongly indicated that the disulfide and noncovalent dimers were phosphorylated on tyrosine residues in the immunecomplex kinase reaction.

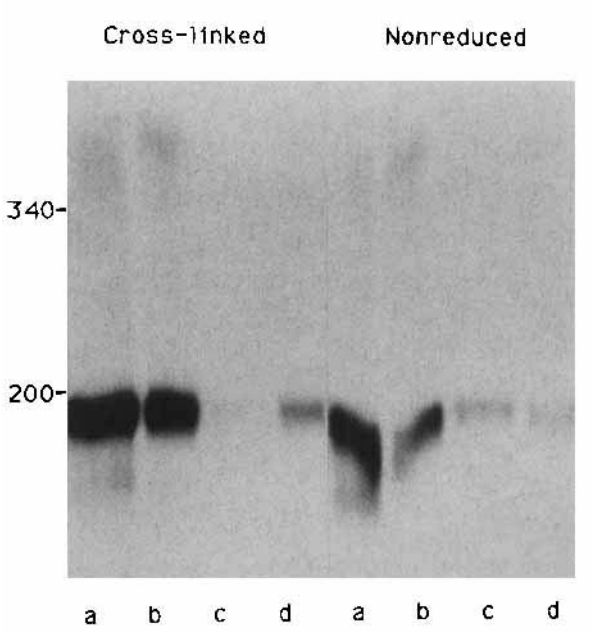

Fig. 4. Immunoprecipitation of $p185^{HER-2}$ dimers with anti-phosphotyrosine antibodies. Cultured SK-BR-3 cells were treated with the cross-linker, BS^3, as described in Materials and Methods or were untreated. Then 1.1 mg of protein from the cross-linked and untreated cultures was immunoprecipitated with anti-peptide antibody against the C-terminus of $p185^{HER-2}$, incubated in an immune complex kinase reaction containing [γ-^{32}P]ATP and the phosphorylated immune complex was washed 3 times with M-RIPA. One-third of the sample from cross-linked cells was directly applied to lane a of an SDS-containing polyacrylamide gel and one-third of the untreated sample was applied under nonreducing conditions. The remainder of the immunoprecipitated proteins was eluted from the immune complex by incubation with 1 ml of RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% deoxycholic acid, 1% NP-40, and 0.1% SDS) containing 8 M urea and 2 M sodium thiocyanate at 100°C for 1 min. The eluted proteins were dialyzed against 1,000 volumes of water overnight. Half of the eluted samples was immunoprecipitated with anti-phosphotyrosine antiserum (lanes b) and proteins in the supernatant that did not react with the antiserum were analyzed in lanes c. The remaining half of each eluted sample was immunoprecipitated with anti-phosphotyrosine antibody that was preincubated with 200 μg/ml of phosphotyrosine (lanes d).

DISCUSSION

The disulfide-linked $p185^{HER-2}$ dimers that we detected in SK-BR-3 cells were eliminated when the alkylating agent, sodium iodoacetate, was added to the cell extraction buffer. Since iodoacetate alkylates cysteines but does not disrupt existing disulfide bonds [Gurd, 1972], it appeared that the intermolecular disulfide bonds were formed after the cell membranes were solubilized. Disulfide bonded dimers have been observed for the rat equivalent of the HER-2, the c-*neu* product, and at enhanced levels for the *neu* oncogene product [Weiner et al., 1989]. It is possible that the covalent dimers were observed because of lower concentrations of alkylating agent used in the extraction buffer (10 μM compared to 25 μM used in our experiments) or because of differences in the structure of the rat versus the human product of the HER-2/*neu* gene.

Goldman et al. [1990] have recently reported EGF-dependent heterodimers between $p185^{HER-2}$ and the EGF receptor in chemically cross-linked SK-BR-3 cells. In contrast to our findings, they did not detect phosphorylated homodimers of $p185^{HER-2}$ in the absence of EGF. We believe that their inability to detect homodimers was caused by the chemical cross-linker that was employed. They used EDC, a cross-linker that catalyzes bond formation between carboxyl and amino groups of neighboring molecules [Ji, 1983], while we used BS^3, a homobifunctional reagent that bonds to primary amine groups of molecules that are within 11 Å of each other [Staros, 1982]. When we used EDC, we also were unable to detect dimers of $p185^{HER-2}$ in SK-BR-3 cells in the absence of EGF [Lin and Clinton, unpublished observations].

If dimers represent the activated form of receptor tyrosine kinases, they may be expected to have enhanced kinase activity indicated by their capacity for self-phosphorylation. In the case of

the PDGF receptor, there is a striking enhancement of autophosphorylation capacity of the dimeric form of the receptor [Heldin et al., 1989]. For the EGF receptor, the difference in autophosphorylation activity of molecules in the dimer compared to the monomer conformation is modest [Schlessinger, 1988; Boni-Schnetzler and Pilch, 1987]. Indeed, Goldman et al. [1990] have observed that within heterodimers, p185HER-2 exhibits enhanced autophosphorylation while the EGF receptor is relatively unaffected in activity. In the intact cell, however, dimerization of the EGF receptor appears to be required for autophosphorylation to occur. This was suggested by recent studies showing that tyrosine phosphorylation occured only in homodimers composed of functional wild type receptors but not in heterodimers containing a defective EGF receptor [Kashles et al., 1991].

Our findings indicate that like the PDGF receptor, cross-linked dimers of p185HER-2 are greatly enhanced in capacity for phosphorylation. While less than 3% of the ^{35}S-labelled HER-2 product was cross-linked into dimers, approximately 30% of the ^{32}P was incorporated into dimers. A difficulty in accurately assessing autophosphorylation capacity is that the amount of cross-linked dimeric molecules was near the limit of detection, which is about 3% of the ^{35}S-labelled monomers. These analyses, however, indicate that dimeric molecules had at least tenfold enhanced phosphorylation levels. Another explanation is that the HER-2 cross-linked dimers only "appeared" to have greatly enhanced autophosphorylation capacity because the monomers may have been phosphorylated in vivo and therefore unable to be further phosphorylated with [γ-^{32}P]ATP. This is unlikely because the phosphotyrosine phosphatase inhibitor, vanadate, was excluded from the cell culture medium and from the cell lysis buffer which usually results in efficient hydrolysis of phosphate from tyrosine residues [Keating and Williams, 1988; Keating et al., 1988; Peles et al., 1991]. We favor the interpretation that the p185HER-2 dimers, cross-linked on the surface of the intact cell, are the catalytically active form of the HER-2 product.

The SK-BR-3 cells used in this study have been reported to secrete low concentrations of a ligand that appears to interact with p185HER-2 [Lupu et al., 1990]. It is possible that the dimers we observed were stabilized by endogenous ligand produced in these cells. In this case, the ligand either was not cross-linked to the p185HER-2 dimer or was sufficiently low in molecular mass as not to affect migration rate of the dimer.

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