The interaction of neu differentiation factor (NDF) with the extracellular domains of Her2 (sHer2) and Her3 (sHer3) have been studied using native gels, light scattering, and sedimentation equilibrium. The full-length NDFβ2 was shown to bind sHer3 with a dissociation constant of 26 ± 9 nM, while it showed a 1000-fold weaker binding to sHer2. Taken together, these results demonstrate that NDF is a high affinity ligand for Her3, but not for Her2. No increase in affinity of the NDFβ2 for sHer3 was observed upon addition of Her2 to the NDFβ2-sHer3 mixture. Binding of NDFβ2 to sHer3 did not induce receptor dimerization or oligomerization, the stoichiometry being one sHer3 per one NDF molecule. This finding suggests that transmembrane and/or intracellular domains of receptor family members or perhaps additional unidentified components may be involved in NDF induced dimerization and autophosphorylation, or alternatively, that dimerization is not the mechanism for Her3 autophosphorylation and signal transduction.

There have been numerous studies showing that high expression of erbB2/Her2 tyrosine kinase receptor correlates with poor prognosis in patients with breast cancer (Slamon et al., 1987; Wright et al., 1989; Gullick et al., 1991; Hartmann et al., 1994). Her2 is a member of the EGF receptor subfamily, which also includes the EGF receptor, and Her3 and Her4 receptors (Kraus et al., 1989; Plowman et al., 1993). EGF and transforming growth factor-α are known as ligands for the EGF receptor but do not stimulate autophosphorylation of Her2, Her3, and Her4 receptors.

Receptor dimerization has been implicated as a mechanism of receptor phosphorylation and signal transduction for a variety of ligand-receptor pairs. Using a recombinant extracellular domain of EGF receptor, dimerization and oligomerization of the receptor, induced by EGF, have been observed with a dissociation constant of 100–200 nM and a stoichiometry of one receptor per one EGF molecule (Hurwitz et al., 1991; Brown et al., 1994).

Recently, neu differentiation factor (NDF) or heregulin and other structurally related ligands have been shown to increase tyrosine phosphorylation of the Her2 receptor and, therefore, was initially assumed as a Her2 ligand (Peles et al., 1992; Wen et al., 1992; Holmes et al., 1992; Marchionni et al., 1993; Falls et al., 1993). However, there is evidence that NDF neither binds directly to Her2 nor stimulates its kinase activity (Tzahar et al., 1994), but rather binds to and stimulates tyrosine phosphorylation of Her3 and Her4 (Plowman et al., 1993; Kita et al., 1994). There is also evidence that NDF's stimulatory activity on Her3 is augmented when Her2 was also coexpressed (Sliwkowski et al., 1994). Here we have studied the interaction of NDF with the extracellular domains of Her2 (sHer2 or Her3 (sHer3) using native gels, light scattering, and sedimentation equilibrium in order to further define the binding strengths and stoichiometries and to explore whether binding specificity is solely a property of the receptor extracellular domains.

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant Chinese hamster ovary cell-derived sHer2 and sHer3 were prepared as described elsewhere. Recombinant E. coli-derived NDF was prepared as described (Tzahar et al., 1994; Lu et al., 1995).

Protein Concentration—Protein concentrations were determined spectrophotometrically using ε280nm = 0.632 for NDFβ2, 0.492 for NDFβ3, 0.870 for sHer2, and 0.991 for sHer3.

Sedimentation Equilibrium—Sedimentation equilibrium experiments were carried out at 25 °C using 6-channel charcoal-Epon cells in a Beckman XL-A analytical ultracentrifuge. Concentration distributions were measured at 280 or 230 nm. Samples were equilibrated at each speed for at least 24 h, and attainment of equilibrium verified by the constancy of scans taken 8 h apart. At the conclusion of a run the rotor was taken to 48,000 rpm for >18 h to force all the protein to the bottom of the cell. Scans of the upper portions of each channel at this high speed were used to establish a baseline absorbance level for each sample, and this baseline offset was subtracted from the data before further processing.

Simultaneous analysis on data from multiple samples and speeds was carried out using nonlinear least squares techniques similar to those in NONLIN (Johnson et al., 1981), using the program KDL-TON developed in-house. Confidence intervals for the parameters were evaluated using the "preferred method" outlined by J. Johnson and Faunt (1992).

The sedimentation data on the mixtures were fitted to heterogeneous association models of the forms A + B → AB or A + 2B → AB + B + AB2, where A is NDF or a sHer3-NDP complex, and B is sHer2 or sHer3, accounting for the different molar extinction coefficients of each species at the measurement wavelength, using techniques described previously (Philo et al., 1994). During the fitting, the buoyant molecular weights of each monomeric species were held fixed at the values determined when run alone. The data for up to 18 samples were globally fitted to determine the association constants. The fitted parameters were constrained to values which correctly account for the total concentrations each protein placed into the cell.

Buffer densities were measured with a Mettler Paar DMA-02 density meter. Polypeptide partial specific volumes of 0.7258, 0.7220, and 0.7272 ml/g, and molar extinction coefficients at 280 nm of 6.04 × 104, 6.76 × 104, and 1.56 × 104 M⁻¹ cm⁻¹ for sHer2, sHer3, and NDFβ2, respectively, were calculated from their amino acid composition (Laue et al., 1992; Gill and von Hippel, 1989).

Light Scattering—The experimental setup, procedures, and data analysis have been described previously (Arakawa et al., 1994; Philo et al., 1994), essentially following Takaishi (1990).

Native Gel Electrophoresis—Binding of NDFβ2 and NDFβ3 to either sHer2, sHer3 or both was analyzed by native gel electrophoresis using

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1 The abbreviations used are: EGF, epidermal growth factor; NDF, neu differentiation factor.

2 Y. Kita, J. Tseng, T. Horan, J. Wen, J. Philo, D. Chang, B. Ratzkin, R. Pacifici, O. Brankow, S. Hu, Y. Luo, D. Wen, T. Arakawa, and M. Nicolson, manuscript in preparation.
of equal amounts of sHer2 and sHer3, addition of excess NDF results with NDF system employed in this study, sHer2 (is formed.

should retard migration of sHer2 or sHer3 if a stable complex they do not migrate into the gel when run alone (

8% polyacrylamide gels (Novex) run in 25 mM Tris, 192 mM glycine, pH 8.6. Prior to loading, 1 μg of sHer2 or sHer3, or 0.5 μg each was incorporated into complexes with NDF2 instead of NDFa2.

Native Gel Results—As shown in Fig. 1, in the native gel system employed in this study, sHer2 (lane 2) migrates faster than sHer3 (lane 1) because of its lower relative pl value. Because the unusually high pl (−10) of both NDF isoforms, they do not migrate into the gel when run alone (lane 3), but should retard migration of sHer2 or sHer3 if a stable complex is formed.

Fig. 1A shows the results with NDFa2. In the presence of 6.8- (lane 4) or 1.7-fold (lane 8) molar excess of NDFa2, sHer2 displayed a new band slightly above the free molecules, indicating formation of a complex. Under identical conditions, sHer2 gave no complex formation (lanes 5 and 9). In the presence of equal amounts of sHer2 and sHer3, addition of excess NDFa2 gave the same band seen with sHer3 alone, i.e. no stable ternary complex of sHer3/NDFa2/sHer2 was observed (lanes 6 and 10). Despite the presence of excess NDFa2 a large portion of sHer3 remained as a free molecule. Fig. 2B shows the results with NDFb2 obtained under exactly the same experimental conditions. Here again, complex formation was observed only when NDFb2 was mixed with sHer3 and no ternary complex was observed when sHer2 was also present. Comparing Fig. 1, A and B, it is apparent that a larger portion of sHer3

Fig. 1. Native gel analysis of NDF binding to sHer2 and sHer3. A, NDFa2 binding to sHer2 and sHer3. Lane 1, 1.0 μg of sHer3; lane 2, 1.0 μg of sHer2; lane 3, 2.5 μg of NDFa2; lane 4, 1.0 μg of sHer3 + 2.5 μg of NDFa2; lane 5, 1.0 μg of sHer2 + 2.5 μg of NDFa2; lane 6, 0.5 μg of sHer3 + 0.5 μg of sHer2 + 2.5 μg of NDFa2; lane 7, 0.625 μg of NDFa2; lane 8, 1.0 μg of sHer3 + 0.625 μg of NDFa2; lane 9, 1.0 μg of sHer2 + 0.625 μg of NDFa2; lane 10, 0.5 μg of sHer3 + 0.5 μg of sHer2 + 0.625 of NDFa2. B, NDFb2 binding to sHer2 and sHer3. Same as lanes in A using NDFb2 instead of NDFa2.

was incorporated into complexes with NDFβ2 than with NDFa2, suggesting that the NDFβ2 has a higher affinity for sHer3 under the experimental conditions used.

Light Scattering—We have also used size exclusion chromatography with on-line light scattering, refractive index, and absorbance detectors to explore the interaction of NDFβ2 with sHer3. As we have shown previously, by using the signals from all three detectors we can obtain the polypeptide molecular weights of glycoproteins and glycoprotein complexes (Arakawa et al., 1994; Philo et al., 1994). A significant complication in these studies is the fact that NDFβ2 interacts strongly with size exclusion chromatography columns, especially at physiological ionic strength (Lu et al., 1995). Therefore, a 0.5 M NaCl + phosphate-buffered saline eluant and a Superose 12 column were selected to minimize this problem when studying sHer3 and NDFb2. When sHer3 was run by itself, its molecular weight from light scattering was 68,000 (our light scattering system normally gives a 3% error in molecular weight measurement). When sHer3 was mixed with NDFβ2 at an 1:1 molar ratio, much of the NDFβ2 eluted as a complex with sHer3, but some free NDFβ2 was also seen. The peak of the complex eluted significantly earlier than the sHer3 control sample, and its molecular weight from light scattering was 80,000. This molecular weight is significantly lower than the theoretical molecular weight of an 1:1 complex (92,900). One possible reason for this is that the binding of sHer3 and NDFβ2 may be weak enough that significant dissociation occurs during chromatography. However, this is inconsistent with sedimentation equilibrium data (next section). A second possibility is that the high ionic strength of phosphate-buffered saline + 0.5 M NaCl may dissociate the complex. However, similar molecular weights were obtained for the complex when it was run using phosphate-buffered saline alone or phosphate-buffered saline +

![Fig. 2. Sedimentation equilibrium of sHer3 + NDF mixtures at 8000 rpm. The natural log of the absorbance at 280 nm is plotted versus (r^2 - r_0^2)/2, where r is the radius and r_0 is the radius of the center of the sample. In such a plot a single species will give a straight line whose slope is proportional to molecular weight. The diamonds are for a sample containing 6 μM sHer3 and 6 μM NDFβ2; the squares are for 6 μM sHer3 + 3 μM NDF. The dashed line illustrates the slope calculated for a complex containing 2 sHer3/NDF. The solid and dotted lines show the calculated slope for a 1:1 sHer3/NDF complex. Only data for the outer half of the cell are shown. Conditions: 25 °C; 10 mM sodium phosphate + 50 mM NaCl, pH 7.9.](image-url)
0.3 M NaCl as eluant. We think the most likely explanation of incomplete complex formation is that the significant, competing interaction of NDFβ2 with the column matrix probably breaks the complex with sHer3 and causes some dissociation. This is supported by the fact that no complexes were observed when Superdex-200 or -75 columns, which interact more strongly with NDFβ2, were used.

NDF Binding to Her3 Studied by Sedimentation Equilibrium—Sedimentation equilibrium provides a non-perturbing means of studying ligand-receptor interactions at true thermodynamic equilibrium. Furthermore, as we have shown previously, this technique is capable of detecting and quantifying binding interactions which are too weak to persist during chromatography (Philp et al., 1994). We therefore carried out sedimentation equilibrium studies of mixtures of NDFβ2 and sHer3 over a wide range of concentration and stoichiometry.

Control studies of NDFβ2 alone were consistent with a monomer at the sequence Mr of 24,694. The data for sHer3 were also well fitted by a single ideal species, with no evidence for self-association to dimers or higher oligomers. From the buoyant molecular weight of 24035 [23976 and 24103] (values within square brackets indicate 95% confidence intervals), the sequence Mr of 68178, and estimates for the carbohydrate partial specific volume (Philp et al., 1994), it is possible to calculate that sHer3 contains 17.0% [15.0%, 18.5%] carbohydrate, for a total mass of 82.2 [80.2, 83.7] kDa.

Fig. 2 shows some data for samples containing 6 μM sHer3 and either 3 or 6 μM NDFβ2. On such a plot, a single species will give a straight line with a slope proportional to molecular weight, and for mixtures of species the slope near the outer portion of the cell will primarily reflect the largest species which are present (only data for the outer half are shown). The dashed line on the plot indicates the slope expected for complexes with two sHer3 bound to one NDF; the solid and dotted lines indicate the slope for one sHer3 per NDF. Clearly both samples contain primarily the 1:1 complex. Furthermore, there appears to be little, if any, 2:1 complex in the sample made at that stoichiometry despite the high protein concentration (which should favor such complexes even if the binding of a second sHer3 is relatively weak).

For more detailed analysis, sedimentation equilibrium data from 13 experiments were simultaneously analyzed to different binding models. These data covered a range of sHer3 loading concentrations from 400 nM to 6 μM, both 2:1 and 1:1 sHer2:NDF stoichiometries, and 2 rotor speeds. Consistent with the simple analysis presented in Fig. 2, a model in which sHer3 and NDF bind to form 1:1 complexes gives a good fit to all the data, with nearly randomly distributed residuals, as shown in Fig. 3, and returns a dissociation constant of 26 [17, 35] nM.

We have also looked for a second sHer3 to the same NDF molecule by analyzing data using a binding model in which NDF has two sites that bind sHer3 independently with different dissociation constants. This analysis clearly showed that no significant amount of 2:1 complex (<1%) exists in these samples, but could not rule out a very weak second binding site with a dissociation constant of 100–300 μM. To further explore the possibility of a second very weak interaction, we carried out further experiments at higher protein concentrations, reaching sHer3 concentrations >35 μM (>2 mg/ml). The inclusion of a second binding interaction in these high concentration data does not significantly improve the fit over the 1:1 stoichiometry model, and the analysis indicates that any such second binding site, if it exists at all, has a dissociation constant >1 nM.

Sedimentation Equilibrium of Mixtures of sHer2 and NDF—Similar sedimentation equilibrium studies were also carried out with sHer2 and NDF. When studied alone, sHer2 again shows no evidence of self-association, and appears to contain 19.3% [16.8, 21.2] carbohydrate, for a total mass of 86.1 [83.5, 88.1] kDa. Preliminary studies at sHer2 loading concentrations of 2–4 μM suggested a possible weak interaction with NDF, so further experiments were done at sHer2 loading concentration up to 23 μM, and at both 1:1 and 2:1 sHer2:NDF stoichiometries.
Simultaneous analysis of 18 data sets gave a reasonably good fit to the 1:1 stoichiometry model with a dissociation constant of 19.6 μM [17.3, 22.0], i.e. about 1000-fold weaker binding than seen for sHer3. This fit does, however, show some systematic deviation from the experimental data, especially at the highest concentrations where the apparent M₁ is somewhat below that predicted by the fit. Consistent with this pattern of deviations, the inclusion of a second interaction (to allow 2:1 complexes) only makes the fit worse, and fits to the two-site independent model will not converge because the fitting routine attempts to make the second dissociation constant become zero. The deviation from the experimental data, especially at the highest concentrations, might also be due to some heterogeneity related to glycosylation. We therefore conclude that the 1:1 binding model is correct.

Sedimentation Equilibrium of Mixtures of sHer2, sHer3, and NDF—Since it has been reported that a coexpression of Her2 and Her3 on the surface of cells reconstitutes a high affinity binding site for NDF (Sliwkowski et al., 1994), it is interesting to ask whether the same phenomenon is seen in solution with the extracellular domains. We have examined the sedimentation equilibrium of equimolar mixtures of sHer2, sHer3, and NDF with each component loaded at 3, 1.5, or 0.3 μM. Control experiments on a mixture of sHer2 and sHer3 did not show any interaction between them in the absence of ligands.

To interpret the data on the ternary mixtures, there are three general cases we need to consider. We have already demonstrated that NDF will bind strongly to sHer3 and weakly to sHer2, but does not induce dimerization of either receptor. Those studies cannot tell us, however, whether sHer3 and sHer2 are binding to the same, or different, sites on NDF. Therefore the first possibility to consider is one where the sites are the same or largely overlapping, in which case the low affinity of sHer2 will make it unable to compete with sHer3, so essentially no interaction with sHer2 will occur. A second possibility is that sHer2 may bind independently to a different "face" of NDF, and then we would expect to see some formation of sHer3:NDF-sHer2 ternary complexes, but the amount of ternary complex would be strongly limited by the weak NDF-sHer2 interaction. The last, and most interesting, possibility is a synergistic binding of sHer2 and sHer3 to the same NDF molecule, giving much more ternary complex than expected based on the weak binding of sHer2 alone.

We can again get a good overall view of the results by looking at Fig. 4, a linearized data plot like that in Fig. 2, for a sample loaded with 3 μM of each protein. The solid line shows the slope expected for a sHer3:NDF complex, while the dashed line shows that for a ternary sHer3:NDF-sHer2 complex. Clearly there is no large amount of ternary complex formed, and the data are consistent with essentially complete formation of sHer3:NDF complexes, as expected at this high protein concentration. Therefore we can eliminate the case of significant synergistic binding of sHer2 and sHer3. On the other hand, if sHer2 is not bound at all, its lower molecular weight would be expected to reduce the slope below that for 100% sHer3:NDF, which is not seen. Therefore the data would be consistent with a very weak formation of ternary complexes.

We have not attempted to directly fit to an appropriate ternary association model. Such a model would require consideration of 6 species with different molecular weights, and at least 3 binding constants, and is therefore exceedingly complex and unlikely to give unique results. However, to further confirm our interpretation that the sHer2 is binding only weakly, if at all, we first tried an analysis of the data for the higher concentration samples as a mixture of two non-interacting species: sHer2, and a sHer3:NDF complex. During this analysis, the concentrations were constrained to give the correct 1:1 molar ratio averaged over the cell. This model gave a moderately good description of the data (not shown), but seemed to somewhat underestimate the average molecular weight, particularly at the highest concentrations.

We then tried a model where sHer2 binds to the sHer3:NDF β2 complex with the same low affinity seen for sHer2 binding to NDF alone. This model clearly predicts too much ternary complex, and gives a variance 3 times higher than the non-interacting model. Last, we allowed the affinity of SHer2 for the sHer3:NDF complex to vary during the fitting. This latter model gives quite a good fit of the data, as shown in Fig. 5, and returns a dissociation constant of 99 μM [77, 128], i.e. 5-fold weaker binding than when sHer3 is not already bound to NDF. Thus this analysis confirms that there is no enhanced binding of sHer2 in the presence of sHer3. Instead, the binding of sHer2 actually appears to be weaker in the presence of sHer3, perhaps as a result of some overlap of the binding sites on NDF, or steric interference between the 2 receptors, or some negative cooperativity through the common ligand.

DISCUSSION

Native gel, light scattering, and sedimentation equilibrium experiments have all demonstrated binding of NDF β2 to sHer3, while native gels and sedimentation equilibrium detected little binding of NDF β2 (or NDF β2) to sHer2. Both native gels and size exclusion chromatography/light scattering showed only partial formation of sHer3:NDF β2 complexes, while sedimentation equilibrium showed stoichiometric 1:1 binding at moderately high (26 nM) affinity. We do not regard these results as contradictory; rather, they probably indicate that NDFβ2 is rather easily dissociated by competing interactions with the polyacrylamide and dextran matrices.

The 26 nM dissociation constant found here for the interaction of sHer3 with NDF β2 is significantly different from the 1–2 nM values reported for the EGF domain of NDF β1 binding to Her3 expressed on insect or COS-7 cells (Carraway et al., 1994). It is interesting,
Her2-expressing cells were stimulated with NDF. All this data suggests that a third component (presumably a protein) might be promoting oligomerization of Her family members on cell surfaces.

In many cases, soluble receptors have been shown to dimerize upon binding to their cognate ligand, although the number of ligand molecules incorporated into the complex differs for different receptors. It is, therefore, quite significant that NDF does not dimerize the soluble receptor at least under the conditions used here. suggesting the possibility that dimerization is not the mechanism of Her3 autophosphorylation and signal transduction. However, if in fact, receptor dimerization is essential for receptor kinase activation, then these results suggest that components required for dimerization are missing in the experimental conditions used here. Alternatively, one or both of the soluble receptors studied here may be altered in such a way to have lost the capability to dimerize without losing the ability to bind ligand. The methods we have used in this study are not able to distinguish this presumed defect from the participation of other receptor domains or ancillary factors.

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**FIG. 5.** Sedimentation equilibrium data and fitted curves for equimolar sHer2/sHer3/NDF mixtures. The curves represent a simultaneous fit to a model in which sHer2 is associating with a pre-formed sHer3/NDF complex (essentially no dissociation of sHer3 from NDF is expected at these concentrations; see Fig. 3). The fitted dissociation constant between sHer2 and the sHer3/NDF complex is 99 μM. The sedimentation constant of Her3 receptor by cross-linking when sHer3 is already bound. This striking difference in binding behavior may indicate that enhanced binding by a Her2/Her3 heterodimer represents a high affinity binding site for NDF. In contrast, our studies using purified receptor extracellular domains show no evidence for enhanced binding by the receptor heterodimer. Indeed, we actually find weaker binding of sHer2 to NDF β2 when sHer3 is already bound. This striking difference in binding behavior may indicate that enhanced binding by a Her2/Her3 heterodimer requires the transmembrane and/or intracellular domains. Alternatively, it could indicate that the enhanced binding requires a third component which is constitutively expressed in COS-7 cells. It is interesting that the cross-linked complexes seen by Sliekowski et al. (1994) have an apparent molecular mass in excess of 500 kDa, much higher than expected for a Her2/NDF-Her3 complex. They also observe complexes of this size when only Her3 is present, whereas we find no formation of Her3/NDF-Her3 or higher complexes even at very high protein concentrations. In this regard, it should be noted that Kita et al. (1994) have shown aggregation of Her3 receptor by cross-linking when...