Communication

An Intramembrane Modulator of the ErbB2 Receptor Tyrosine Kinase That Potentiates Neuregulin Signaling*

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The ErbB2 receptor tyrosine kinase plays a critical role in a variety of developmental processes, and its aberrant activation may contribute to the progression of some breast and ovarian tumors. ASGP2, a transmembrane glycoprotein found on the surface of the highly metastatic ascites 13762 rat mammary adenocarcinoma cell line, is constitutively associated with ErbB2 in these cells and in mammary tissue from pregnant rats. Expression studies indicate that ASGP2 interacts directly and specifically with ErbB2 through one of its epidermal growth factor-like domains and that the co-expression of the two proteins in the same cell dramatically facilitates their direct stable interaction. Ectopic expression of ASGP2 in human melanoma tumor cells potentiates the response of endogenous ErbB2 to the neuregulin-1 growth factor. These observations point to a novel intramembrane mechanism for the modulation of receptor tyrosine kinase activity.

ErbB2 (also known as Neu) is a 185-kDa cell surface transmembrane receptor tyrosine kinase that mediates the growth or differentiation of a variety of cultured cells and contributes to the proper development of cardiac and neural tissues during gestation (1–4). Its overexpression in numerous human tumors, including breast and ovarian tumors, correlates with earlier patient relapse and poor prognosis (5, 6). The observation that ErbB2 overexpression stimulates its protein-tyrosine kinase activity (7), together with the observation that activated alleles of the erbB2 gene induce metastatic tumors when expressed in murine mammary epithelium (8), suggest that the activation of ErbB2 kinase activity may play an important role in tumorigenesis or tumor progression.

The protein-tyrosine kinase activity of ErbB2 may be activated by several soluble, diffusible ligands that possess epidermal growth factor (EGF)-like domains. For example, EGF, transforming growth factor-α, and amphiregulin are all capable of stimulating ErbB2 activity by binding to the related EGF receptor and promoting its heterodimerization with ErbB2 (9, 10). Likewise, the neuregulins (NRGs) bind to the ErbB3 and ErbB4 receptors and stimulate ErbB2 activity through receptor heterodimerization mechanisms (11, 12). However, no molecularly characterized diffusible ligand has been demonstrated to act on ErbB2 directly, and it has been suggested that the primary function of this protein is to augment signaling through the ErB8 receptor network by acting as an auxiliary co-receptor (13–15). In this context factors that influence the activity or availability of ErbB2 could have a significant impact on the strength or specificity of signaling and ultimately the cellular response. Strong candidates for such factors are cell surface proteins that possess EGF-like domains.

The autonomously proliferating and highly metastatic rat ascites 13762 mammary adenocarcinoma cell line expresses a large sialomucin complex in abundance at its cell surface. This complex consists of two noncovalently associated proteins, ASGP1 and ASGP2, which arise from the proteolytic processing (16) of the product of a single gene (17). ASGP1, a ~600-kDa heavily O-glycosylated sialomucin, is an anti-adhesive factor as well as a contributor to the ability of these cells to evade immune recognition (18, 19). The 120-kDa transmembrane subunit ASGP2 tethers the complex to the cell surface. The sequence of ASGP2 includes two EGF-like domains, one of which conserves all of the consensus residues of the active EGF-like growth factors (17). 13762 ascites cells also express, at their surfaces, modest levels of ErbB2. The receptor and several of its associated intracellular signaling proteins are constitutively tyrosine-phosphorylated in the ascites cells, suggesting that ErbB2 is constitutively activated (20). Here we test the hypothesis that ASGP2 influences ErbB2 activity through direct interaction with the receptor.

MATERIALS AND METHODS

Preparation of Ascites Cell Lysates and Mammary Tissue Homogenates—Ascites 13762 adenocarcinoma cells (MAT-C1 subline) were grown intraperitoneally in Fischer 344 rats, and microvillar membranes were prepared under microfilament-depolymerizing conditions as described previously (21, 22). Membranes were solubilized in S buffer (0.2% Triton X-100, 150 mM KCl, 2 mM MgCl2, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM Tris/HCl, pH 7.6) and centrifuged on 7–25% sucrose gradients in S buffer for 15 h at 80,000 × g and 4 °C (23). Gradients were fractionated, and selected fractions were analyzed by co-immunoprecipitation. Mammary gland homogenates were prepared from Fischer 344 rats at day 17 of pregnancy as described previously (24).

Immuno precipitation and Blotting—Immuno precipitation of rat ErbB2 or its deleted variants were carried out with 1 μg of either Ab-3 (intracellular domain) or Ab-4 (extracellular domain) anti-ErbB2 mono-
clonal antibodies (Calbiochem, Oncogene Science) using 2 μg of rabbit anti-mouse IgG secondary antibody (Zymed Laboratories Inc.) to bind to protein A-Sepharose. Immunoprecipitations of ASGP2 were carried out with 3 μl of polyclonal anti-ASGP2 (16). Immunoblotting after transfer to nitrocellulose using Ab-3, polyclonal anti-ASGP2, or monoclonal anti-ASGP2 (Zymed) was performed using enhanced chemiluminescence for detection. In some experiments the levels of precipitated protein were determined by densitometry following staining of the filter with 0.2% Ponceau S (Sigma).

**Insect Cells—**Sf9 insect cell growth, infections, and transfections were carried out as described previously (25, 26). In co-expression experiments where a single protein was expressed as a control, wild type baculovirus was used as the co-infecting virus. Sf9 cell lysis was performed using an Nonidet P-40 lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM orthovanadate, 100 μg/ml leupeptin, 20 KIU/ml aprotenin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine). Lysates were cleared by centrifugation at 12,000 × g for 15 min prior to immunoprecipitation. Infections of High Five cells were performed with 2 × 10⁶ cells/well of 12-well tissue culture dishes and incubation at 27 °C. Cells were incubated for 24 h with baculovirus encoding ErbB2 ECD and the ASGP2 deletion mutants at a multiplicity of infection between 5 and 10 for each virus. The medium was then replaced with Excell 405 serum-free medium (JRH Biological). The conditioned medium was collected after an additional 32 h and clarified by centrifugation at 12,000 × g. An equal volume of 2 × radioimmune precipitation buffer was added prior to immunoprecipitation.

**Analysis of Sialomucin Transfectants—**Construction of A375 human melanoma cell lines expressing sialomucin complex under tetracycline regulation has been described previously (18). Cells were grown to 80% confluency in the presence of tetracycline and concomitantly serum-starved in 0.1% calf serum and treated with and without tetracycline and concomitantly serum-melanoma cell lines expressing sialomucin complex under tetracycline immunoprecipitation.

**RESULTS AND DISCUSSION**

To test whether ASGP2 might act as a modulator of ErbB2 function, the association of the two proteins in 13762 cells was first examined by co-immunoprecipitation. Detergent solubilized plasma membranes were immunoprecipitated with either anti-ASGP2 or anti-ErbB2 under conditions known to efficiently precipitate their targets, and precipitates were immunoblotted with antibodies to the other. ASGP2 was observed in anti-ErbB2 immunoprecipitates, and ErbB2 was observed in anti-ASGP2 precipitates (Fig. 1A). Since the immunoprecipitations were performed using fractions isolated from microvillar plasma membranes (21), these findings indicate that ASGP2 and ErbB2 are present in a complex on the surface of 13762 cells. We have observed previously that during pregnancy the expression of ASGP2 in the mammary epithelium of rats increases dramatically, and a fraction of the expressed sialomucin complex is secreted into milk (24). As with the 13762 cells, ASGP2 and ErbB2 could also be co-immunoprecipitated from lysates of homogenized mammary tissue from animals 17 days pregnant (Fig. 1B; Ref. 24), suggesting that their association is not the result of the aberrant overexpression of ASGP2 in the tumor cells.

Although the observations above indicate that ASGP2 and ErbB2 are constitutively associated at the surface of 13762 cells, the participation of another receptor or other proteins in the interaction could not be ruled out. To test the specificity of the ASGP2-ErbB2 interaction a baculovirus/insect cell expression system was developed. Insect cells were employed because they do not express endogenous ErbB receptors, eliminating confusion arising from potential receptor heterodimerization events. In the first series of experiments Sf9 insect cells were infected with baculovirus encoding ASGP2 alone or co-infected with ASGP2 and each of the known ErbB receptors. The co-immunoprecipitation assay was used to assess association. It was observed that ASGP2 could be co-immunoprecipitated with ErbB2 from cells expressing both proteins, but could not be co-precipitated with the EGF receptor, ErbB3 or ErbB4 proteins (Fig. 2A). Likewise, ASGP2 could be co-immunoprecipitated with ErbB2 when the two proteins were transiently co-expressed in COS cells, but could not be co-precipitated with the endogenous COS cell EGF receptor (data not shown). These observations indicate that the stable association of ASGP2 with ErbB receptors is selective for ErbB2 and does not require another ErbB receptor. Deletion analysis indicated that, as expected, the extracellular domain of ErbB2 is necessary for its interaction with membrane-bound ASGP2. When co-expressed in SF9 cells, ASGP2 could be co-immunoprecipitated with either full-length ErbB2 or the extracellular domain of the receptor, but could not be co-precipitated with the intracellular domain or a transmembrane form lacking most of the extracellular domain (Fig. 2B).

The extracellular domains of the ErbB2 and ASGP2 proteins could also be secreted as a complex when co-expressed in the same cell. In the experiment shown in Fig. 3A, High Five insect cells (cells specifically adapted for the expression of secreted proteins) were infected with baculovirus encoding the extracellular domain of ASGP2 (ASGP2 ECD) or the extracellular domain of ErbB2 (ErbB2 ECD) or were co-infected with both viruses. The co-immunoprecipitation assay was then carried out.

**Fig. 1. In vivo association of ASGP2 and ErbB2 demonstrated by co-immunoprecipitation.** A, cleared lysates containing plasma membrane proteins from ascites 13762 rat mammary adenocarcinoma cells were immunoprecipitated and immunoblotted with anti-ErbB2 or anti-ASGP2 as indicated. B, cleared detergent homogenates from the mammary tissue of lactating rats 17 days pregnant were immunoprecipitated with anti-ErbB2 and blotted with the indicated antibodies. In both experiments lanes marked “None” represent lysates of cells or homogenates used as positive controls for blotting.
out with the cleared conditioned media from infected cells using anti-ErbB2 antibodies. ASGP2 ECD was detected in immunoprecipitates from cells expressing both proteins, indicating that the cells secrete ASGP2 ECD and ErbB2 ECD as a complex. Similar immunoprecipitates from metabolically labeled cells showed no other detectable radiolabeled bands,

\[2\] suggesting that the ASGP2-ErbB2 association occurs through a direct protein-protein interaction. Moreover, resolution of the radiolabeled immunoprecipitated proteins by nonreducing SDS-polyacrylamide gel electrophoresis demonstrated that the association of ASGP2 and ErbB2 is noncovalent in the secreted complex from the High Five cells. Finally, sedimentation analysis of the secreted complex suggested that ASGP2 is capable of associating in a 1:1 complex with monomeric ErbB2 ECD.

To determine the domain within ASGP2 that mediates its interaction with ErbB2, deletion mutants were co-expressed with ErbB2 ECD in high Five cells, and the co-immunoprecipitation assay was employed to determine the extent of interaction between the expressed secreted proteins (Fig. 3B). ASGP2 forms containing EGF1, the EGF-like domain that possesses the consensus residues found in active growth factors (17), could be co-immunoprecipitated with ErbB2 ECD. However, when the EGF1 domain was deleted, the ability of ASGP2 to associate with ErbB2 was almost

\[2\] E. A. Rossi and K. L. Carraway, unpublished observations.
completely abolished. These results indicate that the EGF1 domain of ASGP2 is necessary for its stable interaction with the ErbB2 receptor.

Fig. 3A demonstrates that the co-expression of the ASGP2 and ErbB2 proteins is necessary for their interaction. When the conditioned media from insect cells independently expressing ASGP2 ECD and ErbB2 ECD were mixed, no co-immunoprecipitation of ASGP2 and ErbB2 was observed. This is consistent with our observations that ASGP2 ECD expressed in either insect cells or COS monkey cells will neither bind to nor activate ErbB2 when added exogenously to cultured mammalian cells that express this receptor. The reason for the requirement of co-expression for ASGP2-ErbB2 complex formation is presently unclear. The simplest explanation is that high concentrations of ASGP2 and ErbB2 are necessary for complex formation, a condition that is met in membranes and in cellular compartments, but not by the addition of soluble ligand to cells. Once formed, the complex is very stable and resistant to dissociation during the immunoprecipitation procedures.

The results above indicate that ASGP2 binds directly to the ErbB2 receptor when the two proteins are co-expressed in the same cell. To examine the functional outcome of the interaction, sialomucin complex was expressed in an inducible manner in A375 human melanoma cells (18). These cells express modest levels of ErbB2 and ErbB3 and respond biochemically to the neuregulin-1 (NRG1β) growth factor ligand. When ASGP2 expression was turned on with the removal of tetracycline from the induction, sialomucin complex expression and then treated with and without recombinant NRG1, as indicated. Lysates and anti-tetracycline to induce sialomucin complex expression and then treated without and with recombinant NRG1, as indicated. Lysates and anti-ErbB2, and anti-ErbB3 immunoprecipitates were first blotted with anti-phosphoryrosine (upper panel). The filter was then cut apart and lysate lanes (lower left panel), anti-ErbB2 lanes (lower middle panel), and anti-ErbB3 lanes (lower right panel) were re-blotted with anti-ASGP2, anti-ErbB2, and anti-ErbB3, respectively.

receptor network. Interestingly, ASGP2 affects the extent of NRG1-stimulated receptor tyrosine phosphorylation rather than the dose-response curve of activation, implying that its role is not in the facilitation of ErbB2-ErbB3 heterodimeric complexes. Our results instead suggest that ASGP2 increases either the number of ErbB2 molecules available for activation or the extent of activation of each ErbB2 receptor.

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*4* K. L. Carraway III, unpublished observations.