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ErbB-4 and TNF- α Converting Enzyme Localization to Membrane Microdomains

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Abstract

Sequential proteolytic processing of ErbB-4 occurs in response to ligand addition. Here, we assess the localization of cleavable and non-cleavable ErbB-4 isoforms to membrane microdomains using three methodologies: 1) Triton X-100 insolubility, 2) Brij98 insolubility, and 3) detergent-free density gradient centrifugation. Whereas ErbB-4 translocated to a Triton X-100-insoluble fraction upon treatment of T47D cells with heregulin, it constitutively associated with a Brij98-insoluble fraction and a lipid raft fraction isolated using detergent-free methodology. Comparison of cleavable and non-cleavable isoforms of ErbB-4 revealed that both ErbB-4 isoforms are constitutively localized to either a Triton X-100-soluble or Brij98-insoluble fraction. In contrast, addition of heregulin resulted in translocation of the cleavable isoform to a detergent-free lipid raft. Tumor necrosis factor- α converting enzyme (TACE), the ectodomain secretase for ErbB-4, was present predominantly in its mature active form in most microdomains analyzed. These data suggest the assembly of ErbB-4 ectodomain cleavage apparatus in a membrane microdomain.

Keywords

ErbB-4; receptor tyrosine kinase; tumor necrosis factor- α converting enzyme; lipid rafts; membrane microdomains

INTRODUCTION

As a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, ErbB-4 conforms to the traditional activation pathway of ligand binding, homo- or heterodimerization, tyrosine autophosphorylation, and activation of downstream signaling targets [1]. However, ErbB-4 is unique because, in addition to its canonical signaling regime, it also undergoes sequential proteolytic processing by secretases in response to ligand activation [2]. Both heregulin and betacellulin ligands induce ErbB-4 proteolysis [3], as does 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C (PKC) [4]. Furthermore, tyrosine kinase activity is required for heregulin-stimulated ErbB-4 cleavage [5].

The initial rate-limiting proteolytic event occurs in the ectodomain of ErbB-4 between His651 and Ser652 [5] and is likely executed by TACE [6], a member of the ADAM (a disintegrin and metalloprotease) family of metalloproteases. The resulting membrane-tethered fragment, m80, retains tyrosine kinase activity [7] and serves as a substrate for γ -secretase, which cleaves

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within the transmembrane region of m80 to liberate a soluble 80-kDa intracellular domain (ICD) fragment, termed s80 [2,8].

Cellular properties of the s80 ICD fragment include cytoplasmic, nuclear, and mitochondrial localization plus interactions with Mdm2 [9], WWOX [10], Bcl-2 [11], STAT5A [12,13], YAP [14,15], and ETO2 [16]. Nuclear ErbB-4, presumably the s80 ICD fragment, has been observed in several tumor types [17–19]. Whether s80 ICD acts as a tumor suppressor or an activated oncogene is not clear [20–22].

However, relatively little is known about the initial rate-limiting step, ectodomain cleavage, and how growth factor binding activates this event. Following treatment of T47D breast cancer cells with heregulin, one group determined that ErbB-4 translocates from a Triton-soluble fraction to a Triton-insoluble fraction [3], and that this translocation event requires ErbB-4 tyrosine kinase activity [5]. Others have shown that ErbB-4 moves into neuronal lipid rafts upon heregulin stimulation [23], but migrates out of cardiomyocyte-derived rafts in response to heregulin [24]. The studies of ErbB-4 microdomain localization in T47D breast cancer cells [3,5] and neurons [23] employed Triton X-100, and the influence of detergents on membrane microdomains remains a controversial issue. Therefore, we adopted three separate methods, including a detergent-free method [25], to isolate membrane microdomains and assess ErbB-4 and TACE localization to those domains. This study employs two cell lines, T47D breast cancer cells that express endogenous ErbB-4, and COS-7 cells stably expressing two isoforms of ErbB-4, JM-a and JM-b.

MATERIALS AND METHODS

Materials

Heregulin β -1 was purchased from R&D Systems. ErbB-4 antibody (C-18) was purchased from Santa Cruz Biotechnologies, TACE antibody (807–823) from Calbiochem, and flotillin antibody from BD Transduction Laboratories. All other reagents are from Sigma.

Cell culture

T47D and COS-7 cells were maintained in DMEM supplemented with 10% FBS at 37°C with 5% CO₂. COS-7 cells stably expressing JM-a or JM-b ErbB-4 were selected with 500 μ g/ml G418 for 10 days, then maintained in 200 μ g/ml G418.

Separation of detergent-soluble and -insoluble fractions

For all treatments, cells were starved overnight in serum-free DMEM before treatment. Cells were fractionated as previously described [3]. In brief, cells were lysed for 30 min. on ice with cold TGH (1% Triton X-100, 10% glycerol, 50 mM Hepes, pH 7.2, 100 mM NaCl, 10 mM 1,10-phenanthroline, 5mM NaF, 1mM Na₃VO₄, and Roche Complete-Mini protease inhibitor cocktail tablet) or BGH (TGH substituted with 1% Brij98 in place of Triton). Lysates were centrifuged at 14,000 $\times$ g for 10 min. at 4°C, and the resulting supernatant is the detergent-soluble fraction. The pellet was next washed with TGH and solubilized with RIPA buffer (1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl, pH 7.4, and protease inhibitors). The resulting fraction is the detergent-insoluble fraction. Equal volumes of each lysate were either subjected to SDS-PAGE followed by immunoblotting with TACE or flotillin-1 antibodies, or immunoprecipitated with anti-ErbB-4 and immunoblotted with anti-ErbB-4.

Detergent-free lipid raft isolation

Lipid rafts were isolated from five 150 mm dishes of confluent cells per treatment according to the method of Smart, *et al.* [25]. This technique includes separation of the plasma membrane

fraction and flotation of this fraction on two density gradients: a continuous 20–10% OptiPrep gradient and a discontinuous 25%-15%-5% OptiPrep gradient. Lipid rafts are contained at the 5%–15% OptiPrep interface. Equal volumes of each lipid raft fraction were subjected to immunoblot analysis with ErbB-4, TACE, or flotillin-1 antibodies.

RESULTS AND DISCUSSION

As previously reported [3], treatment of T47D cells with heregulin, but not the PKC activator TPA, promoted ErbB-4 translocation to a Triton-insoluble fraction (Figure 1A). To test the generality of this finding, we stably expressed two ErbB-4 isoforms, JM-a or JM-b, in COS-7 cells. These isoforms were included because the JM-b species is reported to be resistant to ectodomain cleavage due to sequence changes around the cleavage site [26]. As shown in Figure 1B, heregulin treatment did not result in a similar translocation event for either isoform. Thus, these data demonstrate that the ErbB-4 translocation to a Triton-insoluble fraction is not a general phenomenon. While the composition of the Triton-insoluble fraction is not clear, a lipid raft marker, flotillin-1, is present in this fraction in both cell types. The mature form of TACE was detected in the Triton-insoluble fraction as a 100-kDa band in T47D cells (Figure 1A), but not in the Triton-insoluble fractions derived from COS-7 cells (Figure 1B). The enzyme furin processes immature 120-kDa TACE in the Golgi apparatus to a 100-kDa activated form [27].

Since membrane microdomains are often enriched with cholesterol, one frequently used method to disrupt these microdomains is treatment with methyl- β -cyclodextrin (M β CD), which depletes cholesterol from membranes. T47D cells treated with M β CD displayed a loss of basal ErbB-4 in the Triton-insoluble fraction (Figure 2), but not when treated with α -cyclodextrin, which does not deplete cholesterol. Interestingly, M β CD pretreatment did not significantly affect heregulin-stimulated ErbB-4 translocation to this fraction (Figure 2). Therefore, ErbB-4 migration to the Triton-insoluble fraction of T47D cells does not represent association with a cholesterol-dependent microdomain.

A recent report compared EGFR localization to lipid rafts using an array of detergents, including Triton X-100 and two detergent-free methods [28]. The data show that, in the absence of ligand stimulation, EGFR segregates to a lipid raft fraction as determined by detergent-free methods or the use of Brij98, but remains in a non-raft fraction when Triton X-100 is included. Hence, we repeated the experiments in Figure 1 with Brij98. When either T47D (Figure 3A) or COS-7 cells expressing JM-a or JM-b ErbB-4 (Figure 3B) were separated into Brij98-soluble and -insoluble fractions, the resulting analysis revealed that, in both the absence and presence of heregulin stimulation, ErbB-4 was primarily associated with the Brij98-insoluble fraction. Only a small proportion of total receptor localized to the Brij-98 soluble fraction. This is in marked contrast to the results obtained with Triton X-100. Flotillin-1 was enriched in the Brij98-insoluble fractions of T47D cells and COS-7 cells, and equivalent amounts of mature TACE were found in the Brij98-soluble and -insoluble fractions of both cell types (Figure 3).

Data from Figures 1A and 3A indicate that TACE localizes to both Triton- and Brij98-insoluble fractions of T47D cells. In these same cells, however, ErbB-4 localization to membrane microdomains is highly dependent on the detergent used for solubilization. To rule out detergent-dependent effects, we employed a detergent-free methodology developed by Smart *et al.* [25]. Consistent with the Brij98 data, ErbB-4 was present in equal amounts in the raft fraction of control and heregulin-treated T47D cells (Figure 4A). The mature form of TACE was constitutively present in this fraction (Figure 4A), also in agreement with the data in Figures 1A and 3A.

Detergent-free lipid rafts were also isolated from COS-7 cells containing JM-a or JM-b ErbB-4 isoforms. The JM-a isoform migrated into the raft fraction after heregulin treatment (Figure 4B). In contrast, the JM-b isoform was constitutively present in the raft fraction and only slightly increased following heregulin addition (Figure 4B). Also detected were the m80 fragment in the JM-a ErbB-4 raft fractions (in both T47D and COS7 cells) and 80- and 90-kDa fragments in JM-b ErbB-4 raft fractions (Figure 4B, right panel). The m80 fragment was not detected in the Triton-insoluble fraction derived from T47D cells [3]. Mature TACE was also present constitutively in the raft fraction of all cells analyzed (Figure 4).

In summary, using three separate techniques, membrane microdomains were isolated from T47D cells that express endogenous ErbB-4 and COS-7 cells that stably express two ErbB-4 isoforms. Heregulin promoted movement of endogenous ErbB-4 to a Triton-insoluble fraction in T47D cells, but not ErbB-4 expressed exogenously in COS-7 cells. Depletion of cholesterol from T47D cells did not significantly affect heregulin-induced ErbB-4 translocation to the Triton-insoluble fraction, indicating that ErbB-4 movement into the Triton-insoluble fraction is not cholesterol dependent. Rather, this may reflect translocation induced by protein:protein instead of protein:lipid interactions within a lipid raft [29].

ErbB-4 was present at high levels in the Brij98-insoluble fractions of all cells tested regardless of heregulin treatment. Also, the data suggest that the cleavable and non-cleavable isoforms differentially associate with membrane microdomains. Strikingly, only the JM-a isoform of ErbB-4 expressed in COS-7 cells displayed marked heregulin-stimulated translocation to lipid rafts isolated by a detergent-free method. It seems likely that the data obtained using the detergent-free methodology in Figure 4 is the most accurate indicator of ErbB-4 localization in the intact membrane. Therefore, ErbB-4 localization to membrane microdomains is highly influenced by cell type as evidenced by the difference we observed between T47D and COS7 cells as well as previously reported data in neurons [23] and cardiomyocytes [24]. It should be noted that adult cardiomyocytes express only the non-cleavable JM-b isoform [26], and we did not observe a significant heregulin-stimulated JM-b ErbB-4 migration into or out of membrane microdomains in COS-7 cells expressing this isoform. The endogenous ErbB-4 receptor expressed in T47D cells is predominantly the JM-a isoform (Elenius, K., personal communication). ErbB-4 expression and heregulin treatment did not alter TACE localization to membrane microdomains of either cell line. Importantly, the mature form of TACE was the predominant form in all detergent-insoluble or lipid-enriched microdomains tested. While several pieces of data point to the importance of lipid rafts for γ -secretase cleavage of substrates [30], this is the first report to localize the mature form of TACE, an α -secretase, to a membrane microdomain.

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Figure 1.

Heregulin-induced ErbB-4 translocation to a Triton X-100-insoluble fraction. T47D (**Panel A**) or COS-7 (**Panel B**) cells stably expressing JM-a or JM-b ErbB-4 were starved overnight, then treated with heregulin (20 ng/ml) or the phorbol ester TPA (100 ng/ml) for 15 min. Cells were separated into Triton X-100-soluble and -insoluble fractions as described [3]. Equal volumes of soluble and insoluble fractions were either immunoprecipitated with anti-ErbB-4 (upper panels) or directly subjected to SDS-PAGE and blotted with antibodies directed against ErbB-4, TACE or flotillin-1. ErbB-4 bands in the Triton-insoluble fraction were subjected to densitometric analysis, normalized for flotillin-1 levels, and quantitated relative to untreated samples. Arrows indicate immature (upper arrow, 120 kDa) and mature (lower arrow, 100 kDa) forms of TACE (center panels).

Figure 2.

Cholesterol depletion does not inhibit heregulin-dependent translocation of ErbB-4 to a Triton-insoluble fraction. T47D cells were pretreated treated with either methyl- β -cyclodextrin (5mM) or α -cyclodextrin (5mM) for 30 min. followed by heregulin (20 ng/ml) for an additional 15 min. and separated into Triton X-100-soluble and insoluble fractions. The Triton-insoluble fractions were immunoprecipitated with anti-ErbB-4 and blotted for ErbB-4 (upper panel). Lysates were blotted for flotillin-1 expression (lower panel).

Figure 3.

Constitutive ErbB-4 and TACE localization to a Brij98-insoluble fraction. T47D (**Panel A**) or COS-7 (**Panel B**) cells stably expressing JM-a or JM-b ErbB-4 were treated and lysed as described in Figure 1 with the following modification: 1% Brij98 was used instead of 1% Triton X-100. Equal volumes of lysates were either immunoprecipitated with ErbB-4 (Panel A, top) or directly subjected to SDS-PAGE and immunoblotted with anti-ErbB-4, anti-TACE or anti-flotillin-1 as in Figure 1. Arrows denote the mature (100 kDa) and immature (120 kDa) forms of TACE (center panels).

Figure 4.

Detergent-free lipid raft isolation. **(Panel A)** T47D cells were starved overnight, treated with heregulin (20ng/ml) for 15 min. and lipid rafts isolated by the detergent-free method of Smart *et al.*[25]. Equal aliquots of lipid raft fractions were blotted with anti-ErbB-4 (upper panel), anti-TACE (center panel) and anti-flotillin-1 (lower panel). **(Panel B)** Lipid rafts from COS-7 cells expressing JM-a or JM-b ErbB-4 were isolated and analyzed for ErbB-4, TACE, and flotillin-1 localization as in Panel A. Results reflecting two experiments analyzed in duplicate were normalized for flotillin-1 levels and quantitated on the right.