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NDF/heregulin stimulates the phosphorylation of Her3/erbB3

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Abstract

Her3/erbB3 has been identified as a third member of the epidermal growth factor receptor (EGFR) family [(1989) Proc. Natl. Acad. Sci. USA 86, 9193–9197; (1990) Proc. Natl. Acad. Sci. USA 87, 4905–4909]. The natural ligand for Her3 has not been identified. Although recently NDF has been proposed as a specific ligand for Her4 [(1993) Nature 366, 473–475; (1993) J. Biol. Chem. 268, 18407–18410], we report here that Her3 was phosphorylated on tyrosine not only in three breast carcinoma cell lines, MDAMB453, MDAMB468 and SKBR3, but also in Her3-transfected CHO cells in response to NDF stimulation. In further studies, cells were reacted with ^{125}I-labeled NDF and then chemically crosslinked. Immunoprecipitation with anti-Her3 revealed a dense high M_W band, greater than 400 kDa. The results suggest that NDF may be a ligand of Her3 and induces receptor hetero-oligomerization.

Key words: Her3; erbB3; NDF; Heregulin; Tyrosine kinase; EGF receptor family

1. Introduction

Members of the erbB growth factor receptor family are frequently over-expressed in a variety of epithelial carcinomas [5–7]. The four erbB family receptors identified thus far (EGFR, erbB2/Her2, erbB3/Her3 and erbB4/Her4) possess 60–80% homology in their kinase domains [8,9]. EGF, TGFα, amphiregulin, heparin binding EGF and betacellulin have been identified as the EGFR ligands [10–15]. A 25 kDa (p25) peptide from conditioned media of MDP-activated macrophages (MDP-CM) [16], NAF (a *v* protein-specific activating factor) [17], a 75 kDa (P75) peptide from SKBR3 conditioned media [18], NEL-GF from bovine kidney [19], NDF/heregulin [20], ARIA (a factor that stimulates the synthesis of acetylcholine receptors [21] and GGF (glial growth factor) [22], have been proposed as ligands of p185$^{erbB2/Her2}$. Evidence for the interaction of NDF with Her2 was derived from studies with cultured human carcinomas in which the Her2 gene was frequently amplified, but which also expressed other members of the EGFR family. Recent experiments in transfected rodent cells expressing only the Her2 gene have failed to confirm the existence of direct interaction between NDF and Her2 (data not shown). We and others [23] have theorized that the activation of Her2 receptor tyrosine kinase in response to NDF may require the active participation of another receptor, which may initially bind the ligand and secondarily phosphorylate the Her2 through receptor cross-talk [24–26]. We have focused on Her3/erbB3 as the receptor which potentially *trans*-phosphorylates Her2 since both receptors were found to be expressed in the breast cancer cell lines used for these studies (SKBR3 and MDAMB453) [27]. Two groups isolated Her3/erbB3 independently as a third member of the EGFR family [1,2], and a natural ligand for Her3 has not yet been identified. Some doubts initially existed that Her3 possessed tyrosine kinase, due to the presence of unusual amino acids in its catalytic domain. However, in vitro kinase activity has now been demonstrated in Her3 transfected into NIH3T3 cells, and a chimeric EGFR/erbB3 protein was shown to be activated by EGF [27]. We show in this paper that NDF induces tyrosine phosphorylation of Her3 in both breast carcinoma cells and Her3-transfected CHO cells.

2. Materials and methods

2.1. Cells

Her3/CHO cells were prepared by co-transfection of DHFR-deficient CHO cells with two vectors: pJT2 carrying the genes for erbB3 gene and dihydrofolate reductase (*dhfr*) in pDRα2. ErbB3-transfected CHO cells were grown in selective medium which lacked nucleosides (DMEM containing 5% dialyzed fetal bovine serum, 2 mM glutamine and 0.1 mM non-essential amino acids).

2.2. Materials

Human recombinant EGF and NDF isoforms were produced in *E. coli*, and purified to greater than 98% homogeneity by subjecting clarified *E. coli* lysates to anion exchange, cation exchange, hydrophobic interaction and hydroxyapatite column chromatography. Extra methionine residues were added at the N-terminus of NDF as initiation sites for protein translation. Anti-Her3 polyclonal antibodies, 61.3 and 49.3 (affinity purified), were used for immunoprecipitation and Western

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blotting [28]. Anti-Her2 monoclonal antibodies, c-neuAb2 and c-neuAb3, were from Santa Cruz Biotechnology. Anti-phosphotyrosine, PY20, was from ICN. Anti-EGFR monoclonal antibodies, E-2760 and E-3138, were from Sigma.

2.3. Immunoprecipitations

Cells were grown in 10 cm dishes to about 80% confluency, washed twice with DMEM and incubated with ligand or control solution for 5 min at 37°C. Cells were lysed in PBS/TDS buffer, lysates added to the protein-G–agarose–antibody complex and incubated for 2 h at 4°C. The immunocomplex was washed three times with PBS/TDS and 2× SDS sample buffer was added.

2.4. Assay of receptor tyrosine phosphorylation

Cells were grown in 48-well plates and washed with DMEM 2–3 times. Ligand solution was added to the wells and incubated for 5 min at 37°C. The solution was removed and the cells were solubilized with SDS sample buffer. The samples, with or without immunoprecipitation, were subjected to SDS-PAGE followed by Western blotting and probing with anti-phosphotyrosine.

2.5. Crosslinking assay

NDFα2 was iodinated by the method of M.A.K. Markwell, using Iodo-beads (Pierce). Iodinated NDF was purified by Sephadex G-25 column chromatography. Cells in 10 cm dishes were incubated with ^{125}I-labeled NDF for 5–10 min at 37°C. Following a quick wash with DMEM, the cells were incubated with BS3 (3 mM) for 30 min at room temperature. The cells were lysed and Her3, Her2 or EGFR were immunoprecipitated from the lysates. The samples were run on a 4% polyacrylamide gel. Radioactive bands were detected by phosphoimage analyzer (Molecular Dynamics).

3. Results and discussion

Her3 was immunoprecipitated from lysates of the breast carcinoma cell lines MDAMB453, MDAMB468 and SKBR3 using antibodies 61.3 and 49.3 raised to two different synthetic peptides from the cytoplasmic domain of Her3 [28]. These antibodies had previously been shown to identify a p160 erbB3 protein from human embryonal cells (293 cells) transfected with the cDNA encoding c-erbB3 [28]. The antibodies do not crossreact with c-erbB2 or EGFR (data not shown) [29]. In the present studies, utilizing the same two antibodies, we detected a 180 kDa protein in immunoprecipitates of SKBR3 and MDAMB453 cells (Fig. 1) which agree with a recent report [27]. The discrepancy in the molecular weight, 160 kDa vs. 180 kDa, might be explained as either a different degree of glycosylation of the Her3 extracellular region, or different proteolytic processing between the cell lines. NDF stimulated the phosphorylation of a band at 180 kDa, specifically immunoprecipitated by antibodies to Her3 in both MDAMB453, MDAMB468 and SKBR3 (data not shown) cells. Phosphorylated Her2 was also identified in a Her2 immunoprecipitate of NDF-stimulated MDAMB453 cells (Fig. 2) and SKBR3 (data not shown) [23]. Since Her2, Her3 and Her4 are expressed in the MDAMB453 cell line [27], the tyrosine phosphorylation of Her3 in response to NDF isoforms (data not shown) may not be due to direct binding of NDF to Her3 but receptor–receptor cross-talk. Her4, however, is reported not to be expressed in the MDAMB468 cell line [30]. To clarify the nature of the interaction between NDF and Her3, CHO cells which do not express any type 1 receptors were transfected with a plasmid containing the full-length Her3 gene. Selected CHO/Her3 clones, #7 and #12, were used for these experiments. As shown in Fig. 3A, the transfected CHO cells stimulated with NDF showed strong phosphorylation of tyrosine residues in Her3 immunoprecipitates. The results strongly suggest that NDF is a ligand for Her3. It has been reported that NDF binds specifically to the protein tyrosine kinase receptor, Her4

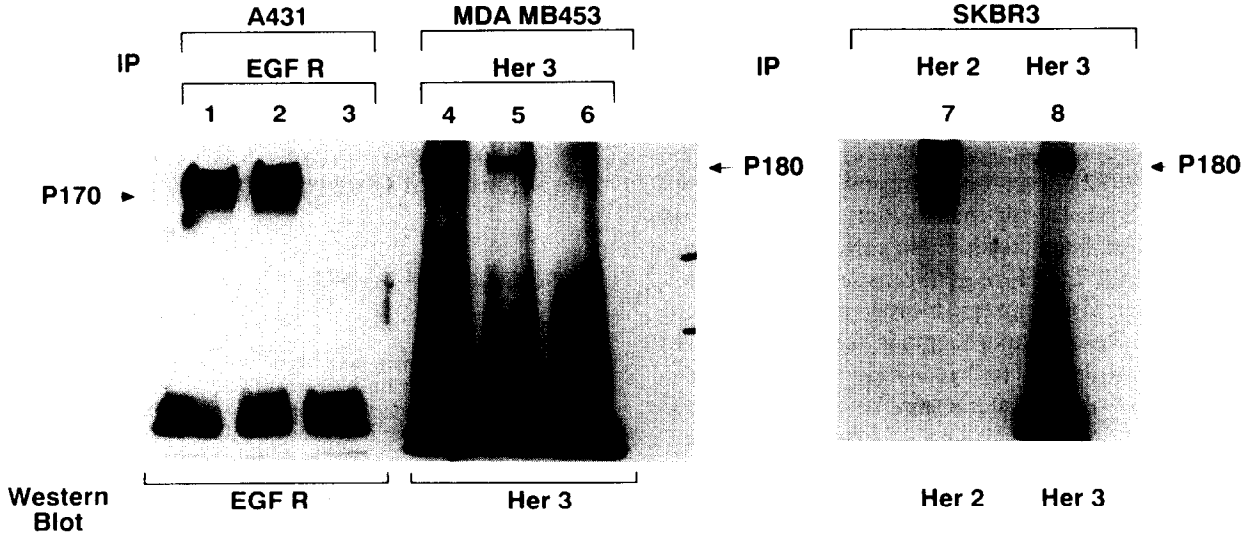

Fig. 1. Her3 (p180) detection with anti-Her3 (61.3 and 49.3) [28] in breast carcinoma cell lines. Her3 was detected by immunoprecipitation from cell lysates of MDAMB453 and SKBR3 with anti-Her3 (61.3) followed by Western blotting and probing with anti-Her3 (49.3). There was no detectable cross-reaction of these antibodies with Her2 (data not shown) [29]. To show differences in electrophoretic mobility between 180 and 170 kDa, EGFR was immunoprecipitated from A431 cell lysates and blotted with anti-EGFR. Lanes 1 and 2 are duplicates. Lanes 3 and 6 are controls, i.e. immunoprecipitation from cell lysing solution instead of cell lysates. Lanes 4 and 5 are duplicates from separate cell cultures. Lanes 7 and 8 are as shown. The low M_w band in lane 8 is non-specific (see lane 6, non-cell lysate control).

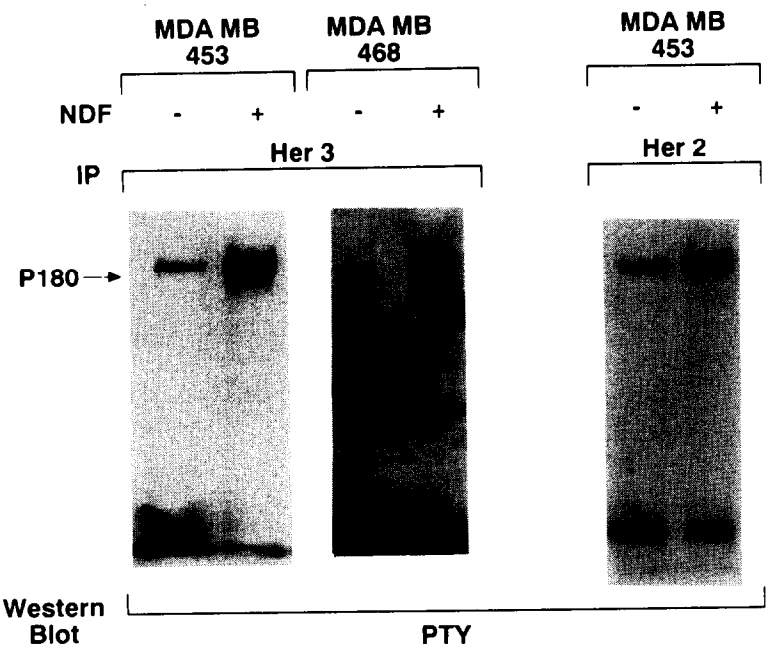

Fig. 2. NDF induces tyrosine phosphorylation of Her3 in breast carcinoma cells. Tyrosine phosphorylated receptors were immunoprecipitated with anti-Her3 or anti-Her2 followed by Western blotting and probing by anti-phosphotyrosine (PTY).

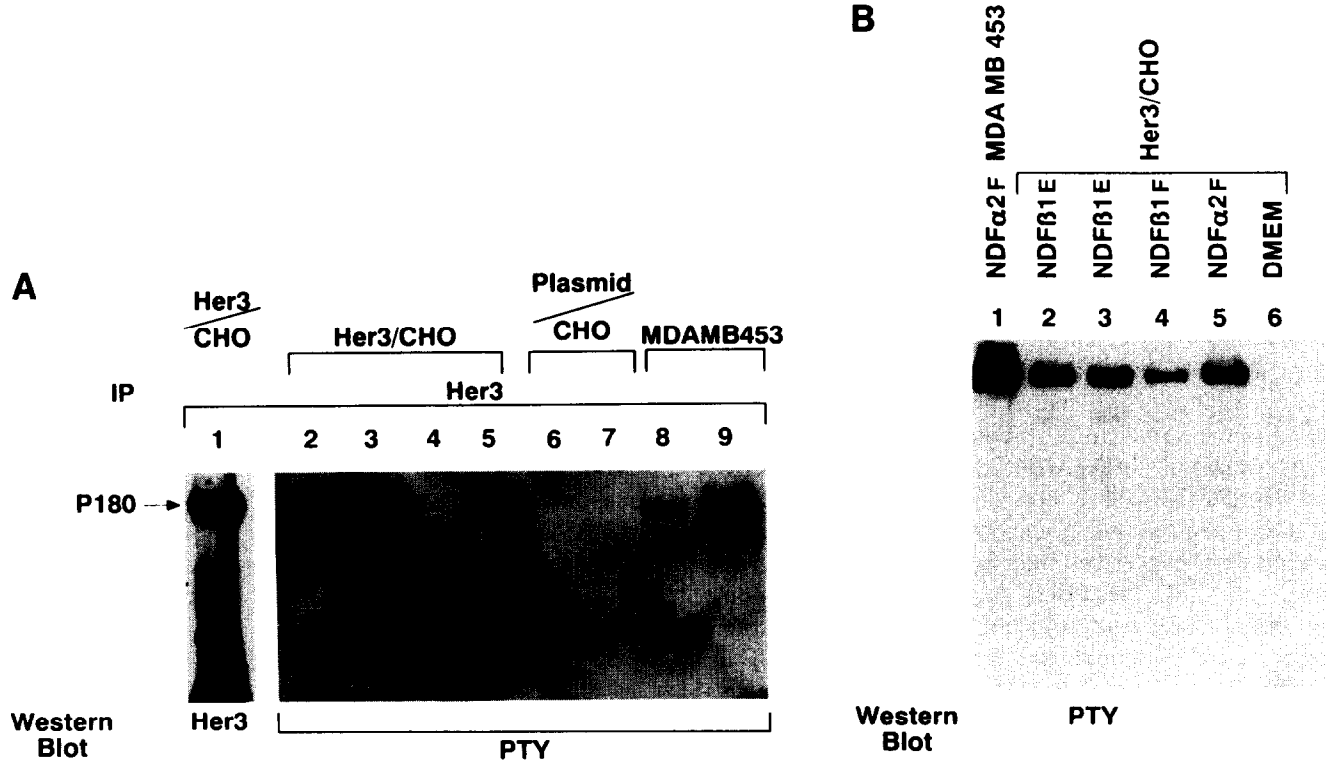

Fig. 3. NDF and the isoforms induce tyrosine phosphorylation of Her3 in Her3/CHO cells. (A) Immunoprecipitate of Her3-positive clone 7, probed with anti-Her3 (49.3) shown in lane 1. Her3/CHO, plasmid/CHO and MDAMB453 cells were incubated with 50 ng/ml NDFα2 (lanes 3,5,7,9) or DMEM (Lanes 2,4,6,8) for 5 min at 37°C, and immunoprecipitated with anti-Her3. Lanes 2 and 3 are clone 7, and lanes 4 and 5 are clone 12. Resulting Western blots were probed with anti-PTY(PY20; ICN). (B) Her3/CHO stimulation by NDF isoforms. Lanes: 1, positive control, $NDF\alpha2_{14-241}$, 50 ng/ml in MDAMB453; 2, $NDF\beta1_{177-246}$, 2 nM; 3, $NDF\beta1_{177-246}$, 6 nM; 4, $NDF\beta1_{14-246}$, 2 nM; 5, $NDF\alpha2_{14-241}$, 2 nM; 6, DMEM, negative control. E in lanes 2 and 3 stands for EGF structural motif. F in lanes 1, 4 and 5 stands for full-length. Her3/CHO cells were seeded in 48 well plates, and after 24 h isoform solutions were incubated for 5 min at 37°C. Cells were solubilized with SDS sample buffer. Solubilized samples were electrophoresed on 8% polyacrylamide gels, followed by Western blotting and probing with anti-PTY.

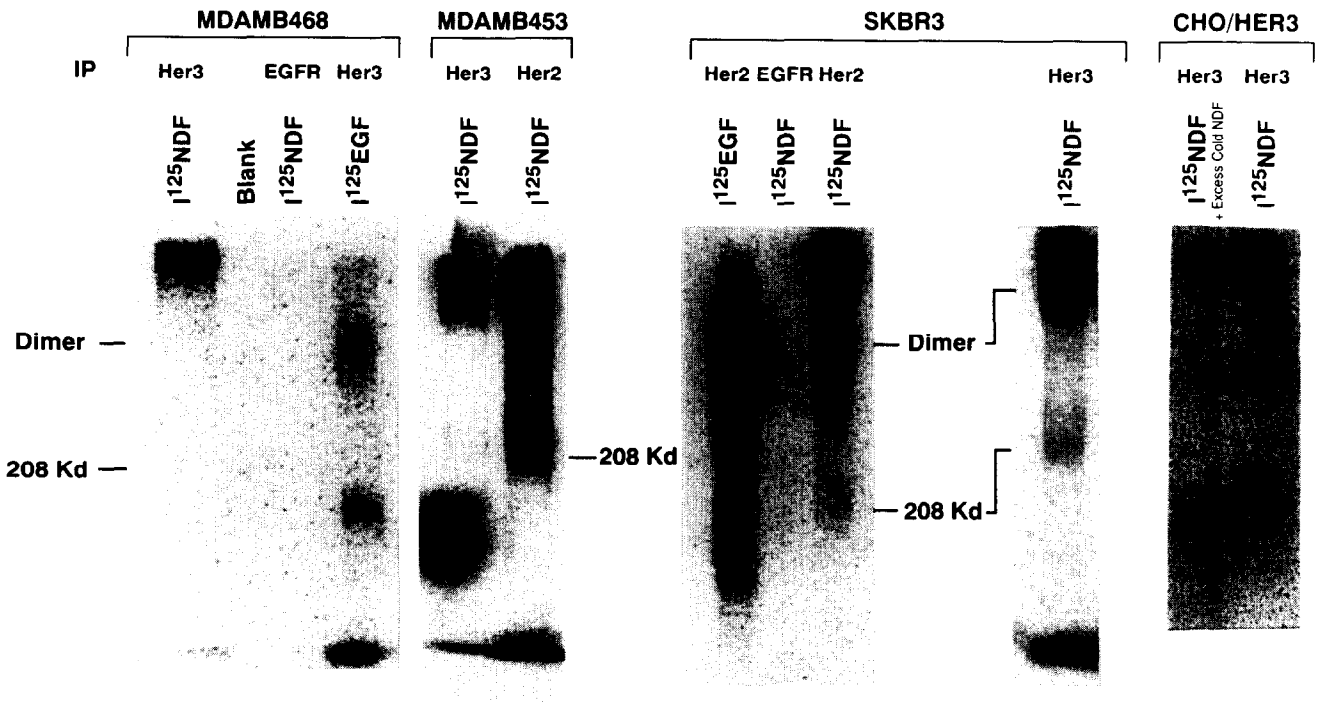

Fig. 4. Crosslinking iodinated NDFα2 to Her3. ^{125}I-labeled NDFα2 or EGF was incubated in MDAMB453, MDAMB468, SKBR3 and Her3/CHO for 10 min at room temperature. Cells were washed with DMEM and then crosslinked with BS3 (Pierce) followed by cell lysis. Lysates were immunoprecipitated with antibodies to Her3, Her2 or EGFR and the samples were run on a 4% polyacrylamide gel. Radioactive protein bands were detected by a phosphoimage analyzer.

[3]. As shown in Fig. 3B, the NDF isoforms examined here all induced tyrosine phosphorylation of Her3 in Her3/CHO cells. In order to confirm the direct interaction of NDF and Her3, ^{125}I-labeled NDF was crosslinked with the chemical crosslinker BS3 in MDAMB453, MDAMB468, SKBR3 (which also lack expression of Her4 [30]) and Her3/CHO cells, followed by immunoprecipitation of cell lysates with either anti-Her3, anti-Her2 or anti-EGFR antibodies (Fig. 4). Immunoprecipitation with either anti-Her3 or anti-Her2 resulted in the appearance of high M_W protein complexes (> 400 kDa) in MDAMB453, MDAMB468, SKBR3 and Her3/CHO cell lysates. The results suggest that NDF induces receptor homo- or hetero-oligomerization in the breast cancer cell lines tested and in Her3 transfected cells. ^{125}I-labeled EGF assayed in the same binding and crosslinking study did not generate a M_W protein complex, but monomer and dimer forms of the EGF receptor were visible in immunoprecipitates. Heterodimers of EGFR and Her2 have been detected previously in SKBR3 cells incubated with ^{125}I-labeled EGF [31].

Thus we show here that several isoforms of NDF induce Her3 tyrosine phosphorylation in Her3/CHO cells. Her3 interaction with NDF and NDF isoforms in Her3-transfected cells may be considered to be a model of naturally occurring cells, although in nature it is rare for cells to possess only a single type of receptor. However, it is more straight-forward to determine individual growth factor/receptor interactions which are not confused by the presence of several receptor types. At least ten NDF isoforms have been identified which were generated from alternative splicing [32]. It is not yet known which of the NDF isoforms bind specifically to Her4. NDF isoforms can be grouped into two types, α and β, based on differences in their receptor binding portions (EGF-like domains). Since all the isoforms examined here bind Her3 in the absence of other receptors, it appears that Her3 may not possess isotype specificity.

In light of the fact that Her3 and Her4 both bind NDF, yet Her2 still appears to be involved in biological responses of some cells to NDF (C. Starnes, unpublished data), we believe that NDF is capable of inducing a variety of receptor hetero-complexes, which may consist of various combinations of Her2, Her3 or Her4 as appropriate to the particular cell line.

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