Cell cycle activation in lung adenocarcinoma cells by the ErbB3/phosphatidylinositol 3-kinase/Akt pathway

Gunamani Sithanandam1,4, George T.Smith2, Akira Masuda3, Takashi Takahashi3, Lucy M.Anderson2 and Laura W.Fornwald1

1Basic Research Program, SAIC Frederick, 2Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick, Building 538, Ft. Detrick, Frederick, MD 21702-1201, USA and 3Division of Molecular Oncology, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan
4To whom correspondence should be addressed
Email: sithanan@mail.ncifcrf.gov

Although ErbB3, a member of the epidermal growth factor receptor family, has been implicated in mammary tumorigenesis, investigation of its role in lung tumorigenesis has been limited. We found that ErbB3 was present at high levels in five of seven human lung adenocarcinoma cell lines examined, along with its ligands, heregulins α and β, whereas ErbB3 was absent from HPL1D, a non-transformed cell line from human pulmonary peripheral epithelium. Interactions and effects of ErbB3 were studied in detail in adenocarcinoma lines H441 and H1373. Complexes containing phosphorylated ErbB2, phosphorylated ErbB3 and the p85 regulatory subunit of phosphoinositidyl 3-kinase were detected by co-immunoprecipitation experiments and were present constitutively even in the absence of serum-stimulated cell division. Serum treatment increased the pErbB3/p85 complexes and also stimulated phosphorylation of Akt and GSK3β, increase in cyclin D1 and cell cycle progression, and these events were blocked by the Akt activation inhibitor LY294002. An ErbB3-specific antisense oligonucleotide reduced amounts of ErbB3 protein and p85 complex in both cell lines, and significantly suppressed cell proliferation. These results together suggest involvement of ErbB3 in growth of lung adenocarcinomas, through activation of phosphoinositidyl 3 kinase and Akt, inactivation of GSK3β and stabilization of cyclin D1 for cell cycle maintenance. It could be a useful therapeutic target.

Abbreviations: EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; HRGs, heregulins; PI3K, phosphatidylinositol 3-kinase.

Introduction

The four members of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, ErbB1(EGFR), ErbB2, ErbB3 and ErbB4, and the dozen or more ligands that activate them, mediate proliferation, differentiation, migration, cell survival or apoptosis through a complex network of signaling, in a cell-specific manner (1,2). These receptors contain an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and an intrinsic tyrosine kinase cytoplasmic domain (3). Of these, ErbB3 lacks intrinsic kinase activity (4). Different isoforms of Neu differentiation factor/herégulin (also known as neuregulin) bind to ErbB3 (5). No direct ligand for ErbB2 has yet been discovered (6). The mature soluble forms of heregulins (HRGs) act as autocrine and paracrine factors while the membrane-associated precursors act through a juxtacrine pathway (7).

Each ErbB receptor has unique properties with regard to ligand affinity, rate of down-regulation and tyrosine kinase activity (8). Specific ligands can elicit distinct signals downstream of a given ErbB receptor, as a function of the specific profile of tyrosine phosphorylation of the receptor (9). Upon ligand binding the ErbB receptors homo- and heterodimerize in various combinations. The transphosphorylation between the receptor subunits in a dimeric complex diversifies the biological response, by recruiting various downstream signaling molecules (10). The type and amplitude of signaling depend upon the co-expression of these receptors and the ligand availability in the cellular context.

This complexity dictates that growth factor/ErbB pathways be examined in detail for every cell and cancer type of interest. In the human fetus, EGFR, ErbB2 and ErbB3 were detected in the epithelium of developing lung, and NRG-1 treatment of fetal lung explants resulted in increased proliferation and reduced differentiation (11,12). In normal adult human lung, EGFR, ErbB2, ErbB3, EGF, TGFα, amphiregulin and heparin-binding EGF were detected by immunohistochemistry in the bronchial epithelium (13,14). Cultured human bronchial epithelial cells expressed TGFα, amphiregulin, betacellulin, heparin-binding EGF and herégulin (15,16).

Less certain is the presence of ErbB receptors and ligands in normal adult alveolar/bronchiolar cells, the precursors of adenocarcinoma. Several immunohistochemical and RNA analyses in human lung noted the absence of EGF from alveolar cells (13,17,18). Immunoelectron microscopy confirmed the presence of EGFR for normal human adult type II pneumocytes, with staining limited to lateral cell membranes at attachments to adjacent epithelial cells (13). Immunohistochemical studies reported moderate staining for ErbB3 in adult alveolar cells (19,20), but absence of ErbB2 (11,19).

There is much evidence that ErbB receptors and ligands contribute to lung tumorigenesis. The EGFR may have high levels of expression in non-small cell lung carcinoma (NSCLC) (21). This over-expression has correlated with poor prognosis in some studies (21–23) but not others (24–26). Over-expression of EGFR was reported at an early stage of basal cell hyperplasia, and persisted through squamous metaplasia, dysplasia and carcinoma in situ (27). Iressa, a small molecular weight inhibitor of EGFR, had activity against non-small cell cancers in clinical trials (28,29).

ErbB2 is over-expressed in ~30% of lung tumors and in up to 100% of lung adenocarcinomas, with associated poor prognosis, especially for lung adenocarcinoma (30–32). Over-expression of ErbB2 and EGFR also correlated with poor prognosis (26). However, over-expression of ErbB2 alone did not transform bronchial epithelial cells (33). Clinical trials of...
an anti-ErbB2 reagent, herceptin, gave negative results for lung cancer (34).

Very limited data are available for ErbB3 in lung cancer. As measured by immunohistochemistry, ErbB3 was highly expressed in some lung adenocarcinomas (35) and associated with poor prognosis (20). Co-expression of ErbB3 with other members of the ErbB family has not been well studied. One immunohistochemical study indicated that ErbB3 co-expression with other ErbB family members was a marker for lung tumor recurrence (36).

New evidence for the importance of ErbB3 in lung tumorogenesis has come from ErbB3 transgenic mice treated with the carcinogen methyl nitrosourea: these developed a high incidence of lung tumors, with reduced latency (37). Furthermore, doubly transgenic mice over-expressing both human ErbB3 and rat ErbB2 had a shorter lung tumor latency compared with the singly transgenic ErbB3 mice (37).

Most investigations of ErbB3-related signaling have been focused on breast and ovarian cancer cells. In these cells ErbB2/ErbB3 heterodimers activate various signaling pathways (38–40). Growth inhibition of ovarian cancer cells with an ErbB3 receptor antibody (41) and of breast cancer cells with dominant-negative ErbB3 (42) have been recently reported. No therapeutic antibodies against ErbB3 are currently available.

In this study, we examined the expression and activation state of ErbB3 in human lung adenocarcinoma cell lines and found that ErbB3 is constitutively activated at a high level in several lung adenocarcinoma cell lines, which also express ErbB2. We demonstrate the presence in these cells of complexes of ErbB3, ErbB2 and the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). Serum treatment augmented amount of ErbB3/p85 complex, activated the PI3K/Akt pathway and promoted cell proliferation. We also provide evidence for ErbB3 mediating growth in these lung adenocarcinoma cells by inhibiting their proliferation with antisense ErbB3 oligonucleotides.

Materials and methods

Cell lines and culture conditions

Human lung adenocarcinoma cell lines were obtained from the American Type Culture collection and were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, penicillin/streptomycin and 10% fetal calf serum. Culture collection and were cultured in RPMI 1640 medium supplemented with 5 mM NaCl, 2 mM glutamine, penicillin/streptomycin and 10% fetal calf serum.

For serum starvation experiments, 70% confluent cultures were maintained with 7 M hydrocortisone (Sigma), 2 mM triiodothyronine (Sigma), 0.05% BSA and 10 mM sodium orthovanadate (Sigma) in serum-free media for 48 h. Cells were then incubated for an additional 72 h and assayed for number of viable cells as per the manufacturer’s instruction. Substrate cleavage was monitored at 570 nm using a Biotech microplate reader. Statistical analysis (paired t-test or non-parametric tests as appropriate) was carried out using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, CA.

Results

ErbB3 is expressed in most human lung adenocarcinoma cells but not in the non-transformed human lung HPL1D cell line

To evaluate any altered expressions in ErbB family members or agonists that may be associated with the transformed phenotype, we compared the non-transformed human lung cell line HPL1D from the peripheral epithelium, with two malignant cell lines from human adenocarcinomas, H441 and H1373, for the expression of ErbB family members by western immunoblot analysis (Figure 1).

ErbB3 was expressed only in the two malignant cell lines; it was absent from HPL1D cells.
Heregulin α (HRGα), and heregulin β (HRGβ), ligands for ErbB3, were found in both transformed and non-transformed cell lines. The co-occurrence of ErbB3 and HRG isoforms in the malignant cells suggested a contribution of an HRG/ErbB3 autocrine loop to maintenance of the malignant phenotype of these cells.

To investigate the generality of the high expression of ErbB3 in human lung adenocarcinoma cells, several additional lines were tested. Of these, three—A549, H1395 and H1355—were positive (data not shown) and two—H23 and H1792—were negative for ErbB3 protein (see Figure 10A).

**ErbB3 and ErbB2 are constitutively active in H441 and H1373 cells**

ErbB3 immunoprecipitates from serum-starved cells were subjected to immunoblot analysis using anti-phosphotyrosine-specific antibodies (Figure 2A). The results indicated that ErbB3 is constitutively activated in these cells in the absence of serum growth factors. Reciprocal anti-phosphotyrosine immunoprecipitations blotted with ErbB3 antibody confirmed this observation (Figure 2B).

Western blotting with phospho-specific ErbB2 antibody indicated that ErbB2 is also constitutively phosphorylated in these cells (Figure 2C), with the highest levels in H441 and least in HPL1D. Immunoprecipitations with anti-ErbB2 antibody followed by immunoblotting with anti-phosphotyrosine antibody confirmed this observation (Figure 2D). These high levels of phosphorylated ErbB2 and ErbB3 in the absence of serum indicate that an HRG/ErbB2/ErbB3 autocrine loop may be active in these cells.

The p85 regulatory subunit of PI3K co-immunoprecipitates with ErbB3 in proliferating and serum-starved H441 and H1373 cells

Since ErbB3 is highly expressed in these cells, and activated ErbB3 has high affinity for the p85 regulatory subunit of PI3K, we investigated ErbB3/p85 interaction by co-immunoprecipitation with either anti-p85 or anti-ErbB3. In both adenocarcinoma cell lines, the p85 subunit of PI3K co-immunoprecipitated with anti-ErbB3 (Figure 3A) and reciprocally ErbB3 co-immunoprecipitated with anti-p85 (Figure 3B). This confirmed that a protein complex between ErbB3 and p85 is indeed formed in these malignant cells. Even under serum-starvation conditions, both cell lines contained a complex of ErbB3 and the p85 subunit of PI3K, which was immunoprecipitated by either anti-ErbB3 (Figure 3A) or anti-p85 (Figure 3B). The ErbB3/p85 complex was especially prominent in H441 cells.

Next, we examined whether the ErbB3 present in the complex was indeed the activated ErbB3. The blot with the complex immunoprecipitated by anti-ErbB3 was probed with anti-phosphotyrosine antibody; strong bands indicated that the ErbB3 receptor in 441 and 1373 cells was tyrosine phosphorylated (Figure 3A). Immunoprecipitation with

---

**Fig. 1.** Immunoblots for ErbB receptors and ligands. Aliquots of total lysates with 50 μg protein from subconfluent H441, H1373 and HPL1D cultures were subjected to immunoblot analysis for different ErbB family receptors and for heregulin α (HRGα) and heregulin β (HRGβ). Results are representative of three assays.

**Fig. 2.** Constitutive phosphorylation (activation) of ErbB3 and ErbB2. (A) Total lysate (1 mg protein) from serum-starved HPL1D, H441 and H1373 cells were immunoprecipitated with 4 μg of anti-ErbB3 and immunoblotted initially with anti-ErbB3 (1:1000) and subsequently stripped and reblotted with anti-phosphotyrosine (1:1000). IP indicates the antibody that was used for immunoprecipitating the cell lysates, and IB indicates the antibody used for probing. (B) Immunoprecipitation of lysates (1 mg protein) with anti-phosphotyrosine was followed by probing with anti-ErbB3. (C) Total cell lysates containing 50 μg protein were immunoblotted with phospho-specific ErbB2 antibody. (D) Total lysate (1 mg protein) were immunoprecipitated with 2 μg anti-ErbB2, probed initially for ErbB2, then probed for anti-phosphotyrosine after stripping. All results are representative of three assays.
anti-phosphotyrosine followed by immunoblotting with anti-ErbB3 confirmed this observation (Figure 3C). The levels of activated ErbB3 in the p85 complex appear to be higher in the serum-starved cells than in those rapidly proliferating, again suggestive of operation of a vigorous autocrine loop.

**ErbB2 is a heterodimer partner for ErbB3 in H441 and H1373 cells**

In some breast cancer cells, HRG activates ErbB2 by mediating its direct binding to ErbB3. In these cells ErbB2 forms heterodimers with ErbB3 with particular avidity, and stimulates transphosphorylation events (38,39). An association between ErbB2 and ErbB3 was detected both in quiescent and serum-stimulated H441 and H1373 cells (Figure 4). Immunoprecipitates obtained with anti-ErbB3 contained ErbB2 as well as p85 (Figure 4A). Anti-ErbB2 immunoprecipitate included ErbB3 and p85 (Figure 4B). In confirmation, anti-p85 immunoprecipitates included ErbB3 and ErbB2 as well (data not shown).

**Serum augments ErbB3/p85 complex and activates the PI3K/Akt pathway in human lung adenocarcinoma cells**

The complex of ErbB3 and the p85 subunit of PI3K was greatly increased by serum stimulation of starved H441 and H1373 cells (Figure 5A). Emerging evidence implicates the PI3K/Akt pathway as a major driver of proliferation in a number of normal and cancerous epithelial cells (44–47), including those of lung (48–50). Therefore, we examined the level of activated Akt, a downstream target of PI3K, in serum-stimulated H441 and H1373 cells using a phospho-serine-specific anti-Akt antibody. Phosphorylated Akt was detected in serum-starved cells, and treatment with serum led to an ~4-fold (H1373) to 10-fold (H441) increase in activated phosphorylated Akt in these cells (Figure 5B). Time course experiments indicated that the phosphorylation of Akt in H1373 cells increased within 10 min of stimulation and remained active at least up to 6 h, the longest time point tested (Figure 5C; 6 h time point not shown). Amounts of phosphorylated Akt were reduced by ~50–80% to constitutive levels or below when the specific LY294002 inhibitor was included (Figure 5B and C).

**The ErbB3/PI3K/Akt pathway is implicated in cell cycle progression and cell survival**

In order to assess the importance of PI3K pathway in the growth of these malignant lung cell lines, we studied the impact of the specific PI3K inhibitor, LY294002, on cell cycle progression by FACS analysis. Serum starvation of H441 and H1373 cells led to a 50–60% reduction in percentage S phase cells and a doubling of those in G0/G1 (Figure 6). Treatment of proliferating cells with the LY294002 for 24 or 48 h had a similar suppressive effect on proliferation (data not shown). This was studied further in cells serum-starved for 48 h, followed by re-stimulation with serum (Figure 6). Twenty hours after re-feeding of serum-starved cells, high percentages of H441 and H1373 cells had entered S + G2/M phases, 46% for H441 and 69% for H1373. This re-activation of the cell cycle was completely blocked by inclusion of LY294002, resulting in S + G2/M values less than those for serum-starved H441 cells (Figure 6A) and similar to those for serum-starved H1373 cells (Figure 6B). Thus, PI3K activity is essential for serum-induced cell cycle progression in these human lung adenocarcinoma cells.

Levels of apoptosis were low (0.5–1.1%) in these cell lines, as determined by FACS analysis. No consistent increases in these percentages occurred after treatment of the cells with LY294002 (data not shown).

**Serum treatment increases inactivating phosphorylation of glycogen synthase kinase 3β and levels of cyclin D1 in human lung adenocarcinoma cell lines**

Akt is known to inactivate glycogen synthase kinase 3β (GSK3β) through phosphorylation. GSK3β is a negative regulator of cyclin D1, targeting it for proteosomal degradation.
Fig. 4. Co-immunoprecipitation of ErbB3, p85 and ErbB2. (A) Total lysate (1 mg protein) from serum-starved and serum-stimulated H441 and H1373 cells were immunoprecipitated with anti-ErbB3, probed initially for ErbB2 and then probed in succession, after stripping for ErbB3 and p85. (B) Total lysates (1 mg protein) from serum-starved cells were immunoprecipitated with anti-ErbB2, probed initially for ErbB2, and then probed in succession, after stripping for ErbB3 and p85. Results are representative of three experiments.

Fig. 5. Akt in serum-stimulated human H441 and H1373 cells. Cells were serum-starved for 48 h and then serum-stimulated for 10 min. (A) Total cell lysates containing 1 mg of protein were immunoprecipitated with anti-p85 and immunoblotted with anti-ErbB3, then stripped and blotted with anti-p85. (B) Cells were pre-treated with 50 μM LY294002 for 30 min prior to serum stimulation for 30 min. Total cell lysates containing 50 μg protein were immunoblotted for pAkt and then after stripping for total Akt. (C) H1373 cells were collected at the times indicated and total cell lysate containing 50 μg protein were immunoblotted with pAkt or Akt. Results are representative of three experiments.
through phosphorylation. Serum treatment of H441 and H1373 cells indeed resulted in an increase in phosphorylated GSK3β within 30 min after serum stimulation (Figure 7A) and the levels of pGSK3β were reduced to constitutive levels or below when LY294002 inhibitor was included (Figure 7A).

In these human lung carcinoma cells levels of cyclin D1 were increased within 30 min of serum stimulation, reaching a maximum after 4 h (data not shown for 4 h). This serum-induced cyclin D1 up-regulation was inhibited by 60% in LY294002 treated cells (Figure 7B). These results indicate that the PI3K/Akt pathway may have stimulated the cell cycle via Akt inactivation of GSK3β, permitting increased stability and higher levels of cyclin D1.

**Anti-sense ErbB3 oligonucleotide reduces proliferation of human lung adenocarcinoma cell lines**

An anti-sense (AS) oligonucleotide specific for human ErbB3 was used to evaluate the effect of inhibiting ErbB3 in these human lung cancer cell lines. Scrambled and sense oligonucleotides were used as controls. Treatment of H441 and H1373 lung cancer cells with 1 μM ErbB3 AS oligonucleotide resulted in significant, 60–75% reduction in ErbB3 protein levels whereas control oligonucleotides had no significant effect (Figures 8A and 9). The expressions of ErbB2, p85 and Akt were not markedly altered, indicating that the effects of AS ErbB3 were sequence-specific and did not involve general toxicity (Figure 8A; ErbB2 data not shown). Co-immunoprecipitation experiments with the ErbB3 AS treated cell lysates indicated that the down-regulation of ErbB3 protein correlated with reduced p85 pull down (Figure 8B).

In order to assess growth-suppressive effects of the AS directly, the MTT assay for viable cells was used. The growth of cell lines H441 and H1373, which highly express ErbB3, was inhibited by ErbB3 AS treatment (Figure 9A). Compared with Lipofectin transfected or sense oligonucleotide treated cells, a highly significant reduction in the average number of cells was observed after treatment with 1 μM AS oligonucleotide, in both H441 and H1373 cells (Figure 9A). The decreases in average amount of ErbB3 protein are shown in Figure 9B. Both average cell numbers and ErbB3 protein levels were slightly, non-significantly lower in the scrambled antisense control compared with the sense control; this could be due to non-specific toxicity and/or retention of some affinity of the scrambled-antisense oligonucleotide for the mRNA. All of the data were combined for linear regression analysis (Figure 9C). This illustrated strikingly the clear significant association between the ErbB3 protein level and the cell numbers in H441 and H1373 ($P = 0.011$).

To confirm the specificity of the effects of ErbB3 AS oligonucleotide treatment on proliferation, we examined adenocarcinoma lines H23 and H1792, which express little or no ErbB3 protein (Figure 10A). RT–PCR experiments have indicated that the ErbB3 gene is present in H23 and H1792 cells, but full-length ErbB3 transcripts are lacking (data not shown). Proliferation of these cells was not affected by any of the oligonucleotide treatments (Figure 10B). These results strongly confirm that ErbB3 plays a major role in the proliferation of lung carcinoma cells expressing high levels of this protein.

**Discussion**

The present results provide possibly the first mechanistic evidence for a significant role for ErbB3 in human lung adenocarcinomas, and confirmation of the importance of the PI3K/Akt pathway in this type of cancer. We have found that ErbB3 was highly expressed in the majority of lung adenocarcinoma...
cell lines tested, in contrast to its absence from a non-transformed cell line derived from human lung peripheral epithelium. The results may indict ErbB3 over-expression in the genesis and maintenance of many lung adenocarcinomas. ErbB2 amplification is not common in lung cancer (51). It may be that over-expression of ErbB3 is the critical event leading to up-regulation of the ErbB pathway in many cases of this type of cancer.

The H23 and H1792 cell lines are examples of the minority of adenocarcinoma cell lines, which express very low ErbB3; ErbB2 was reported to be not expressed in H23 cells and ErbB2 anti-sense was without effect (52). We found that ErbB3 anti-sense did not inhibit H23 or H1792 growth, an important control confirming the specificity of the effect. Mitogenic advantage during neoplastic transformation evidently is provided by an alternate pathway in these cells. For future studies, these cell lines can be used as negative controls for study of the specificity of therapies targeting ErbB3.

The constitutive ErbB2 and ErbB3 phosphorylation and ErbB2/ErbB3/p85 complexes demonstrated here in the lung adenocarcinoma cells are of particular interest. The ErbB2/ErbB3 heterodimer is the most potent ErbB signaling complex in terms of in vitro growth and transformation (8,38), and can enhance drug resistance (53). This high potency may be related to the C-terminal sequences of ErbB3 which contain binding motifs for several downstream novel signaling molecule

---

**Fig. 7.** Inactivation of GSK3β and up-regulation of cyclin D1. Cells were serum-starved for 48 h and then serum-stimulated for 30 min. Cells were pre-treated with 50 μM LY294002 for 30 min prior to serum stimulation for 30 min. (A) Total cell lysates containing 50 μg protein were immunoblotted for total and pGSK3β. (B) Serum-stimulated cell lysates (50 μg protein) with and without LY294002 were immunoblotted for cyclin D1.

**Fig. 8.** ErbB3 protein down-regulation by ErbB3-specific AS oligonucleotide in human lung carcinoma cells. Cells were treated with 1 μM antisense (AS), scrambled antisense (SC) or sense (SE) oligonucleotides for 4 h in the presence of Lipofectin in OptiMEM media, followed by replacement with regular media. L: Lipofectin-only treated cells. (A) Thirty hours after transfection 20 μg of total protein were analysed for ErbB3, p85 and Akt protein expression. (B) Complex of ErbB3 and p85 in AS and control oligonucleotide treated H1373 and H441 cells as demonstrated by co-immunoprecipitation experiments. Results are representative of three experiments; quantitative results are shown in Figure 9.
candidates (8), and also to the low rate of internalization and characteristic recycling pathway of ErbB2-containing complexes, which prolong signal (54, 55). ErbB3 contains at least 8 SH2 binding tyr-x-x-met motifs that, when tyrosine phosphorylated, form the high affinity binding site for the regulatory domain p85 of PI3K. Co-expression of ErbB3 with activated ErbB2 greatly augmented the transforming capability of ErbB2 in genetically engineered Rat 1 fibroblast cell lines (56).

Fig. 9. Quantification of effects of ErbB3-specific AS oligonucleotides on proliferation and ErbB3 protein expression of lung carcinoma cell lines H441 and H1373. (A) Cell numbers following AS, SC or SE treatment are expressed as a percentage of the numbers of viable cells observed in H1373 and H441 cells compared with cells treated with Lipofectin alone. 5 x 10^3 cells were plated and transfected with 1 μM concentrations of oligonucleotides in the presence of Lipofectin. MTT assay was performed 72 h after transfection. The data for cell number are mean ± SE from three independent experiments. (B) Densitometric analysis of the effects of AS, SC or SE oligonucleotides on ErbB3 protein in H1373 and H441 cells. Levels of ErbB3 protein following AS, SC or S oligonucleotide treatment were analyzed by western blotting. Western blots were quantified by densitometry. The results shown are relative to the level of ErbB3 protein expression in Lipofectin control treated cultures. Data represent mean ± SE (n = 5 for H1373 and n = 4 for H441). (C) Regression analysis showing correlation of the level of ErbB3 protein with the cell number in H1373 and H441 cells. These parameters correlate with a high degree of statistical significance.
In the context of lung, HRG was shown to be a mitogen in human fetal lung epithelial cells that express both ErbB2 and ErbB3 (12) and also in rat neonatal distal lung airway epithelial cells (57). A 4-fold increase in ErbB2 and ErbB3 phosphorylation was noted when freshly isolated fetal human lung in serum free media was stimulated with Hrg-1 (12). Constitutive ErbB2/ErbB3 heterodimer has been reported in HRG-expressing, non-tumorigenic, TGFα-negative human bronchial epithelial cells E6TA, and in TGFα/ErbB2 overexpressing tumorigenic E6T cells (58). NRG-1(HRGα) induced cell proliferation in H441 cells and in H520 cells (a squamous lung carcinoma cell line) through activation of Janus kinase (JAK) and the STAT pathway (59). ErbB2-specific antibody, 2C4 treatment, which inhibits ErbB2 heterodimerization, blocked NRG1 induced JAK phosphorylation, further indicating the importance of ErbB3 and ErbB2 heterodimer in HRG-induced proliferation of these cells.

Strong in vivo evidence for the importance of ErbB3 and cooperative ErbB3 and ErbB2 signaling in lung tumors was provided by a new transgenic ErbB3 mouse lung cancer model. Lung tumors developed by these transgenic mice, the majority of which were adenocarcinomas, had not only elevated transgenic human ErbB3 expression but also high levels of endogenous ErbB2. Double transgenic rat ErbB2/human ErbB3 mice developed lung tumors with shorter latency (37). Thus, both cell culture and animal model studies are fully consistent with our findings pointing to a central role for ErbB3 in driving proliferation in lung cancer cells.

Our data indicate that PI3K, activated by its regulatory subunit p85, and Akt transmit the cell proliferation signal from the ErbB2/ErbB3 complex. These results support studies of primary human lung cancers indicating the importance of PI3K and Akt. Over-expression of PI3K p110 mRNA by northern blotting and up-regulation of p85 and p110 protein detected by immunohistochemistry correlated with poor differentiation in NSCLC lung tumors; p85 was up-regulated in 77% of primary lung carcinomas (60). Akt is itself a proto-oncogene that can transform lung epithelial cells (61).

Fetal calf serum potently activated the PI3K pathway in SCLC cells and was the most potent growth factor for SCLC.
cells compared with stem cell factor or insulin-like growth factor (50). The prolonged activation of Akt by serum (up to 6 h) reported for SCLC cell lines have been very similar to that reported here for lung adenocarcinoma cells. Our results confirm the importance of PI3K/Akt pathway in the proliferation of transformed lung epithelial cells.

Published results with cultured lung cells of various types are consistent. Hepatocyte growth factor induced proliferation in mink lung epithelial cells through the PI3K pathway (49). Serum, stem cell factor and insulin like growth factor 1 activated the PI3K pathway and promoted proliferation in primary bronchial epithelial cells, MRC-5 pulmonary fibroblast and SCLC cell lines (50). LY 294002 inhibited the proliferation induced by these growth factors and by serum in a dose-dependent manner (50). Constitutively high PI3K levels mediated anchorage independent proliferation through Akt and a P70S6 kinase-dependent pathway in small cell lung cancer cell lines (48). Inhibition of the PI3K pathway blocked SCLC growth in liquid cultures and colony formation in semisolid medium (62). An inducible constitutively active Akt was utilized to distinguish the proliferative from anti-proliferative effects of the PI3K/Akt pathway in SCLC cells and indicated that a threshold level of PI3K/Akt pathway activation is essential for the proliferation of SCLC cells (50). Expression of Akt1 antisense oligonucleotide inhibited the proliferation of a lung cancer cell line, H460, and blocked its ability to grow in soft agar (63). In addition to cell proliferation, the PI3K/Akt pathway also contributed to cell survival and therapeutic resistance in the SCLC cells.

Constitutive Akt activity was found in 16 of 17 of NSCLC cell lines and was associated with cell survival and therapeutic resistance (64). Our results suggest that complex of p85 with ErbB3 may contribute to these effects. Dominant-negative Akt was utilized to confirm the specificity of the inhibitory effect of LY294002 (50,64). Apoptosis resulting from Akt inhibition was observed to varying degrees in different cell lines. Treatment of LY294002 did not induce apoptosis in four lung adenocarcinoma cell lines even though it efficiently blocked the Akt activation (65). We observed no marked increase in apoptosis in adenocarcinoma cells after treatment with LY294002, consistent with the known lower frequency of apoptosis in this cancer type (66).

Constitutive and growth factor-dependent PI3K recruitment by tyrosine phosphorylated ErbB3 has also been observed in other types of cancer and in fact was first evidenced in breast cancer cells (38,67,68). Since then, the cancer related interactions of ErbB family members and agonists with PI3K-controlled pathways have been extensively studied in mammary cells, and the results have been very similar to those reported here for lung carcinoma cells. A complex of ErbB2, ErbB3 and p85 was reported in a breast carcinoma cell line (39). Constitutive ErbB3/p85 complexes either due to ligand independent over-expression of ErbB2 or HRG/ErbB3 autocrine loop have been reported in a number of ErbB3 expressing breast cancer cells (38,39,67,68). Intracellular retention of ErbB2 (69) or transfection with dominant-negative ErbB3 (42) led to dramatic decrease in ErbB3 phosphorylation, its association with PI3K, and proliferation in breast cancer cells. Treatment with Her2 blocking antibody, herceptin, blocked the constitutive phosphorylation of ErbB3, disrupted the ErbB3/p85 association, and inhibited cell proliferation in BT-474 cells (70). An anti-ErbB3 receptor antibody inhibited growth factor independent growth and also blocked the HRG1β induced proliferation in ovarian tumor cells (41).

Our results indicate that cyclin D1 could be an important nuclear target of the ErbB3 activated PI3K pathway in lung adenocarcinoma. Elevated cyclin D1 expression was observed in human lung carcinomas (71) and associated with poor prognosis in some studies (72,73). Cyclin D levels correlated with growth in mouse lung cancer lines (74). Stable introduction of anti-sense cyclin D1 into H441 and A549, another adenocarcinoma cell line which also expresses ErbB3, resulted in marked reduction in their proliferation rate (75). Although cyclin D1 levels are regulated in a variety of ways in cells, altered protein stability has been repeatedly demonstrated to be a critical event in lung epithelial and cancer cells (76–78). Our observation of an increase in the phosphorylation-associated inactivation of GSK3β, which would lead to increased cyclin D1 stability, is consistent with such a mechanism. Doxycyclin-regulated expression of constitutively active Akt induced phosphorylation of GSK3β and stimulated growth in SCLC cells in the absence of growth factors (50).

In summary, these findings implicate ErbB3 in lung adenocarcinoma, via an activatable complex involving PI3K and leading, downstream, to activation of Akt, inactivation of GSK3β, increase in cyclin D1, and cell cycle progression. Thus, cancer cells that over-express ErbB3 are dependent on an ErbB3-related pathway for proliferation. This discovery could have potential for lung adenocarcinoma prevention, intervention and therapy.

Acknowledgements

We thank Janet Fields for cell culture, Refika B.Turnier and Louise R.Finch for flow cytometric analysis and Dr Y.-H.Shiao for critical comments on this manuscript. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. This project has been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO1-CO-12400.

References

ErbB3 in lung adenocarcinoma


Received March 20, 2003; revised June 17, 2003; accepted July 18, 2003