Abstract

ErbB receptor tyrosine kinases can transit to nuclei in tumor cells, where they have been shown to regulate gene expression as components of transcriptional complexes. Quantitative analysis of a human bladder cancer tissue microarray identified nuclear epidermal growth factor receptor (EGFR) in tumor cells and also showed an increased frequency of this histologic feature in cancer relative to normal tissues. This observation suggests a potential role for nuclear EGFR in bladder cancer. We confirmed that EGFR could be induced to transit to nuclei in cultured human bladder cancer cells in response to the urothelial cell growth factor and EGFR ligand heparin-binding EGF-like growth factor (HB-EGF). Mass spectrometric analysis of EGFR immune complexes from a transitional carcinoma cell line (TCCSUP) identified the phosphoinositide kinase, PIKfyve, as a potential component of the EGFR trafficking mechanism. RNA silencing indicated that PIKfyve is a mediator of HB-EGF–stimulated EGFR nuclear trafficking, EGFR binding to the cyclin D1 promoter, and cell cycle progression. These results identify a novel mediator of the EGFR transcription function and further suggest that nuclear EGFR and the lipid kinase PIKfyve may play a role in bladder oncogenesis. [Cancer Res 2007;67(19):9229–37]

Introduction

The epidermal growth factor receptor (EGFR) tyrosine kinase (EGFR/ErbB1/HER1) is a signaling protein that plays a prominent role in the maintenance of epithelial tissues and in malignant transformation at many organ sites (1), with the result that the EGFR is now a major focus of targeted pharmacotherapy (2). Pharmacologic inhibitors of the EGFR are in clinical use for several malignancies, including non–small cell lung cancer and metastatic colon cancer, often in combination with other agents (3). Increased expression of the EGFR is associated with disease progression in transitional cell carcinoma (TCC) of the bladder and is an independent predictor of tumor stage and disease-specific mortality in this disease (4, 5). Activation of the EGFR occurs in response to binding of the extracellular domain by one or more soluble proteins containing an EGF-like motif. Expression of transforming growth factor-α (TGF-α), an EGFR ligand, was shown to correlate with recurrence of superficial bladder cancer (6).

Another EGFR ligand, heparin-binding EGF-like growth factor (HB-EGF), is an autocrine urothelial cell mitogen that is synthesized normally by bladder urothelium and smooth muscle cells (7, 8). HB-EGF is up-regulated in bladder smooth muscle cells in vivo in response to hypertrophic stimuli (9), suggesting a physiologic role for HB-EGF/EGFR signaling in the urinary tract.

Several growth factors and growth factor receptors have been reported to localize to noncanonical subcellular compartments (10–12). Fibroblast growth factor (FGF)-2 accumulating in the nucleus was implicated in regulation of tumor cell survival and metastatic potential in vivo (13, 14). The EGF-like growth factors HB-EGF (15, 16), TGF-α, and amphiregulin have all been reported to accumulate in nuclei in several cell types. The HB-EGF precursor was reported by our laboratory to localize to tumor cell nuclei in human TCC in a manner that positively correlated with disease progression (15). This result was recently confirmed independently by another group in a larger bladder cancer series (5). Nuclear-localized HB-EGF in TCC was also shown to be mobilized by oxidative stress into an autocrine loop involving transport of the growth factor from the nucleus to the cell surface followed by regulated ligand shedding and EGFR activation (16).

EGFR and related receptor tyrosine kinases (RTK), ErbB2, ErbB3, and ErbB4, have similarly been found in nuclei, where these proteins have been shown to do roles distinct from their function as plasma membrane signal transducers (10, 17–20). This noncanonical signaling function of RTKs is still poorly understood. Nuclear EGFR has been detected in hepatocytes in regenerating liver (21), and nuclear ErbB4 was recently identified as a component of transcription complexes involved in astrocyte differentiation in the mouse (22). Expression levels of nuclear EGFR were recently reported to correlate with the cell proliferation markers cyclin D1 and Ki-67 in a large breast cancer series (23), in which the extent of localization of EGFR to tumor cell nuclei was inversely correlated with patient survival. EGFR has been shown to contain a functional nuclear localization sequence within the juxtamembrane region (24) and to act as a transcriptional coactivator by participating in the formation of a transcription complex at the cyclin D1 (17) and inducible nitric oxide synthase (iNOS) promoters (18), resulting in the activation of gene expression from these regulatory domains.

When ErbB receptors translocate to nuclei, they likely associate with accessory proteins specialized for receptor transit, stability, or assembly into functional chromatin complexes. In its nuclear regulatory role, ErbB4 has been shown to complex with the mitogen-activated protein kinase (MAPK) kinase kinase–interacting protein TAB2 and the corepressor N-CoR (22, 25). However, the precise regulatory mechanisms of EGFR/ErbB receptor trafficking to nuclei, and the manner in which RTKs are stabilized and turned...
over in nuclei, are still poorly understood. To date, a direct mediator of EGFR nuclear trafficking from the cytosol to the nucleus has not been identified.

In this study, we present evidence for a role for nuclear EGFR in human bladder cancer by providing the first identification of nuclear EGFR in patient-derived tumor tissues. In addition, we have applied a mass spectrometric approach toward analysis of EGFR translocation complexes in TCC cells. Here, we report the identification of the lipid kinase PIKfyve as a novel regulator of EGFR translocate to nuclei.

Materials and Methods

Immunostaining and automated quantitative immunohistochemical analysis of human tissues. A human tissue microarray (TMA) consisting of normal bladder tissues (n = 3) and bladder cancer tissues (n = 18) from Brigham and Women’s Hospital (Boston, MA) was stained with an anti-mouse EGFR antibody (Biosource, Inc.) at a dilution of 1:50. Sections (4 μm) from the TMA paraffin block were transferred to an adhesive-coated slide system. Standard indirect immunoperoxidase procedures were used for immunohistochemistry after antigen retrieval in citrate buffer (pH 6.0) as described (26). Nuclear localization of EGFR was analyzed quantitatively using the automated quantitative analysis (AQUA) system (27). TMA sections were processed for immunofluorescence using a rabbit anti-cytokeratin polyclonal antibody (1:500 dilution; WWS, DAKO Corp.) to localize epithelial cells and the cytosolic compartment. An anti-mouse EGFR (Biosource), used at a dilution of 1:50, was applied to the sections simultaneously. Cytokeratin was visualized using a secondary anti-rabbit FITC-conjugated antibody. EGFR was detected using Cy5-tyramide (NEN Life Science Products) as described (27). 4,6-Diamidino-2-phenylindole (DAPI) was used to identify the nuclear compartment. Using this approach, subcellular compartments were identified based on molecular colocalization. The cytokeratin compartment corresponds to all epithelial cells in the 0.6-mm TMA spot. The tumor mask was determined by gaging the pixels in this image, in which an intensity threshold was set by visual inspection, and each pixel was recorded as “on” (tumor) or “off” (nontumor) by the software based on the threshold. The DAPI image, which was used to identify the nuclei, was subjected to a rapid exponential subtraction algorithm that improves signal to noise ratio by subtracting the out-of-focus image from the in-focus image. DAPI is the area defined as cell nuclei. Images of the TMA core sections were captured with an Olympus AX51 microscope and analyzed with the AQUA software (IPLabs, Scanalytics, Inc.). A detailed description of this method has been described (28). Commercially available software Statistical Package for the Social Sciences 11.1 (SPSS, Inc.) was used for statistical analysis.

Indirect immunofluorescence microscopy of cultured cells. Cells seeded at low density in chamber slides were treated with HB-EGF, fixed with 3% paraformaldehyde, and incubated with various antibodies in 1% iodoacetamide, respectively. Gel bands were dehydrated in acetonitrile on April 15, 2017. © 2007 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from www.aacrjournals.org on April 15, 2017. © 2007 American Association for Cancer Research.

CAGCAUAUCUU (Dharmacon). The siPIKfyve-l contains sense sequence GAUAUGGAGUUGGCAAUAC and antisense sequence 5′-P UGAUCCUGAAUCCAUU. The siPIKfyve-2 contains sense sequence GGAUAUCUUCCUCUGCAGAAU and antisense sequence 5′-P UUUGGAGCAGGAGAGUUCUU. The siPIKfyve-3 contains sense sequence UGAAGAGGUGAGC-GAUAAUU and antisense sequence 5′-P AUAAUGUCACCCUACUU. The siCONTROL nontargeting siRNA was obtained from Dharmacon.

Chromatin immunoprecipitation assay. Cells were serum starved for 16 h and stimulated with HB-EGF (100 ng/mL). At the indicated time points, formaldehyde (1% final concentration) was added for 10 min to cross-link proteins to DNA. Cells were washed with ice-cold PBS and resuspended in 500 μL of hypotonic buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L KCl, 1 mmol/L DTT] followed by passage through a 25-gauge needle (20 needles). Nuclear pellets were resuspended in 200 μL SDS lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0), protease inhibitors]. After sonication, the supernatant was diluted 1:10 with immunoprecipitation buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP40]. Immunoprecipitation using anti-EGFR antibody was done and immunoprecipitates were washed with radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, 1.0% NP40], high-salt buffer [500 mmol/L NaCl, 1.0% NP40, 0.1% SDS, 50 mmol/L Tris-HCl (pH 8.0), LiCl buffer [250 mmol/L LiCl, 1.0% NP40, 0.3% sodium deoxycholate, 1 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0)], and TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA]. DNA was harvested by the following steps: incubation with RNase solution (50 mg/mL, 30 min, 37°C) and proteinase K/SDS solution (0.25% SDS, 250 mg/mL proteinase K, 37°C, overnight) and heating (65°C, 6 h), extraction with phenol/chloroform (1:1), and precipitation with ethanol. The cyclin D1 promoter in the immunoprecipitates was detected by PCR with sense primers (5′-GAGGGACTAATATTCTCCAGCAA-3′) and antisense primers (5′-TAAAGGGATTCAGCTTACG-3′; ref. 17).

Cell cycle analysis by fluorescence-activated cell sorting. Cells were serum starved overnight and stimulated with 100 ng/mL HB-EGF for the indicated times. After harvesting, cells were stained with propidium iodide and assessed by flow cytometry (16).

Immunoprecipitation and Western blot analysis. For whole lysates, proteins were extracted into lysis buffer [1% NP40, 50 mmol/L Tris (pH 7.4), 10 mmol/L NaCl, 1 mmol/L NaF, 5 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, COMPLETE protease inhibitor cocktail tablet (Roche)] and centrifuged at 12,000 × g for 15 min. Supernatants were applied to each immunoprecipitation after protein measurement. Each 1 μg of antibodies, including control IgG, was incubated (16 h, 4°C) with 500 μg protein samples followed by incubation with protein A/G beads for 1 h. Immunocomplexes were washed with modified lysis buffer thrice, subjected to SDS-PAGE, and transferred into nitrocellulose membranes. Target proteins were assessed by Western blot using specific antibodies. Cytoplasmic and nuclear fractionation and blotting were done as described (16).

Protein identification by mass spectrometry. Mass spectrometric analysis with EGFR immunoprecipitates from TCCSUP cells was done to search for binding partners of the translocating EGFR. The protein bands from native SDS-PAGE gels were reduced and alkylated by DTT and iodoacetamide, respectively. Gel bands were dehydrated in acetonitrile followed by trypsin digestion (500 ng/band) in NH4HCO3 buffer (50 μL/ band) for 16 h at 37°C. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis was done using a Voyager-DE STR mass spectrometer (Applied Biosystems). α-Cyano-4-hydroxy-cinnamic acid matrix (0.5 μL, saturated in 50% acetonitrile and 0.1% trifluoroacetic acid) was used for each sample (0.5 μL). In the reflector mode, 20,000 V of accelerating voltage and 200 ns of extraction delay time were applied under the acquisition mass range of 750 to 4,500 Da with 600 Da low mass gate. The ExPaSy Proteomics Tool6 and ProteinProspector7 were applied for peak

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6 http://www.expasy.org
7 http://prospector.ucsf.edu

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analysis and protein identification using one trypsin peptide (molecular weight = 842.5099) as the internal standard.

Statistical analysis. Data were compared using a paired Student’s t test. P values of <0.05 were considered significant.

Results

Nuclear localization of EGFR in bladder cancer cells and tissues. The discovery that HB-EGF localizes to nuclei in TCC, and that this subcellular feature can have functional consequences (5, 15, 16), led us to question whether the EGFR, the primary cognate receptor for HB-EGF, might also localize to nuclei in bladder cancer. To test this hypothesis, we analyzed EGFR expression and localization in a bladder cancer TMA consisting of 18 tumors and 3 normal tissues. Using standard immunohistochemistry, we identified sporadic nuclear localization of EGFR, which otherwise exhibited a predominantly membrane/cytosolic pattern (Fig. 1A). AQUA (27) of this TMA after immunostaining with antibodies against EGFR and keratin showed that nuclear EGFR was identifiable and quantifiable in this series of tissues (Fig. 1A and B). Nuclear EGFR levels were increased in bladder cancer relative to benign tissues (P = 0.003). Cytosolic EGFR was also detected and quantitated by the AQUA system; however, no correlation with disease status was observed (P = 0.12; Fig. 1B).

We next attempted to test whether EGFR could be detected in nuclei in TCCSUP bladder cancer cells, a cell line that previously was shown to accumulate HB-EGF in nuclei (15, 16). EGFR localization was initially assessed by subcellular fractionation and Western blot analysis. In these experiments, we were able to show that the intact (170 kDa) form of the EGFR was detectable in the nuclear fraction (Fig. 1C). No smaller fragments were seen in the blots, consistent with previous findings that intact EGFR can transit to nuclei (30). The specificity of the antibody detection result was shown by RNA interference. Liposome-mediated transfection of TCCSUP cells with control and anti-EGFR siRNA duplexes showed that the intensity of the EGFR immunoreactive signal in the nuclear subcellular fractions was reduced by the EGFR siRNA but not by the control siRNA (Fig. 1C). Consistent with published data (17), we showed association of the nuclear EGFR with the endogenous cyclin D1 promoter in a chromatin complex by chromatin immunoprecipitation (ChIP) using a specific probe.
anti-EGFR antibody (Fig. 1D), strongly suggesting that the protein does a similar role in cyclin D1 regulation in the bladder cancer cell background.

**Translocation of EGFR to the nucleus is followed by association of the receptor with the cyclin D1 promoter.**

Indirect immunofluorescence cell staining indicated that cell surface EGFR moved to the nucleus within 30 min after TCCSUP and 253J bladder cancer cells were treated with HB-EGF (Fig. 2A). A Western blot time course showed that the EGFR accumulated and declined in the nuclear fraction with similar kinetics (Fig. 2B). The specificity of the antibody-derived signal detecting the trafficking event was confirmed by RNA interference (Fig. 2B). These results suggest that nuclear EGFR is degraded in TCCSUP cells on HB-EGF treatment, consistent with previous observations showing proteasome- and ubiquitin-mediated EGFR degradation following receptor activation and clearance from the cell surface (31, 32). The cytoplasmic and nuclear fractions used in this and subsequent experiments showed enrichment for the cytoplasmic marker, β-tubulin, in the cytoplasmic fraction and the nuclear marker, lamin, in the nuclear fraction, with no detectable cross-contamination.

To assess the potential function of nuclear EGFR in bladder cancer cells, we attempted to follow EGFR association with the cyclin D1 promoter kinetically following receptor activation with HB-EGF. The expression level of cyclin D1 has been positively correlated with high proliferation rate in tumor cells (33, 34). Treatment of TCCSUP cells with HB-EGF enhanced the association of the EGFR with the cyclin D1 promoter in a dose- and time-dependent manner as assessed by ChIP (Fig. 2C). The effect of HB-EGF on association of EGFR with the cyclin D1 promoter reached a maximum level at 100 ng/mL after 30 min of incubation with the growth factor. Incorporation of EGFR into the chromatin complex was inhibited by EGFR siRNA but not by control siRNA, verifying the authenticity of the ChIP result (Fig. 2C).

**EGFR signaling pathways involved in EGFR transit to nuclei.**

As an independent confirmation of the effect of EGFR activation on EGFR nuclear trafficking and association of the receptor with the cyclin D1 promoter, we attempted to confirm dependence of these events on EGFR pathway activation. Immunofluorescence staining using a specific phosphorylated EGFR antibody showed that nuclear EGFR is phosphorylated in response to HB-EGF (Fig. 3A). Treatment of TCCSUP cells with either one of two different pharmacologic inhibitors of the EGFR kinase activity (AG1478 and PD153035) inhibited HB-EGF–stimulated nuclear translocation of EGFR (Fig. 3B). In addition, AG1478 suppressed complex formation of the EGFR with the cyclin D1 promoter in a dose-dependent manner as assessed by ChIP (Fig. 3B). A time course experiment showed that HB-EGF induced increased expression of cyclin D1 (Fig. 3C). We showed in a published study that HB-EGF increases the rate of cell cycle transit in an EGFR kinase-dependent manner in TCCSUP cells (data not shown; ref. 16). These data verify that EGFR activation is sufficient to induce EGFR transit to nuclei and association of the receptor with chromatin complexes, leading to enhancement of cell proliferation.

**Figure 2.** EGFR nuclear trafficking stimulated by HB-EGF in bladder cancer cells. A, immunofluorescent localization of EGFR in response to 100 ng/mL HB-EGF for 30 min (green, EGFR; blue, nucleus) in TCCSUP and 253J bladder cancer cells. B, time-dependent localization of EGFR to nuclear fractions in response to HB-EGF (100 ng/mL, 30 min). EGFR siRNA reduced EGFR levels substantially. C, time- and dose-dependent ChIP experiments were done using anti-EGFR antibody. Increased association of nuclear EGFR and the cyclin D1 promoter region following HB-EGF treatment is shown. Down-regulation of EGFR by gene silencing suppressed the association of EGFR with the cyclin D1 promoter.

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Consistent with this interpretation, when TCCSUP cells were pretreated with inhibitors of signal transduction pathways known to be downstream from the EGFR [LY294002, phosphatidylinositol 3-kinase (PI3K) inhibitor; PD98059, MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor] before addition of HB-EGF, EGFR trafficking to the nucleus was substantially suppressed, based on both Western blot analysis of nuclear fractions and ChIP (Fig. 3D). Both the PI3K/Akt and ERK/MAPK pathways were activated in TCCSUP cells in response to HB-EGF (Fig. 3D).

TCCSUP cells engineered to stably express the membrane-anchored, precursor form of HB-EGF (proHB-EGF) were also analyzed (15, 16). proHB-EGF transfectant cells contain EGFR on plasma membrane and nucleus under steady-state conditions (Fig. 4A). Membrane EGFR was lost by treatment with H2O2, a source of reactive oxygen species that promotes cleavage and secretion of membrane-associated proHB-EGF. Western blot analysis confirmed that EGFR accumulated in nuclei in response to H2O2 (Fig. 4B), suggesting that membrane EGFR trafficked to nucleus. Treatment of these cells with other known inducers of proteolytic cleavage of proHB-EGF and liberation of soluble HB-EGF [the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (TPA), and ionomycin] also induced EGFR accumulation in the nucleus (Fig. 4B). These data indicate that nuclear trafficking of the EGFR occurs following activation of the receptor by multiple routes.

Identification of PIKfyve as a binding partner of cytoplasmic EGFR. The mechanism that mediates transit of the EGFR across the complex vesicular trafficking network in the cytoplasmic space during translocation of the receptor to the nucleus is unknown. We sought to approach this question by identifying proteins that bind EGFR during the translocation process. Whole-cell lysates from TCCSUP cells were subjected to immunoprecipitation with a monospecific anti-EGFR antibody as described in Materials and Methods. Mass spectrometric analysis of immunoprecipitated proteins was then done. These experiments identified a phosphoinositide kinase, PIKfyve, as a member of the cytoplasmic EGFR immunocomplex in five independent trials. PIKfyve synthesizes phosphatidylinositol 3,5-bisphosphate (35, 36) and has been linked to endosomal dynamics and intracellular trafficking (35, 37–41), suggesting the hypothesis that PIKfyve is a direct mediator of EGFR transit to nuclei.

To validate the association of EGFR with PIKfyve in TCCSUP cells, whole-cell lysates were subjected to coimmunoprecipitation followed by Western blot. The data showed that endogenous EGFR associated with endogenous PIKfyve (Fig. 5A), When TCCSUP cells were treated with HB-EGF, and EGFR complexes were isolated with anti-EGFR antibody, the binding of EGFR and PIKfyve increased ~10-fold compared with the unstimulated condition. Consistent with previous reports about PIKfyve, the protein was detected exclusively in the cytoplasm, not nuclei, by immunofluorescent cell staining (Fig. 5B).

Enforced expression of PIKfyve was done to examine the effect on EGFR trafficking to nuclei. Transfection of wild-type PIKfyve

Figure 3. Signaling pathways required for EGFR nuclear trafficking in TCCSUP cells treated with HB-EGF. A, immunofluorescence micrographs showing active (phosphorylated) EGFR (p-EGFR) in the nucleus. Green, phosphorylated EGFR; blue, nucleus. B, left, effect of EGFR kinase inhibitors [AG1478 (AG) and PD153035 (PD)] on EGFR trafficking under conditions where cells were treated with HB-EGF for 30 min; right, effect of the EGFR inhibitor AG1478 on assembly of the nuclear EGFR/cyclin D1 promoter complex. C, Western blot analysis was done and showed that HB-EGF treatment increased expression of cyclin D1. SF, serum-free control. D, effect of PI3K [LY294002 (LY)] and MEK [PD98059 (PD)] inhibitors on nuclear trafficking of EGFR. Top, nuclear extracts were subjected to Western blot analysis after 30 min of HB-EGF treatment in the absence or presence of inhibitors; bottom, ChIP analysis was done under the same experimental conditions as above. Right, both PI3K and ERK/MAPK signal pathways were activated by HB-EGF; p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt.
increased the level of nuclear-resident EGFR (Fig. 5C), whereas EGFR translocation to nuclei was significantly inhibited in transfectants with a dominant-interfering, kinase-dead PIKfyve mutant, PIKfyveK1831E (Fig. 5D; ref. 29). These data suggest that PIKfyve plays a role specifically in the trafficking mechanism. Binding of EGFR and PIKfyve was not significantly affected in PIKfyveK1831E-transfected cells (data not shown).

To further investigate the regulatory role of PIKfyve, gene silencing experiments were done using three different PIKfyve siRNAs as well as pooled siRNA PIKfyve oligos (Fig. 6A and B). Nuclear accumulation of EGFR was inhibited significantly when expression levels of PIKfyve were knocked down by siRNAs differentially targeting PIKfyve (Fig. 6A). No effect on levels of PIKfyve and nuclear EGFR was observed using two sets of control oligos (Fig. 6A). Cell cytometry analysis showed cell cycle transit was arrested at the G0-G1 phase when 70% to 80% of endogenous PIKfyve in TCCSUP cells was down-regulated by RNA interference (Fig. 6B). As assessed by immunofluorescent cell staining, HB-EGF enhanced translocation of EGFR from the cell surface to the nucleus, and knockdown of PIKfyve protein expression significantly attenuated this effect (Fig. 6C). ChIP analysis showed that silencing PIKfyve RNA also reduced complex formation between EGFR and the cyclin D1 promoter under conditions of log-phase growth as well as under HB-EGF–stimulated conditions (Fig. 6D). We conclude from these data that transient depletion of PIKfyve significantly reduced the effects elicited by HB-EGF on cell cycle transit, EGFR relocalization to nuclei, and complex formation between EGFR and chromatin.

Taken together, our findings indicate that nuclear EGFR plays a significant role in cell cycle progression in bladder cancer cells and that PIKfyve is an essential mediator of EGFR transit to nuclei and association of the receptor with chromatin.

**Discussion**

In this study, we provide the first evidence from human tissue that EGFR/HER1 accumulates in tumor cell nuclei in TCC and we provide evidence that nuclear EGFR plays a role in bladder cancer cell proliferation by identifying a novel mediator of the EGFR nuclear trafficking mechanism and showing that this protein is involved in cell cycle progression. Quantitative analysis of EGFR localization patterns in a human bladder cancer TMA verified the presence of EGFR in tumor cell nuclei and identified an apparently significant quantitative increase in nuclear EGFR associated with TCC in comparison with benign bladder tissue. Further analysis is necessary to assess the possibility that nuclear EGFR is an independent prognostic indicator capable of providing information of clinical value. At least one previous study that confirmed the
presence of nuclear HB-EGF in TCC specimens, originally reported by our group (15, 16), did not find significant nuclear EGFR (5). Although in our study we did find nuclear EGFR in bladder cancer tissues using conventional immunohistochemical staining, our study benefited from the use of AQUA, a highly sensitive and quantitative system for localizing immunoreactive signal to subcellular compartments in tissue. Nuclear EGFR has been reported in human breast cancers and in normal tissues, including regenerating liver (21).

We also found that nuclear EGFR could be shown in human bladder cancer cells in culture. We showed that receptor activation by the cognate EGFR ligand, HB-EGF, stimulates translocation of full-length EGFR to the nucleus in TCCSUP bladder cancer cells and that this results in binding of the receptor to chromatin within 30 min. The kinetics of nuclear transit we observed are consistent with the kinetics reported by other groups for nuclear translocation of the EGFR (17, 18). Notably, the rapid translocation of EGFR seems distinct from the slower translocation process seen with ErbB4, where proteolytic cleavage of the intracellular domain of the receptor precedes translocation of the cleaved fragment to active chromatin. In the present study, we also identified the lipid kinase PIKfyve as a component of an EGFR cytosolic trafficking complex. Down-regulation of PIKfyve by RNA interference inhibited EGFR trafficking to nuclei, association of the receptor with the endogenous cyclin D1 promoter shown by ChIP. Top, log phase growth; bottom, HB-EGF–stimulated conditions.

Ligand activation of the EGFR promotes numerous downstream signal transduction processes, leading to cell proliferation, resistance to apoptosis, aspects of development, and migration (42, 43). Modulation of receptor activity through endocytosis, degradation, and vesicular trafficking regulates the downstream targets of EGFR. The physiologic roles for nuclear trafficking of RTKs, including EGFR, FGFR receptor, and other receptor families, are currently under intense scrutiny (10, 13, 17, 44–46). Vesicular and other trafficking devices, such as involvement of the exportin CRM1, are likely to be important elements of the regulatory mechanisms of nuclear-localized RTKs (47–49). EGFR has been shown to colocalize and interact with the nuclear pore protein Nup358 as well as the nuclear importing cofactors importins α1/β1 (49). Nuclear EGFR also interacts with signal transducers and activators of transcription 3, leading to transcriptional activation.
of iNOS (18). Current evidence suggests that RTKs from several protein families use similar strategies for transit to nuclei. Despite some information about likely candidates for cofactors in the translocation mechanism, nothing has been published previously about the mechanism whereby the ligand-activated EGFR in transit to the nucleus navigates the cytosolic vesicular compartments.

Our finding that PIKfyve is a facilitator of EGFR nuclear trafficking has shed light on this question. PIKfyve is the mammalian orthologue of the yeast protein Fab1p. The principal in vivo activity of both enzymes is the production of phosphatidylinositol 3,5-bisphosphate from phosphatidylinositol 3-monophosphate (35, 36). PIKfyve has been implicated in vesicular trafficking in mammalian cells, and direct comparisons between the yeast and mammalian forms of the protein indicate that their predominant role seems to be conserved across a great evolutionary distance (39–41, 50). Perturbations in the PIKfyve function by protein silencing or expression of a dominant-negative, kinase-deficient form in human cells did not significantly affect internalization, recycling, or degradative sorting of the EGFR (40, 50). However, PIKfyve suppression did result in defective exit from early endosomes and trafficking between the early endosomal compartment and the trans-Golgi network (40, 41, 50). Our data are consistent with these findings and further implicate PIKfyve in the transport of EGFR from the cell surface through the cytoplasmic vesicular space to the nucleus. We generated multiple lines of evidence that support this conclusion. Endogenous PIKfyve coprecipitated with EGFR from TCCSUP cytosolic fractions, indicating that the two proteins are associated within a cytosolic multiprotein complex. siRNA knockdown of PIKfyve and/or enforced expression of the dominant-negative mutant PIKfyve<sup>K1831E</sup> inhibited multiple end points associated with EGFR nuclear trafficking, including progression through the cell cycle in response to EGFR activation. These data indicate that PIKfyve kinase activity is a requisite process for relocation of surface EGFR to the nucleus. In addition, our data show that intracellular signaling pathways, including PI3K and ERK/MAPK, are also involved in the HB-EGF–stimulated nuclear localization of EGFR. It remains to be elucidated how PIKfyve lipid products are related to these pathways in the context of EGFR nuclear transit.

In summary, we have identified a novel mediator of nuclear shuttling of the EGFR and have provided a new pathologic context for this phenomenon. Because EGFR can be overexpressed and is believed to be functionally involved in some bladder cancers (4–6), our findings indicate that EGFR likely plays a role in both conventional cell signaling initiated from the plasma membrane and also by direct intervention, at the level of chromatin, in transcriptional regulatory mechanisms of tumor cell proliferation in this disease. Because nuclear HB-EGF can be mobilized into an autocrine loop leading to EGFR activation (16), our conclusions are consistent with those of Kramer et al. (5) with respect to the point that disease progression in TCC may be related to the mode of HB-EGF signaling. We propose a model in which PIKfyve facilitates transit of EGFR from the cytosolic vesicular compartments to the nucleus, where EGFR rapidly and reversibly associates with chromatin to regulate gene expression. This study provides the first evidence of functional involvement of PIKfyve in mechanisms of TCC oncogenesis, suggesting that targeting this lipid kinase may be fruitful as a novel therapeutic strategy in circumstances where changes in EGFR or HB-EGF expression or subcellular localization are evident histologically.

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References

The Phosphoinositide Kinase PIKfyve Mediates Epidermal Growth Factor Receptor Trafficking to the Nucleus


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