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Novel phosphotyrosine targets of FGFR2IIIb signaling

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Abstract

In partnership with exclusively the epithelial FGFR2IIIb isotype and a structurally-specific heparan sulfate motif, stromal-derived FGF7 delivers both growth-promoting and growth-limiting differentiation signals to epithelial cells that promote cellular homeostasis between stromal and epithelial compartments. Intercompartmental homeostasis supported by FGF7/FGFR2IIIb is subverted in many solid epithelial tumors. The normally mesenchymal-derived homologue FGFR1 drives proliferation and a progressive tumor-associated phenotype when it appears ectopically in epithelial cells. In order to understand the mechanism underlying the unique biological effects of FGFR2IIIb, we developed an inducible FGFR2IIIb expression system that is specifically dependent on FGF7 for activation in an initially unresponsive cell line to avoid selection for only the growth-promoting aspects of FGFR2IIIb signaling. We then determined FGF7/FGFR2IIIb signaling-specific tyrosine phosphorylated proteins within 5 min after FGF7 stimulation by phosphopeptide immunoaffinity purification and nano-LC-MS/MS. The FGF7/FGFR2 pair caused tyrosine phosphorylation of multiple proteins that have been implicated in the growth stimulating activities of FGFR1 that included multi-substrate organizers FRS2 α and IRS4, ERK2 and phosphatases SHP2 and SHIP2. It uniquely phosphorylated CDK2 and phosphatase PTPN18 on sites involved in the attenuation of cell proliferation, and several factors that maintain nuclear-cytosolic relationships (emerin and LAP2), protein structure and other cellular fine structures as well as some proteins of unknown functions. Several of the FGF7/FGFR2IIIb-specific targets have been associated with maintenance of function and tumor suppression and disruption in tumors. In contrast, a number of pTyr substrates associated with FGF2/FGFR1 that are generally associated with intracellular Ca²⁺-phospholipid signaling, membrane and cytoskeletal plasticity, cell adhesion, migration and the tumorigenic phenotype were not observed with FGF7/FGFR2IIIb. Our findings provide specific downstream targets for dissection of causal relationships underlying the distinct role of FGF7/FGFR2IIIb signaling in epithelial cell homeostasis.

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Keywords

receptor tyrosine kinases; signaling networks; stromal-epithelial homeostasis; tumor suppression; tyrosine phosphatases

1. Introduction

Fibroblast growth factor (FGF) signaling plays essential and diverse roles in embryonic development and adult tissue homeostasis [1,2]. The ubiquitous transmembrane signaling system consists of 18 receptor-mediated FGF ligands, 4 transmembrane tyrosine kinase FGF receptors (FGFRs) with a variety of alternative splicing isoforms [1,3], and cofactors that include heparan sulfate (HS) chains, klothos and cadherin [4-8]. These factors combine to result in a wide range of context-specific endpoints that include both stimulation and inhibition of cell growth, cell death, cell migration, differentiation, and differentiated functions. Consequently aberrations in FGF signaling result in diverse tissue-specific defects and diseases [1,9-11].

The stoichiometry, structural relationships and order of assembly of participants in the oligomeric FGFR signaling complex are unclear as well as the mechanism of activation of the complex from extracellular to intracellular domain [12]. Two irreconcilable models have emerged from crystallographic analyses [13,14]. Both models, one comprised of 2 FGF:2 HS:2 FGFR [13] and another comprised of 2 FGF:1 HS:2 FGFR [14], propose diffusion-limited proximity of the three participants that controls the tyrosine phosphorylation-dependent trans-activation (derepression) between neighboring tyrosine kinases and recruitment and activation of downstream receptor substrates. FGF and HS by different mechanisms in the two models serve to stabilize interactions in the extracellular domain that bring kinases together in the intracellular domain. An alternative model that is conformationally activated through rearrangements that relieve restrictions on the enzyme-substrate relationship between two FGFR kinases that unifies both structural and biochemical analyses has also been proposed [1,5,15-17]. In this model a free FGF or a complex of FGF and HS oligosaccharide docks into a preformed inactive dimeric complex of FGFR and HS that is dependent on and restricted by the membrane context. HS chains in the ectodomain and membrane context limit enzyme-substrate proximity of the kinases. HS motifs and other cofactors control access and ability of free FGF or a complex of FGF-HS oligosaccharide to enter and activate the preexistent FGFR-HS complex by induction of conformational rearrangements transmitted from ectodomain to intracellular domain.

Phosphorylation of tyrosines in the intracellular sequence of FGFRs serves initially to change the conformation of the tyrosine-containing repressor domain that blocks access of external substrates to the active sites of the kinases [18-21]. In contrast to other receptor tyrosine kinases whose internal pTyr sites serve as docking sites for diverse signaling mediators for activation and signal relay [22,23], only one of the phosphotyrosines clearly recruits a functionally significant external signal-mediating substrate (PLC) by direct interaction [24]. Instead, pathway adaptors that other receptors recruit to diverse phosphotyrosine sites are largely assembled on sites on a single membrane-anchored adaptor, FRS2. Although phosphorylation state of FGFR affects the affinity of FRS2 that varies among different FGFR isoforms, FRS2 appears to associate with the intracellular juxtamembrane domain of FGFR through a non-phosphorylated sequence [25-27]. In the conformational model, conformational events in the extracellular domain of the oligomeric FGFR complex are transmitted to a largely preexistent membrane-proximal intracellular environment poised for activation via derepression by conformational rearrangements within the oligomeric complex. The communication of the membrane proximal ectodomain with membrane proximal signaling mediators in the

intracellular domain provide potential for combinations of different FGFR isotypes, FGFs and co-factors in the ectodomain to elicit different signaling outputs from the same preassembled complexes in the intracellular domain.

The exclusive expression of FGFR2IIIb is a hallmark of a variety of epithelial cells in multi-compartment parenchymal tissues [28-30]. FGFR2IIIb in partnership with a structurally specific HS octasaccharide motif, 7,8-S-OctaF7, is activated by FGF7 which is partitioned in the stroma [5,16]. The epithelial cell resident FGFR2IIIb mediates stromal-derived signals promoting net intercompartmental homeostasis including restraints on malignant progression of tumor cells [31-33]. This is in marked contrast to effects of FGFR1 when it appears ectopically in epithelial cells to drive proliferation and malignant transformation [34-39]. Ectopic expression of FGFR1 is a common property of tumor epithelial cells coincident with reduction in FGFR2IIIb expression [32,39]. How the two homologous FGFR isotypes exert such cell/tissue context-dependent and different endpoints is of considerable interest.

Maps of changes in tyrosine phosphorylated proteins (pTyr-proteins) facilitated by improvements in separation technologies and sensitivity of downstream mass spectrometric analysis potentially can provide clues to FGFR isotype-specific signaling [40]. A tyrosine phosphoproteomic profile of transiently overexpressed FGFR1 in the common cultured human embryonic kidney (HEK293) cell host for test of transfected genes on phenotype has been reported [41,42]. Cultured cells are plagued by intrinsically high basal levels of pTyr-proteins due to their importance in support of growth and survival. Moreover, the negative regulation of proximity of FGFR kinase subunits and thus FGF-dependence of the signaling complex is subverted by increasing levels of overexpression over the time. In addition most cultured cells constitutively express activated FGFR1 that is normally silent in quiescent cells (unpublished observation). In cells with high intrinsic background of phosphoproteins even after serum/stimuli withdrawal, differential labeling of basal and stimulated pTyr-proteins has been employed as one of the approaches to overcome these difficulties [42,43]. After failure to overcome these problems in epithelial cells in which FGFR2IIIb is resident and specifically stimulated by stromal-derived FGF7 such as in mouse keratinocyte (MK) and prostate epithelial cells (DT3) [16,32,33,44], we examined HEK293 cells as a naïve host to better understand potentially unique aspects of FGF7/FGFR2IIIb signaling, which exhibited undetectable tyrosine phosphorylation background after serum withdrawal. A repertoire of 22 tyrosine phosphopeptide sites that were completely dependent on stimulation of FGF7 within a 1 to 5 min period over a negligible background were identified by phosphopeptide immunoaffinity purification (IAP) followed by nano-LC nano-ESI MS/MS analysis. A subset of tyrosine-phosphorylated targets overlapped with those previously reported for FGFR1, but larger subset of targets generally associated with FGFR1 was not detected in the FGFR2 proteome. In addition a novel subset of heretofore unreported targets was observed in response to activation of the FGFR2IIIb complex. These differences provide clues to the specific cellular activities observed for epithelial FGFR2IIIb in maintenance of compartmental homeostasis between stroma and epithelium and its subversion during tumorigenesis.

2. Materials and Methods

2.1. Establishment of the Tet-inducible FGFR2IIIb-responsive System

The two-Ig loop isoform of rat FGFR2IIIb (FGFR2 β IIIb) starting from coding sequence MVSWGRF was inserted into the Tet-on mammalian expression vector pCDNA4/TO at the BamH 1 and Xba 1 sites. A 6xHis tag was introduced into the N-terminus immediately after the signal peptide sequence which is between Ala-21 and Arg-22 as predicated by the SignalP program. This was accomplished by PCR using two pairs of primers: 5'-GCGGATCCGCCACCATGGTCAGCTGGGGGCGC and 5'-(atggtgatggtgatgatg) GGGCCGGGCCAGGGACAA, and 5'-CCC(catcatcaccatcaccat)

TCCTTCAGTTTAGTTGAA and 5'-CACCGTGGAGGAATTTGC (the underlined sequence indicates a BamHI site, italics indicate the Kozak sequence and lower case indicates coding sequence in both reverse and forward directions for 6xHis). The correct sequence was confirmed by nucleotide sequencing.

The resulting 6xHis-FGFR2βIIIb-pCDNA4/TO construct was transfected into Tet-off T-Rex HEK293 cells using Lipofectamine 2000 to generate tet/on inducible cell lines. 6xHis-FGFR2βIIIb positive clones were selected by 300 µg/ml Zeocin for 15 days and verified by induction with 1 µg/ml tetracycline followed by analysis with both RT-PCR and Western blot analysis using anti-6xHis tag antibody (Sigma-Aldrich, MO). Positive clones exhibiting the highest level of 6xHis-FGFR2βIIIb protein expression in the presence of tetracycline, but with the least background of 6xHis-FGFR2βIIIb in the absence of tetracycline were selected for study (Fig. 1). Moreover, experimental cell lines were further selected for minimal background level tyrosine phosphorylation after short-term serum restriction.

2.2. Cell Culture, FGF7 Priming, Stimulation and Preparation of Proteome

Before tetracycline induction, tet/on cells were maintained in high-glucose DMEM medium supplemented with 10% tetracycline-free FBS and 100 µg/ml zeocin and 1 µg/ml blasticidin. Upon 80-90% confluence, cells were induced for 6xHis-FGFR2βIIIb expression by addition of 1 µg/ml tetracycline for 1 day. Thereafter, cells were primed by 10 ng/ml FGF7 and 1 µg/ml heparin overnight, and then starved in serum- and FGF7-free medium containing 1 µg/ml tetracycline for 4 to 6 hrs for reduction of backgrounds. After stimulation with 10 ng/ml FGF7 for 5 min, cells were immediately lysed in a 6 M urea-modified buffer of 20 mM HEPES, pH 7.0 containing 0.15 M NaCl, 1% NP-40, 2 mM sodium orthovaladate and 2 mM sodium fluoride on ice for 10 min. The control group transfected with vector alone or unstimulated 6xHis-FGFR2βIIIb-expressing cells was also prepared. Lysates were then sonicated and clarified by centrifugation at 12000 rpm at 4 °C for 10 min. Protein concentration was determined by the Bradford assay with Coomassie brilliant blue G-250 staining.

2.3. Preparation of Peptide Pools

40 mg proteomes from control and FGF-7-stimulated cells were adjusted to equal volume with lysis buffer. DTT prepared in 50 mM NH₄HCO₃ was added to a final concentration of 10 mM for reduction of disulfide bond at ambient temperature for 1 hr. The free -SH groups were then blocked by alkylation with 20 mM iodoacetamide for 1 hr in the dark. The proteomes were then diluted to 2 M urea by addition of 0.1 M NH₄HCO₃, digested by TPCK-treated trypsin at an enzyme to protein ratio of 1:100 overnight. The digestion was monitored by 12% SDS-PAGE for completeness.

The resulting peptide mixture was acidified by addition of TFA to a final concentration of 0.2%, and the precipitate was removed by centrifugation at 12000 g for 10 min. Peptides in the clear supernatant were extracted and desalted by 3 Sep-Pak reverse phase C18 cartridges (500 mg C18 stationary phase each). Briefly, a dry cartridge was wetted with 50% acetonitrile (ACN), 0.1% TFA and equilibrated by 0.1% TFA. Clear supernatant containing peptides from about 5-10 mg protein was loaded on each cartridge, which was then washed by 3 ml 0.1% TFA 4 times. Peptides were eluted sequentially by 18%, 40% and 80% ACN solvent containing 0.1% TFA. The resulting fractions were then dried by centrifugal evaporation.

2.4. Immunoaffinity Purification of Phosphopeptides

A slurry of 150 µl of protein G Sepharose beads was washed once with 0.5 ml 1 × PBS, and twice with 0.5 ml Immunoaffinity Purification (IAP) buffer comprised of 20 mM Tris-HCl (pH 7.2), 10 mM sodium phosphate, 50 mM NaCl, 0.01% NaN₃ and 200 µg anti-phosphotyrosine antibody P-Tyr-100 (Cell Signaling Technology, MA) was mixed with

protein G Sepharose, incubated gently by slow rotation for 6 hrs at 4°C for noncovalent coupling of antibody, and then washed as described above.

The dried peptides were reconstituted together in 3 ml IAP buffer. After removal of precipitate, the mixture was incubated overnight with the protein G Sepharose complexed with P-Tyr-100 antibody, by slow rotation at 4°C. The resulting beads were washed with 0.4 ml IAP buffer and 2 mM NH_4HCO_3 for three times each. The bound peptides were eluted by incubation with 100 μl 0.1% TFA for 5 min for three times and 50% ACN, 0.1% TFA twice. Eluants were combined and briefly dried by centrifugal evaporation.

2.5. Nano-LC nano-ESI MS/MS

The dried peptides were reconstituted in 0.1% aqueous formic acid (FA), injected into a capillary reverse phase (RP) column (7 cm $\times$ 75 μm) packed with Magic C18 (3 μm silica sphere, 100 Å pore size, Michrom Bioresources Inc. Auburn, CA) with a pressure injection cell device (NextAdvance Inc, NY). The column ends immediately with a pulled tip to 10 μm inner diameter in order to eliminate dead volume, which also served as the electrospray emitter to the MS inlet. The distal end of the column was connected to a solvent transfer line via a liquid conjunction called uncoated tip module (UTM) (New Objectives, MA) where high charge was applied for peptide ionization. The Agilent 1100 HPLC binary pumps were used to generate the mobile phase with ACN as the organic modifier with variable flow rates of 0.3 to 0.75 ml/min. Such flow rates were further reduced to 100 nl/min by online incorporation of an adjustable flow splitter (Upchurch Scientific, WA), which was then guided to the transfer line. The optimum variation of the flow rate was established by pilot trials to compensate for the pressure drop resulting from the viscosity change of the flowing mobile phase with varying concentration of acetonitrile in order to maintain a constant pressure (33 bar) over the nanoflow capillary RP column and thus maximal ESI spray stability. The column was washed with 2% acetonitrile (ACN), 0.1% FA, and developed by a linear gradient of ACN from 2% to 75% over 45 min, and isocratic elution by 95% ACN, 0.1% FA for 5 min. The column was then regenerated by 2% ACN and 0.1% FA.

The Esquire3000plus Ion Trap mass spectrometer (Bruker Daltonics, MA) was interfaced online with a nano-electrospray ionization (Nano-ESI) source (New Objective, MA), which houses the capillary RP column with its emitter tip 2 mm away from the MS inlet co-axially. The MS/MS data acquisition was controlled by EsquireControl (version 5.0). The capillary charge was set to 1550 volts via the liquid conjunction for ionizing peptides. The nitrogen drying gas flow was 3 l/min with a temperature of 160 °C. The Esquire3000plus was run in the online nano-ESI positive ion mode with an enhanced scan resolution of 5500 m/z/s. The mass spectrometer was calibrated with ES Tuning Mix with reference masses of 118.09, 322.05, 622.03, 922.01, 1521.97, 2121.93 and 2721.89 in positive ion mode (Agilent Technology, Santa Clara, CA). The helium gas was used for collision-induced dissociation (CID) tandem MS. The data-dependent auto-MS/MS mode was activated with a full scan range of 60-2400 m/z and a precursor scan range of 400-1800 m/z with an average of four spectra. The three most abundant precursor ions were monitored simultaneously from every MS survey scan and isolated with Maximum-Resolution-Scan mode with an isolation width of 4 m/z and then actively excluded after three spectra. The ion charge control target in ion trap was set to 50000 with a maximum accumulation time of 200 ms. The MS/MS fragmentation amplitude was set to 1.1 volts with a fragmentation time of 40 ms. Product ions scan was at an average of 6 spectra. Experiments were repeated at least three times with samples from independent preparations. Subsets of identified tyrosine-phosphorylated peptides and corresponding proteins in data-dependent mode were further verified by targeted MS/MS analysis.

2.6. Database Analysis

The MS/MS compound list was generated by Bruker Daltonics DataAnalysis™ (Version 3.0). The raw TIC data were extracted into Base Peak Chromatogram (BPC) with “All MS” as the filter and “Full Scan” as the scan mode. Compounds were detected with an intensity threshold of 100000 and a retention time window of 1 min. APEX mode was used to create MS and MS/MS mass list with S/N (defined as the height of the mass peak above baseline relative to the noise ($5 \times$ standard deviation)) threshold of 0.1 and peak width (minimum full width at half height) of 0.1 m/z. Charge state calculation and peak de-isotoping were done with the mode of resolved-isotope deconvolution with a maximum allowed charge of 3 for MS full scan and 2 for MS/MS given that the adduct ion is H^+ . The resulting compound peak list was exported as mgf file format with a global charge limitation of 2+ and 3+, and 30 most abundant non-deconvoluted ions for each MS/MS spectrum and 4 most abundant ions for each MS spectrum, and was subjected to MASCOT MS/MS Ion search (version 2.1, Matrix Science) against the composite non-identical MSDB_20060831 protein database (3239079 sequence entries) in mammalian species (339491 entries) with one maximum missed cleavage by trypsin. The mass tolerance for peptide and fragment was set to 1.5 and 0.8 Da respectively, with carbamidomethyl of Cys as fixed modification and oxidation of Met and phosphorylation of tyrosine as variable modifications. A minimum peptide ion score was set to 25 and expected value ≤ 0.05 . Minimum Mascot protein score of 50 was used for confident identification. A decoy database with reversed sequences was searched simultaneously to assess the false discovery rate, which is set to 0-10% for acceptance for peptide matches above identity threshold. The de novo peptide sequencing was performed in BioTool (version 2.1) (Bruker Daltonics, MA).

2.7. Immunoprecipitation and Western Blot Analysis

Cell culture, priming and stimulation conditions were described above. Total urea-lysed proteomes (50 μ g) were analyzed by SDS-PAGE followed by Western blot analysis with antibodies for 6xHis tag and phosphotyrosine. For immunoprecipitation (IP), stimulated cells were lysed by RIPA buffer and supernatant was collected from lysates by centrifugation. Antibodies for SHP2, FRS2, SHIP2, PTPN18 and IRS4 were conjugated non-covalently to protein A/G Sepharose, and then added to the lysate supernatant containing an equal amount of protein from stimulated and unstimulated cells for IP overnight. The immunoprecipitates were analyzed by western blotting with p-Tyr-100 or the indicated antibodies. Where antibodies specific for phosphotyrosines on proteins were available, the results were further confirmed by immunoblot analysis. Antibodies for pERK(Y186) (same as Y204) and pSHIP2 (Y986) were from Cell Signaling Technology (MA).

3. Results

3.1. Development of a FGF7/FGFR2IIIb Response System with High Signal to Noise Ratio

Cultured mammalian cells exhibit intrinsically high levels of tyrosine phosphorylation presumably because of constitutively activated adaptive pathways required for growth and survival in vitro [42]. A screen of several epithelial cell lines in which FGFR2IIIb is the single resident FGFR isotype [16,29,30,32,33,44] indicated an unacceptably high basal level of FGF7-independent pTyr-proteins that were resistant to suppression by various manipulations of environmental conditions. HEK293 cells express detectable mRNA levels of all FGFR isoforms except FGFR4 and exhibit activation of ERK in the presence of FGF2 (unpublished results). However, they exhibited undetectable basal tyrosine phosphorylation levels after serum depletion and were unresponsive to FGF7 in respect to pTyr-proteins (Fig. 1). This indicated that functional FGFR2IIIb was not present. We therefore employed it as a host to test for FGF7-dependent FGFR2IIIb pTyr targets before adaptation of the cell population to the constitutive presence of active FGFR2IIIb, selection for the growth and survival effects

and selection against the growth controlling effects of FGFR2IIIb. This was accomplished by preparation of cell lines bearing an FGFR2 β IIIb expression vector under control of a dose-dependent tetracycline (Tet)-inducible promoter. Cells were maintained in Tet-free culture medium without any detectable leaky expression (data not shown), and then FGFR2IIIb expression was induced by tetracycline for 6 to 12 hrs, exposed to FGF7 stimulation for up to 30 minutes and analyzed for total pTyr-proteins. Surprisingly, there was a minimal increase in FGF7-stimulated phosphorylated proteins indicating that the cells were incapable of responding to the FGF7/FGFR2IIIb pair. Therefore, we adapted a strategy of “response priming” followed by reset of backgrounds by medium restriction. Cells were temporarily incubated in the presence of FGF7 for 6 hr, and then levels of pTyr-proteins were reduced by serum restriction and withdrawal of FGF7 for a period of 4 to 6 hr. Subsequently cells exhibited a response to FGF7 over a period of 5 to 10 min with maximum signal to noise ratio (Fig. 1). This makes the SILAC approach unnecessary, which is used to detect the differential signals when the intrinsic background is persistently high. An FGF7/FGFR2IIIb-dependent tyrosine phosphoproteome indicated by 22 phosphopeptides was apparent at about 1-5 min after stimulation by FGF7. These were identified by immunoaffinity purification (IAP) of phosphopeptides followed by nano-LC nano-ESI MS/MS analyses (Table 1). None of these were detected in parent cells or transfected cells without Tet induction of FGFR2IIIb expression. Reproducibility was established in at least three additional independent preparations. These 22 phosphopeptides listed in Table 1 appeared only in the FGF7/FGFR2IIIb-stimulated proteomes among all the repeats and were never observed in the control proteomes.

Prolonged stimulation by FGF7 of constitutive FGFR2IIIb-expressing cells exhibited a 10 to 20% depression in cell population growth rates with little apparent morphological changes (data not shown).

3.2. Autophosphorylation of FGFR2IIIb in Response to FGF7

Ten of the tyrosine phosphopeptides contained Y466, Y586, Y588, Y656 and Y657 that are within the intracellular domain of FGFR2 (Table 1, Fig. 2). These are the homologous sites to Y463, Y583, Y585, Y653 and Y654 within the catalytic core of FGFR1 that have been shown to be autophosphorylated in temporal order [21]. FGFR2 Y656 and Y657 (Fig. 3) are homologues of the two key sites, Y653 and Y654 for maximal derepression and activation of the FGFR1 kinase [19-21]. Notably all three peptides containing pTyr656, pTyr657, or both were detected. Counterparts of FGFR2 pY733 and pY769 in FGFR1 have also been reported [18,19,24], but did not appear in our analysis. Phosphoproteomic analysis of FGFR1 using the SILAC method in HEK293 cells also failed to detect phosphopeptides containing these two sites in FGFR1 [42]. This was possibly due to the high molecular weight (>3 kDa) of the Y769 containing peptide [41] or loss of solubility in sample preparation steps and the nano-reverse phase HPLC analysis. Phospho-Y766 in FGFR1 is essential for FGF-stimulated tyrosine phosphorylation of PLC γ in cultured cells and has been demonstrated to be a direct binding site for PLC γ via its SH2 domain [18,24]. Despite the lack of detection of the phosphopeptide containing Y766 in FGFR2, multiple phosphopeptides from PLC γ were detected in immunoprecipitated proteins by SDS-PAGE in the FGFR1 proteomic analysis [41,42]. A separate analysis of immunoprecipitates with anti-PLC γ antibody from cell extracts revealed that PLC γ was tyrosine phosphorylated upon activation of FGFR2IIIb by FGF7 in the current study (Fig. 4).

Phosphorylation of Y344 at the splice junction of Ig IIIb and IIIc in the FGFR2 ectodomain was also observed (Fig. 2). Phosphorylation of tyrosines in the ectodomain of FGFR1 have been previously reported [18,41]. However, the phenomena has been attributed to non-specific autophosphorylation by soluble FGFR1 kinase as a consequence of liberation from its

membrane context in non-denaturing conditions [18,41]. In the current study, stringent denaturing conditions were employed to minimize non-specific phosphorylation by solubilized FGFR2IIIb, dephosphorylation by phosphatases and degradation by proteases. The potentially novel tyr phosphorylation in the ectodomain deserves further study.

3.3. Tyr-phosphorylation of phosphotyrosine Phosphatases in Response to FGF7/FGFR2IIIb Signaling

Activity of pTyr phosphatases is thought to maintain a low level of basal tyrosine phosphorylation in pathways poised for amplification by external signals, but also play a role in attenuation of amplifying signals through dephosphorylation of pTyr proteins [45,46]. In addition pTyr phosphatases may play a role in activation through dephosphorylation of repressors [47]. We observed pTyr-phosphorylation of three FGF7/FGFR2IIIb-dependent intracellular phosphatases (Table 1), the SH2-containing protein-tyrosine phosphatase 2C (SHP2/PTPN11) (Fig. S1), SH2-containing inositol polyphosphate 5-phosphatase (SHIP2) (Fig. S2) and protein-tyrosine phosphatase non-receptor type 18 (PTPN18/PTP20/BDP1) (Fig. 5A), that were phosphorylated on Y62, Y986 and Y387, respectively. FGF7/FGFR2IIIb-dependent tyrosine phosphorylation of the three phosphatases was confirmed separately by immunochemical analysis with phosphotyrosine antibody (Fig. 4). Separate analysis with pTyr986-specific SHIP2 antibody further confirmed the identification (Fig. 4).

Phosphorylation of SHP2 bound to FRS2 recruits additional Grb2 molecules to the cell membrane-proximal FGFR1 complex resulting in strong activation of the canonical RAS-MAPK pathway [25,48,49]. pTyr-SHP2 also has been shown to inhibit Sprouty, a repressor of FGFR1-activated signaling [47]. Activated pTyr-SHP2 may also play a role in attenuation of phosphotyrosine-mediated signaling [50]. Although it has received little study in FGFR signaling, SH2-domain substrate SHIP2 is recruited to the activated EGF receptor and insulin receptor and has been shown to attenuate both EGF and insulin signaling by reducing PtdIns (3,4,5)P3 levels and depressing PI3K (phosphoinositide 3-kinase)-linked pathways [51,52]. PTPN18/PTP20/BDP1 is a less studied phosphatase, which has been shown to down-regulate HER2 and Tec signaling by dephosphorylation of pTyr [53,54].

3.4. Other pTyr Targets Elicited by FGF7-activated FGFR2IIIb

FGF7-induced activation of FGFR2IIIb elicited tyrosine phosphorylation of the insulin receptor substrate 4 (IRS4) at Y921 (Fig. S3) (Table 1), the canonical extracellular signal-regulated kinase 2 (ERK-2, MAPK2) at Y186, and cyclin-dependent protein kinase (CDK2) on Y15 (Fig. 5B). IRS4 and ERK2 (Fig. S4), but not CDK2 were also detected in FGF2-stimulated FGFR1 pathways [42]. Separate analysis with pY186-specific antibody for ERK2 also confirmed presence of the phosphorylation site (data not shown). IRS4 is a non-myristylated, non-membrane bound PTB-domain and pTyr-site-containing organizer of downstream signaling mediators. It has been most widely studied as a substrate of the insulin/insulin-like growth factor (IGF) receptor kinases and most commonly linked to the growth promoting activities of the two receptors [55]. IRS4 appears to act independently of FRS2 in respect to FGFR1 signaling [42]. Phosphorylation of Y15 on CDK2 has been associated with depression of CDK2 activity in the G1-S transition in promotion of cell cycling [56].

Notably and similar to the FGFR1 studies [42], the major FGFR proximal signaling mediator and tyrosine kinase substrate, FRS2, was not detected among the pTyr peptides. However, immunoprecipitation with anti-FRS2 and then development with anti-pTyr antibody indicated that phosphorylation of FRS2 was stimulated by the FGF7/FGFR2IIIb pair (Fig. 4).

Other targets elicited by FGF7/FGFR2IIIb were M-phase phosphoprotein 11 (ZRF1) (pY569) (Fig. S5), a ribosome-associated nascent polypeptide chaperone [57] and three targets involved

in organization of nuclear, membrane and cytoskeletal elements, emerlin (pY94) (Fig. 6), lamina-associated polypeptide 2 (LAP2) (Y275) (Fig. 7) and synapse-associated protein 102 (SAP102) (pY673) (Fig. S6). The analysis further suggested ZNF598, a predicted zinc finger protein (pY306) (Fig. S7), PDGFA-associated protein 1 (PDAP1) (pY17) (Fig. S8) and gene expression product FLJ20625 (Y40) as novel pTyr targets of the FGF7/FGFR2IIIb pair. These targets were unique to FGF7/FGFR2IIIb and not reported in the FGFR1 analyses [41,42].

4. Discussion

FGFR2IIIb is the resident FGFR isotype in differentiated epithelial cells in most multi-compartment adult squamous and glandular epithelia. Exclusive expression of exon IIIb instead of IIIc gene in epithelial cells confers specificity of the ectodomain of the FGFR2 transmembrane receptor tyrosine kinase for FGF7 that fails to activate other FGFR isoforms [29,44]. Unlike numerous other FGFs, the expression of FGF7 is limited to the stromal cell compartment in adult tissues where FGFR2IIIb is expressed in the epithelium [28-30]. Among the cellular FGFs, access of stromal-derived FGF7 to epithelial FGFR2IIIb is stringently regulated by abundant heparan sulfate oligosaccharides with a spectrum of charge density in the extracellular matrix between stroma and epithelium. The structural requirements for a heparan sulfate oligosaccharide to form an active oligomeric complex of FGFR2IIIb and FGF7 is the most stringent of those studied to date [5,16]. Consistent with stringent co-factor control and its resident status in differentiated epithelial cells, FGFR2IIIb plays a role in maintenance of cellular and compartmental homeostasis in epithelial tissues [30,39]. FGFR2IIIb is capable of supporting epithelial cell proliferation, but the proliferation is self-limited and often concurrent with differentiation [28,29,32,33,39]. This property has been exploited for application of FGF7 (Palifermin, Kepivance™), the first FDA approved FGF for clinical use, as an adjuvant in cancer chemotherapy to alleviate severe mucositis and restoration of damaged epithelia [58]. The net homeostasis-promoting activity of FGFR2IIIb in epithelial cells is in marked contrast to the normally mesenchymal or stromal cell-associated homologue FGFR1 that largely has been implicated in cell proliferation and ancillary cellular activities most commonly associated with mesenchymal-derived cells as motility and migration. Ectopic expression or appearance of FGFR1 is observed in numerous epithelial-derived tumor cells and in a variety of tumor progression models, which imparts properties associated with normal mesenchymal cells constitutively to epithelial cells [34-39,59]. These properties are predominantly proliferation and multiple phenotypes that drive malignancy. In contrast, loss of stromal-epithelial compartmental homeostasis concurrent with loss of FGFR2IIIb or FGFR2 expression altogether is often associated with progression to malignancy [29,39]. Restoration of FGFR2 to tumor epithelial cells restores responsiveness to stroma, differentiation in the epithelial compartment, and balance between stroma and epithelium that results in a net limitation in tumor growth and malignancy [32,37]. Consequently, FGFR2IIIb may serve as a “tumor suppressor” and a potential avenue for anti-tumor therapy by restoration of normal compartmental homeostasis or even cell death [31-33].

In this study, we have produced new clues and new potential downstream targets that shed light on molecular aspects of the multiple functions of growth stimulation, growth inhibition and induction of differentiation supported by FGFR2IIIb signaling relative to FGFR1 through tyrosine phosphoproteomic analysis. In addition to the “priming” strategy, we employed the same cell model for a better comparison to oncogenic FGFR1, the HEK293 cells that were used in an FGFR1 phosphoproteomic study. These cells appear to have undetectable or very low tyrosine phosphorylation background after short-term serum restriction. FGFR2IIIb was introduced into the naïve HEK293 cells with no previous history of expression of FGFR2IIIb by controlled expression of an inducible stably transfected FGFR2IIIb gene. This preserved an absolute dependence on and quick response to exogenous FGF7, a ligand that only activates FGFR2IIIb. This prevented adaptation and selection for only the growth stimulatory elements

of FGFR2IIIb signaling. An FGF7-dependent response of the pTyr proteome to FGFR2IIIb required “priming” of the naïve HEK293 cells to the transient presence of activated FGFR2IIIb followed by reduction of pTyr proteins to negligible levels by medium restrictions. The mechanism underlying this short-term priming phenomenon in cells originally unresponsive to the FGF7/FGFR2IIIb pair is of considerable interest. The priming occurs in an insufficient time frame for cell division and evolution via selection of a responsive population. Although all isoforms of FGFRs including ectodomain splice variants IIIb and IIIc are expressed at the mRNA level, our preliminary results further indicate that FGFR1 is the predominant endogenous FGFR isoform expressed, albeit at very low but a still functional level in HEK293 cells. Thus it is likely that the cell population has undergone long-term adaptation and selection for the proliferative response driven by FGFR1 stimulated pathways even though it appears to be no longer dependent on it (unpublished results).

FGFR2 exhibits all seven homologous tyrosines that have been observed to be phosphorylated in the intracellular domain of FGFR1 [19] (Fig. 2). Five FGFR1 pTyr counterparts [21] were observed in FGFR2 in our analysis compared to three in the FGFR1 analysis [42]. The role of FGFR1 tyrosines other than the counterparts of FGFR2 Tyr 656, 657 and 769 in FGF signaling is unclear. FGFR1 counterparts of FGFR2 Tyr 656 and 657 are involved in repression/derepression via transactivating FGFR kinase phosphorylation rather than recruitment of diverse signaling mediators [19-21]. The FGFR1 counterpart of FGFR2 Tyr 769 is the only direct binding site for a signal mediator (PLC γ) validated to date. Although FGFR2 pTyr 769 was observed neither in our analysis nor FGFR1 Tyr766 in the FGFR1 analysis [42], FGF-stimulated pTyr-PLC γ was observed in both. Although low yield of the pTyr 769-containing peptide cannot be eliminated in both the FGFR1 and FGFR2 analyses, the results raise the possibility that PLC γ may access FGFR by mechanisms other than recruitment to pTyr 769. There are no clear differences between tyrosine phosphorylations on FGFR2 and FGFR1 that would suggest differences in signaling between the two FGFR isoforms.

Unlike other receptor tyrosine kinases, FGFR signaling is largely mediated through a single membrane-bound tyrosine phosphorylated substrate, FRS2, that organizes diverse signal pathway mediators that are recruited to its pTyr sites directly or bind to it independently of these sites [25,27,48,49]. Although we did not detect FRS2 pTyr peptides in the proteomic screen, separate immunoprecipitation indicated that FRS2 α is phosphorylated in response to FGF7. Detection of pTyr-SHP2 that is a major substrate of specific pTyr residues in pTyr-FRS2 α [48] further suggested that FRS2 α is a major mediator of FGF7/FGFR2IIIb signaling in cells including the HEK293 cells utilized as host in this study. In contrast, the FGFR1 proteomic study in the same cells also failed to detect pTyr-FRS2 α causing the authors to suggest that FRS2 α may be deficient in the HEK293 cells [42]. Separate results not shown here also indicate that FRS2 α levels are significant in this cell line.

Consistent with its growth promotion properties and overlapping with FGF2/FGFR1 [42], the FGF7/FGFR2IIIb pair induced tyrosine phosphorylation of the canonical ERK2, a central mediator of the RAF-RAS pathway that is a hallmark correlate of both compensatory growth and constitutive oncogenic growth [60]. In addition the FGF7/FGFR2IIIb pair elicits tyrosine phosphorylation of IRS4. IRS4 has been implicated in mediating the growth-associated elements of growth hormone, insulin and IGF-1 in diverse tissues and models [55]. Notably the FGF7/FGFR2IIIb pair elicited phosphorylation of cell cycle promoter CDK2 ser/thr kinase on Y15 which was not apparent in the reported FGFR1 analyses [42]. pTyr15-CDK2 is an inhibitory event in respect to CDK2 ser/thr kinase activity and thus an inhibitory event on cell cycle in respect to the role of CDK2 [56]. Therefore phosphorylation of Tyr15 on CDK2 may contribute to the growth controlling aspects of the FGF7/FGFR2IIIb pair observed in epithelial cells [33].

The FGF7/FGFR2IIIb pair elicited tyrosine phosphorylation of tyrosine phosphatases SHP2, SHIP2 and PTPN18. SHP2 and SHIP2, but not PTPN18 were also detected in the FGF2/FGFR1 analyses [42]. pTyr phosphatases intuitively are predicted to attenuate positive aspects of phosphotyrosine signaling and in particular tyrosine kinase driven cell growth cascades. However, through activation of its phosphatase activity via pTyr 62, SHP2 is a strong enhancer of the canonical RAS-MAP kinase pathway and hyperactivation has been shown to be oncogenic [49,61]. Although pTyr-SHP2 is predicted to be a part of the growth promoting function of FGFR2IIIb along with the RAS-MAP kinase pathway, participation of SHP2 and the associated pathway in the growth attenuation and differentiation functions of FGFR2IIIb cannot be eliminated. The selective phosphorylation of PTPN18 may further add to the selective growth attenuation function of FGFR2 through downregulation of growth-stimulatory signaling including that of the EGFR family [53,54].

A number of pTyr proteins observed in FGF2/FGFR1 signaling were notably absent from the FGF7/FGFR2IIIb pTyr proteome. These substrates included a number of kinases or kinase-modifying factors (RSK2, PIX, GIT, FAK, PI3K, ShcA) as well as cytoskeletal components or modifiers (ODIN, paxillin, p130Cas, Nck-associated protein, vimentin, annexin) that largely are related to cell adhesion and motility. Alterations in most are generally associated with tumorigenic phenotypes. Instead the FGF7/FGFR2 pair caused tyrosine phosphorylation of several factors that maintain nuclear structure and nuclear interactions with cytosolic elements (emerin and LAP2), transcription regulation, protein folding and other cellular fine structures as well as novel proteins of unknown function. Among them are several pTyr-proteins associated with growth limitation and tumor suppression and their disruption is associated with tumor promotion [62-64].

In summary, our results indicate that FGF7-activated FGFR2IIIb shares a limited subset of phosphotyrosine targets generally associated with FGFR1 and other growth promoting kinases, but also exhibit novel FGFR2IIIb-specific targets associated with growth limitation in the same context. This is consistent with observed differences among the two isoforms in growth, migration, growth limitation and differentiation. Our results provide specific targets for further study of differences between the two FGFRs in diverse biological contexts and associated diseases. The “induction and prime” approach used here should be useful for dissection of differences in the phosphoproteome elicited by combinations of FGFs, FGFR and co-factors heparan sulfate and klothos associated with the family’s increasingly diverse roles in both cellular and metabolic homeostasis [6,8]. A major challenge is to dissect how the overlapping and unique subsets of pTyr substrates observed among the FGFR isoforms are generated from co-factor and cell context-dependent conformational changes within the ecto- and intracellular domains of the transmembrane FGFR signaling complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FGF7, fibroblast growth factor 7; FGFR2IIIb, fibroblast growth factor receptor 2 isotype IIIb; FRS2, FGF receptor substrate 2 (SNT1); IAP, immunoaffinity purification; IP, immunoprecipitation; IRS4, insulin receptor substrate 4; Nano-LC nano-ESI MS/MS, nano-flow HPLC coupled to nano-electrospray tandem mass spectrometry; PTPN18, protein-

tyrosine phosphatase non-receptor type 18; SHIP2, inositol polyphosphate 5-phosphatase; SHP2, protein tyrosine phosphatase 2C.

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Fig. 1.

Dependence of pTyr-proteins on induced FGFR2IIIb and FGF7. **A.** Flowchart for FGFR2IIIb-inducible, FGF7-dependent generation of phosphoproteome analysis. **B.** T-Rex 293 cells stably transfected with FGFR2IIIb construct were induced by Tet, primed and then stimulated by FGF7 as described in Experimental Procedures and **A.** Total proteomes (50 μ g) extracted with urea-modified buffer were subjected to western blot analysis (WB) with antibodies against phosphotyrosine (pTyr) and the 6XHis tag under the indicated conditions. β actin was used as loading control.

Fig. 2.

Tyrosine phosphorylation sites on FGFR2. FGFR2 used in the study consisted of the splice variant encoding the two Ig-like domains (II and III). The transmembrane (TM), kinase domains 1 and 2 (K1, K2) and the interkinase domain (IK) are shown. Counterpart tyrosine phosphorylation sites to those in FGFR1 and the detected phosphopeptide sequences in FGFR2 are indicated.

Fig. 3.

Nano-LC Nano-ESI MS/MS identification of double tyrosine-phosphorylation in the activation loop of FGFR2 kinase. Data-dependent MS/MS was used to determine the phosphorylation of Y656 and Y657 and the amino acid sequence of the peptide containing them from the FGFR2IIIb kinase activation loop upon FGF7 stimulation. The detected b and y ions from the helium-assisted collision induced dissociation (CID) of peptide DINNIDpYpYKK (m/z 723.2, 2+) is indicated. Some of the a-type fragment ions and water-loss ions are also shown.

Fig. 4.

Immunochemical analysis of specific pTyr-proteins in response to FGF7 stimulation of FGFR2IIIb. T-Rex 293 cells bearing the FGFR2IIIb construct or vector alone were induced by Tet for FGFR2IIIb expression, primed by FGF7 and then starved and stimulated by FGF7 as described in Methods. Cell lysates of equal amount of total protein were used for immunoprecipitation with anti-SHP2, SHIP2, PTPN18, PLC γ , IRS4 and SNT1/FRS2 antibodies with protein A/G beads. The immunoprecipitated proteins were further analyzed with p-Tyr-100 antibody, and specific pSHIP2(Y986) antibody. β actin was used as utility and loading control for the analyzed proteome.

Fig. 5. FGF7/FGFR2IIIb stimulation of tyrosine phosphorylation of PTPN18 and CDK2. The CID fragmentation of the phosphopeptide from PTPN18 and CDK2 and determination of phosphotyrosine and other amino acid sequence are indicated. Some of the a-type and water-loss ions are also shown.

Fig. 6. FGF7/FGFR2IIIb signaling causes phosphorylation of emerlin on tyr94. The b and y ions resulting from the CID and pTyr residue and the amino acid sequence of the phosphopeptide from emerlin are indicated.

Fig. 7.

Phosphorylation of LAP2 on tyr275. The determination of b and y ions, pTyr residue and the amino acid sequence of the phosphopeptide from LAP2 are indicated. The oxidized methionine and carbamidomethylated cysteine are underlined.

Table 1

^aFGF7/FGFR2IIIb-dependent tyrosine phosphorylation sites

m/z	Phosphopeptide	pTyr site	Ion score	PubMed accession number
1. FGF2IIIb				
683.4	DINNIDpYYKK	656	55	L19107
1237.6	DINNIDYpYK	657	32	L19109
723.2	DINNIDpYpYKK	656, 657	51	
683.4	DINNIDYpYKK	657	45	
950.1	RPPGMEpYSpYDINRVPEEQMTFK	586, 588	33	
840.2	RPPGMEpYSYDINR	586	50	
840.2	RPPGMEYSpYDINR	588	44	
847.7	RPPGMEpYSYDINR	586	35	
847.7	RPPGMEYSpYDINR	588	39	
1082.5	LSSTADT ^a PMLAGVSe ^a pYELPEDPKWEFPR	466	31	
1720.2	HSGINSSNAEVLALFNVTEDAGEYICKVSNpYIGQANQSAWLTVLPK	344	27	
2. Phosphatases				
908.8	IQNTGDpYYDLYGGEK	62	63	Q06124
SHIP2	NSFNNPpYVVEGVPHQLLPPEPPSPAR	986	46	Q13577
PTPN18	SAEEAPLpYSK	387	40	CAA56105
3. Signaling Mediators				
ERK2	VADPDHDHTGFLTe ^a pYVATR	186	52	Q499G7
IRS4	EADSSSDpYVNMDFTK	921	66	O14654
CDK2	IGEGTpYGVVYK	15	42	CAA43985
4. Others				
ZRF1	FEGPpYTDFTPWTTTEEQK	569	74	CAA66913
Emerin	GYNDdpYEEsYFTTR	94	51	Q5HY57
LAP2	EMFPpYEASTPTGISASCR	275	34	Q59G12
SAP102	DNEVDGQDpYHFVVSr	673	32	Q92796
ZNF598	RNEGvVGGEDpYEEVD ^a R	306	51	Q86UK7
PDAP1	QpYTSPEID ^a QLQAEK	17	57	AAH00684
FLJ20625	ALNGAEPNpYHSLPSAR	40	72	Q6IAA8

^aUnderlined M or C indicates oxidized methionine or carbomidomethylated cysteine.