Expression and muscarinic receptor coupling of Lyn kinase in cultured human airway smooth muscle cells

Thomas Pertel,1 Defen Zhu,1 Reynold A. Panettieri,2 Naoto Yamaguchi,3 Charles W. Emala,1 and Carol A. Hirshman1

1Department of Anesthesiology, College of Physicians and Surgeons of Columbia University, New York, New York; 2Pulmonary and Critical Care Division, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; and 3Department of Molecular Cell Biology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

Submitted 8 August 2005; accepted in final form 13 October 2005

The most widely expressed members of the Src family include c-Src, Yes, Lyn, Lck, Hck, Blk, Fgr, and Frk (22). Nonreceptor Src family tyrosine kinases share a common NH2-terminal Src homology 4 (SH4) acylation site, followed by a region unique to each family member, an SH3 domain, an SH2 domain, a protein kinase domain (SH1), and finally, a COOH-terminal regulatory domain (32). Two important tyrosine phosphorylation sites are vital for Src kinase activity: a tyrosine located within the activation loop of the SH1 domain and another in the COOH-terminal regulatory domain. Src family kinases are inactivated by phosphorylation of a COOH-terminal tyrosine residue by CSK (COOH-terminal Src kinase) and are activated by dephosphorylation of this tyrosine by a tyrosine phosphatase, but autophosphorylation of the tyrosine in the SH1 domain is required for full activation (17, 41).

Nonreceptor Src family tyrosine kinases are well-known components of signaling pathways utilized by growth factor receptors. However, evidence is accumulating that Src family tyrosine kinases are also components of G protein-coupled receptor signaling pathways (18) mediating such processes as cellular migration, integrin signaling, and actin reorganization (8, 9, 20, 33). Lyn, c-Src, Yes, and Lyn are activated by either lysophosphatidic acid, endothelin-1, or the cholinergic agonist acetylcholine (19, 30, 36) in non-smooth muscle cells. Fyn couples to M2 muscarinic receptors in cultured canine colonic smooth muscle cells and cells transduced with wild-type human Lyn kinase, carbachol increased Lyn kinase activity. Pertussis toxin pretreatment failed to block carbachol activation of Lyn kinase but did attenuate the carbachol-induced increase in ERK/MAPK phosphorylation. Moreover, carbachol inhibited adenyl cyclase but failed to increase total inositol phosphate synthesis in these cells. The present study shows that Lyn kinase is expressed in human cultured airway smooth muscle cells at both the mRNA and protein levels and that carbachol, an M2 muscarinic receptor agonist in these cells, activates Lyn kinase by a pertussis toxin-insensitive signaling pathway.

Src kinase; lentivirus; carbachol; immunoblot; cell culture

Although cultured human airway smooth muscle cells express mainly M2 muscarinic receptors (39), intact airway smooth muscle cells express both M2 and M1 muscarinic receptors (21). Activation of M3 and M2 muscarinic receptors on intact airway smooth muscle leads to contraction and inhibition of relaxation, respectively, whereas activation of M2 receptors on cultured cells leads to actin polymerization. M1 muscarinic receptors couple to Gq to stimulate phospholipase C and inositol phosphate synthesis, whereas M2 receptors couple to Gi and inhibit adenyl cyclase. Multiple kinase cascades are involved in these signaling pathways, and in some systems activation of Src family tyrosine kinases is an upstream early postreceptor event.
ylation of the COOH-terminal Tyr508 by Csk and activated by both dephosphorylation of Tyr508 and autophosphorylation of Tyr979 in the SH1 domain (41).

Despite the wealth of information about the expression and function of nonreceptor Src family tyrosine kinases and their function in lymphocytes and neoplasms, little is known about the expression and signaling of these kinases other than c-Src in airway smooth muscle. Lyn kinase may be expressed more widely than previously thought, and many G protein-coupled receptors activate Src family tyrosine kinases. Therefore, the goal of the present study was to determine whether Lyn is expressed and whether muscarinic receptor activation is coupled to Lyn kinase activation in human airway smooth muscle cells.

METHODS

Materials. Enhanced Chemiluminescent (ECL) Plus, protein G-Sepharose 4 Fast Flow, ECL horseradish peroxidase-linked donkey anti-rabbit IgG, ECL horseradish peroxidase-linked sheep anti-mouse IgG, and Hyperfilm ECL chemiluminescence film were purchased from Amersham Biosciences. Immun-Blot polyvinylidene difluoride (PVDF) membranes, 2× Laemmli sample buffer, Tris·HCl 4–20% gradient, 10% polyacrylamide precast Ready Gels, and certified molecular biology agarose were purchased from Bio-Rad. Complete, Mini, EDTA-free protease inhibitor cocktail tablets were from Roche Applied Science. Radiolabeled adenine-5'-triphosphate, [γ-32P]ATP (4,500 Ci/mmol, Lyn kinase assay), [α-32P]ATP (800 Ci/mmol, adenyl cyclase assay), and [3H]myo-inositol (20 Ci/mmol) were from MP Biomedicals. Carbamylcholine chloride (carbachol), hexadimethrine bromide (Polybrene), bovine carboxybiotin (Biotin), peroxidase, and phosphatase inhibitor cocktail 2 were from Sigma-Aldrich. DMEM/F-12 1:1, RPMI 1640, trypsin-EDTA solution, l-glutamine, PBS, HEPES buffer solution, certified FBS, Opti-MEM I reduced serum medium, and Lipofectamine 2000 were from Invitrogen.

For muscarinic M2 and M3 receptor RT-PCR analysis, total RNA was extracted as described above from nontransformed cultured human airway smooth muscle cells, and performed RT-PCR on total RNA isolated from nontransformed cultured human airway smooth muscle cells using gene-specific primers (Table 1) for the nine major Src family kinases: BLK, Fgr, Frk, Fyn, Hck, Lyn, Lck, c-Src, and Yes. Total RNA from human spleen, Jurkat, and Ramos cells was used as positive controls. Water was used as a negative control to rule out sample contamination.

Cells were pelleted (5 × 10^6) at 250 g for 5 min and washed once with cold PBS. Total RNA was extracted using an RNAsky kit (Qiagen), following the manufacturer’s instructions, and quantified by absorption at 260 nm. RT-PCR was performed using the SuperScript III One-Step RT-PCR System (Invitrogen) following the manufacturer’s instructions. Briefly, 50 ng of total RNA were reverse transcribed at 55°C for 30 min, followed by PCR using gene-specific primers (0.2 μM for sense and antisense primers). Forty cycles of denaturing (94°C for 15 s), annealing (60°C for 30 s), and extension (68°C for 2 min) were used, followed by a 10-min extension at 68°C. PCR products were electrophoresed through a 0.8% agarose gel, stained with ethidium bromide, and scanned under ultraviolet illumination using a Fluor-S MultiImager (Bio-Rad).

For muscarinic M2 and M3 receptor RT-PCR analysis, total RNA was extracted as described above from nontransformed cultured human airway smooth muscle cells and from CHO cells stably transfected with the human M2 or M3 muscarinic receptor. RT-PCR was performed with the following modifications: 32 cycles of denaturing (94°C for 15 s), annealing (60°C for 30 s), and extension (68°C for 2 min) were used, followed by a 10-min extension at 68°C. PCR products were electrophoresed through a 0.8% agarose gel, stained with ethidium bromide, and scanned under ultraviolet illumination using a Fluor-S MultiImager (Bio-Rad).

For muscarinic M2 and M3 receptor RT-PCR analysis, total RNA was extracted as described above from nontransformed cultured human airway smooth muscle cells and from CHO cells stably transfected with the human M2 or M3 muscarinic receptor. RT-PCR was performed with the following modifications: 32 cycles of denaturing (94°C for 15 s), annealing (60°C for 30 s), and extension (68°C for 2 min) were used, followed by a 10-min extension at 68°C. PCR products were electrophoresed through a 0.8% agarose gel, stained with ethidium bromide, and scanned under ultraviolet illumination using a Fluor-S MultiImager (Bio-Rad).

M2 and M3 receptor RT-PCR analysis, total RNA was extracted as described above from nontransformed cultured human airway smooth muscle cells and from CHO cells stably transfected with the human M2 or M3 muscarinic receptor. RT-PCR was performed with the following modifications: 32 cycles of denaturing (94°C for 15 s), annealing (60°C for 30 s), and extension (68°C for 2 min) were used, followed by a 10-min extension at 68°C. PCR products were electrophoresed through a 0.8% agarose gel, stained with ethidium bromide, and scanned under ultraviolet illumination using a Fluor-S MultiImager (Bio-Rad).

Table 1. Src family RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLK</td>
<td>ATGGGCTCTGTAATGACAAAGGGCAGCAGGCGAACGAGG</td>
<td>CTagGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,518</td>
</tr>
<tr>
<td>FGR</td>
<td>ATGGGCTCTGCTGTTGCTGGGCGGCGGCGG</td>
<td>CTagGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,590</td>
</tr>
<tr>
<td>FRK</td>
<td>ATGGGCAAGACCATCTGAGGCAGCCTGAGGAGTACC</td>
<td>TCATCTTGAGAATTTGCACAGGATCTGGAAGG</td>
<td>1,614</td>
</tr>
<tr>
<td>FYN</td>
<td>ATGGGCTGCTGAGTAATGACAAAGGGCAGCAGGCGAACGAGG</td>
<td>TTAGGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,518</td>
</tr>
<tr>
<td>HCK</td>
<td>ATGGGCTGCTGAGTAATGACAAAGGGCAGCAGGCGAACGAGG</td>
<td>TTAGGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,590</td>
</tr>
<tr>
<td>LCK</td>
<td>ATGGGCTGCTGAGTAATGACAAAGGGCAGCAGGCGAACGAGG</td>
<td>TTAGGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,614</td>
</tr>
<tr>
<td>LYN</td>
<td>ATGGGCTGCTGAGTAATGACAAAGGGCAGCAGGCGAACGAGG</td>
<td>TTAGGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,518</td>
</tr>
<tr>
<td>SRC</td>
<td>ATGGGCTGCTGAGTAATGACAAAGGGCAGCAGGCGAACGAGG</td>
<td>TTAGGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,590</td>
</tr>
<tr>
<td>YES</td>
<td>ATGGGCTGCTGAGTAATGACAAAGGGCAGCAGGCGAACGAGG</td>
<td>TTAGGGCTGACGTGATCTGCTGCTGCTG</td>
<td>1,614</td>
</tr>
</tbody>
</table>
5 min and loaded onto a 10% polyacrylamide precast Ready Gel (Bio-Rad). After protein separation by SDS-PAGE, proteins were transferred onto a PVDF membrane, and the membrane was blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. Mouse α-Lyn (H6; 1:1,000), rabbit anti-Fyn (15) (1:500), mouse anti-Src (GD11; 1:1,000), mouse anti-Yes (F-7; 1:100), or rabbit anti-G-α (1:1,000) antibodies were added in 5% milk TBST and rotated for 2 h at room temperature. After three quick washes with TBST, horseradish peroxidase-linked sheep anti-mouse or anti-rabbit IgG was diluted 1:10,000 in 5% milk TBST and incubated at room temperature for 1 h. The membrane was washed three times for 10 min each with TBST, and proteins were visualized using Enhanced Chemiluminescent (ECL) Plus (Amersham) and exposed to Hyperfilm ECL chemiluminescence film (Amersham).

Immunoprecipitation before immunoblotting. Human airway smooth muscle cells were plated in a T-75 culture flask and allowed to reach confluence. Cells were washed twice with cold PBS and lysed by the addition of 1 ml of cold radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Trizma-HCl), pH 8.0, 150 mM sodium chloride (NaCl), 1% octylphenyl-polyethylene glycol (Igepal CA-630), 0.1% SDS, 0.5% sodium deoxycholate, with protease (Mini-complete, EDTA-free, Roche) and phosphatase (inhibitor cocktail II, Sigma) inhibitors]. Cells were scraped, transferred to a prechilled microcentrifuge tube, and incubated on ice for 10 min. Cells were homogenized, and cellular debris were pelleted by centrifugation at 16,000 g for 10 min at 4°C. Equal amounts of protein (~500 μg), determined by BCA Protein Assay (Pierce), were transferred to a fresh, prechilled microcentrifuge tube, and 5 μg of mouse anti-Lyn (H6) were added. Samples rotated overnight at 4°C, and immune complexes were recovered by the addition of 25 μl of protein G-Sepharose beads (Amersham) for 1 h at 4°C. Beads were pelleted, washed three times with 1 ml of RIPA lysis buffer, and resuspended in 50 μl of 2X Laemmli sample buffer (Bio-Rad) containing 715 mM 2-ME for subsequent immunoblot analysis.

Production of lentiviral vectors and transduction of cultured human airway smooth muscle cells. Wild-type human Lyn cDNA was amplified from pcDNA4/TO (12) using primers to incorporate BamHI and EcoRI sites at the 5’ and 3’ ends of the opening reading frame for human wild-type Lyn (forward: 5’-CTTCGAG[GAATTC]ACCATGGATGTAAATAATC-3’ [BamHI recognition sequence]; reverse: 5’-GCCGCG[GAATTC]CTACTAGGGGCTGCTGCGTGG-3’ [EcoRI recognition sequence]). This PCR product containing the human wild-type Lyn coding sequence was cloned into the lentiviral vector pFUW (16) after plasmid digestion with BamHI and EcoRI, which simultaneously removed the enhanced green fluorescent protein coding region. Vesicular stomatitis virus glycoprotein (VSVG)-psuedotyped human immune-deficiency virus (HIV) vectors were generated by cotransfecting the transfer vector pFUW-wtLyn, the HIV-1 packaging vector pCMVΔR8.91, and the pMD.G plasmids (27) encoding the VSVG transfer vector pFUW-wtLyn, the HIV-1 packaging vector pMD.G (27) encoding the VSVG transfer vector pFUW-wtLyn, the HIV-1 packaging vector pMD.G plasmids were kindly provided by Jeremy Luban (Columbia Univ.).

Immunoprecipitation and Lyn kinase activation assay. In vitro Lyn kinase activation assays were performed following the method of Platsnik et al. (30), substituting the Src substrate peptide (Upstate) for acid-denatured enolase as an exogenous substrate for Lyn kinase. Nontransformed and wild-type Lyn-transduced cultured human airway smooth muscle cells were rendered quiescent by serum starvation for 24 h. Cells were treated as indicated, washed with cold PBS, and lysed with 50 mM Trizma-HCl, pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.1% SDS, and 2 mM EDTA, with protease and phosphatase inhibitors. For each sample, 500 μg of protein (determined by BCA Protein Assay, Pierce), typically in 1 ml of lysis buffer, were incubated with 5 μg of mouse anti-Lyn (H6) monoclonal antibody for 1 h at 4°C. Protein G-Sepharose beads (25 μl) were added and incubated at 4°C for 1 h. Beads were pelleted and washed once with lysis buffer and three times with kinase buffer (25 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, and with protease and phosphatase inhibitors). Beads were resuspended in 25 μl of kinase reaction buffer (25 mM HEPES, 50 mM MgCl2, 10 mM MnCl2, 10 μM L-[32P]ATP, 10 μg Src substrate peptide) and incubated at 30°C for 30 min with agitation. Reactions were terminated by the addition of 45 μl of 2X Laemmli sample buffer with 2-ME, incubated at 100°C for 5 min, and loaded onto a 4–20% gradient Polyacrylamide Ready Gel. Proteins were separated by SDS-PAGE, and the gel was dried and exposed to a phosphor screen (Molecular Dynamics) overnight and analyzed with a PhosphorImager SI (Molecular Dynamics). Densitometry was performed using Image Quant software (Amersham).

Phospho-Lyn (Tyr508) analysis. Wild-type Lyn-transduced cultured human airway smooth muscle cells were plated on 12-well cell culture plates and allowed to reach confluence, whereupon they were deprived of serum for 24 h before treatment. Quiescent cells were washed once with PBS and treated as indicated. Treatment medium was removed, and cells were washed once with cold PBS. Cells were lysed by the addition of 200 μl of 2X Laemmli sample buffer with 2-ME. Whole cell lysates were transferred to a microcentrifuge tube and incubated at 100°C for 5 min, and 45 μl of lysate were loaded onto a 10% polyacrylamide precast Ready Gel. After protein separation by SDS-PAGE, proteins were transferred onto a PVDF membrane and blocked with 5% BSA in TBST for 1 h at room temperature. Rabbit anti-phospho-Lyn (Tyr507) or rabbit α-phospho-p44/42 MAP kinase (Thr202/Tyr204) polyclonal antibodies were diluted to 1 μg/ml in 5% BSA TBST and incubated with the membrane for 4 h at room temperature. After three quick washes with TBST, horseradish peroxidase-linked donkey anti-rabbit IgG was diluted 1:10,000 in 5% BSA TBST and incubated at room temperature for 1 h. The membrane was washed three times for 10 min each with TBST, and proteins were visualized using ECL Plus and exposed to Hyperfilm ECL chemiluminescence film. Membranes were reprobed with mouse anti-GAPDH monoclonal antibody (Abcam) to confirm equal protein loading. Densitometry was performed using Quantity One software (Bio-Rad).

Insoluble phosphate assays. Synthesis of total [3H]inositol phosphates in response to carbachol was performed on nontransformed cultured human airway smooth muscle cells and CHO cells stably expressing the human M1 muscarinic receptor grown to confluence in 24-well tissue culture plates. Briefly, after overnight loading with [3H]myo-inositol (10 μCi/ml, 20 Ci/mmol) in inositol-free and serum-free DMEM, plates were washed three times (37°C Hanks’ balanced salt solution with 10 mM LiCl). Incubation of cells in parallel wells in a final volume of 300 μl at 37°C for 30 min was performed in the absence or presence of carbachol (1–100 μM) or bradykinin (10 μM). Reactions were terminated, and total [3H]inositol phosphates were recovered by chromatography as described (10).

Adenylyl cyclase assays. Adenylyl cyclase activity was determined by measuring the conversion of [α-32P]ATP to [βγ]cAMP according to the method of Salomon et al. (34). In brief, adenylyl cyclase assays were performed in intact nontransformed cultured human airway smooth muscle cells...
smooth muscle cells in 24-well cell culture plates for 15 min at 37°C in a total volume of 150 μl containing 50 mM HEPES, pH 8.0, 50 mM NaCl, 0.4 mM EGTA, 1 mM cAMP, 7 mM MgCl2, 0.1 mM ATP, 7 mM creatine phosphate, 50 U/ml creatine phosphokinase, 0.1 mg/ml BSA, and 10 μCi/ml [γ-32P]ATP (10). Preliminary experiments confirmed the linearity of adenylyl cyclase activity at the cell density and incubation times used. Cells were treated with 10 μM forskolin in the presence or absence of 1 μM carbachol. The reactions were terminated by the addition of 100 μl of stop buffer (50 mM HEPES, pH 7.5, 2 mM ATP, 0.5 mM cAMP, 2% SDS, and 1 μCi/ml [3H]cAMP). The synthesis of [32P]cAMP was determined by sequential column chromatography over Dowex (Bio-Rad, Hercules, CA) and alumina (34). Recovery of [3H]cAMP from each column was used to calculate column recovery rates that ranged from 75 to 90%. Data were expressed as percent of forskolin-stimulated activity.

Statistical analysis of data. All data are presented as means ± SE. Immunoblot and phosphorimage intensities were compared by ANOVA with repeated measures and Bonferroni posttest comparisons when appropriate or by unpaired t-test with Instat software (Graph Pad, San Diego, CA). Inositol phosphate dose responses were analyzed by one-way ANOVA. Adenylyl cyclase activity was analyzed by Student’s t-test. P < 0.05 was considered significant.

RESULTS

To determine the expression of Src Family kinases in human airway smooth muscle cells, RT-PCR was performed using total RNA isolated from these cells for nine major Src family kinases: Blk, Fgr, Frk, Fyn, Hck, Lyn, Lck, c-Src, and Yes (Fig. 1). RT-PCR revealed mRNA transcripts for FYN, c-SRC, YES, FRK, and LYN in nontransformed cultured human airway smooth muscle cells. Transcripts for BLK, FGR, HCK, and LCK were not amplified in these cells; however, they were amplified from either Jurkat or Ramos cell lines or from human spleen RNA used as positive controls (Fig. 1).

Fyn (59 kDa), c-Src (60 kDa), Yes (62 kDa), and Lyn were identified in nontransformed cultured airway smooth muscle cells by immunoblot analysis (Fig. 2). Lyn appeared as two bands representing the two isoforms commonly expressed (56 and 53 kDa). To test the specificity of the Lyn antibody for use in vitro kinase assays, Lyn was immunoprecipitated from the cultured nontransformed human airway smooth muscle whole cell lysates. Lyn-immunoprecipitated samples were probed for Lyn, as well as Fyn, Yes, and c-Src. Lyn was immunoprecipitated, whereas Fyn, Yes, or c-Src were not, demonstrating that these Src family tyrosine kinases were not nonspecifically contributing to the in vitro kinase assay (Fig. 3).

To study the role of Lyn kinase in G protein-coupled receptor signaling, wild-type human Lyn was overexpressed in human airway smooth muscle cells using an HIV-1-based lentivirus transduction system. High-transduction efficiency was demonstrated by the substantial increase in wild-type Lyn expression compared with empty vector-infected cells (Fig. 4).

To determine whether Lyn kinase played a role in G protein-coupled muscarinic receptor signaling in airway smooth muscle, Lyn kinase activity was assayed in both nontransformed human cultured airway smooth muscle cells and human cultured airway smooth muscle cells transduced with wild-type human Lyn exposed to carbachol (10 μM) for 5 and 10 min. In cells transduced with wild-type Lyn, carbachol increased γ-32P

Fig. 1. Representative images of RT-PCR assays for mRNA encoding Src family members. Cultured human airway smooth muscle cells expressed mRNA encoding Frk, Fyn, Lyn, Yes, and c-Src. mRNA encoding Blk, Fgr, Lck, and Hck was not identified in human airway smooth muscle cells but is shown in human lymphocyte positive controls.

Fig. 2. Representative images of immunoblot analyses for Src family members identified in cultured human airway smooth muscle cells. A: Fyn; B: Lyn; C: c-Src; and D: Yes.
incorporation into the Src family kinase substrate peptide to 1.53 ± 0.20-fold and 1.65 ± 0.26-fold above levels in untreated cells at 5 and 10 min of carbachol treatment, respectively (P < 0.05; n = 4; Fig. 5). In nontransformed native cells, carbachol increased Lyn kinase activity 1.41 ± 0.14-fold (P < 0.05; n = 3). These results demonstrate that carbachol activates Lyn kinase in both nontransformed cells and in cells transfected with wild-type Lyn.

Cultured human airway smooth muscle cells are thought to express mainly M2 muscarinic receptors that are coupled to Gι protein (39). To determine whether Lyn activation by carbachol was mediated via Gι proteins coupled to muscarinic receptors, studies were conducted using an antibody that recognizes phosphorylation of Tyr508 on Lyn kinase. Decreased phosphorylation of this residue is indicative of Lyn kinase activation. Human airway smooth muscle cells transfected with wild-type Lyn were either untreated or pretreated with pertussis toxin (100 ng/ml for 4 h) and exposed to carbachol (10 μM for 5 min). Carbachol decreased the band intensity to 67 ± 6.2% of untreated values (n = 6, P < 0.01; Fig. 6A). Pretreatment with pertussis toxin failed to inhibit carbachol-mediated decreased phosphorylation of Lyn Tyr508 as cells treated with both pertussis and carbachol yielded relative band intensities that were 56.7 ± 5.2% of untreated values (n = 6, P < 0.01) (Fig. 6A). Pertussis toxin alone was without effect on LynTyr508 phosphorylation, yielding relative band intensities of 90 ± 8.8% of untreated values (n = 5, P > 0.05 compared with control; Fig. 6A).

As pertussis toxin failed to inhibit carbachol-mediated activation of Lyn kinase, additional studies were performed to confirm effective pertussis toxin pretreatment. Carbachol increased ERK/MAPK phosphorylation 374 ± 75% in the absence (P < 0.01) but only 171 ± 30% in the presence (P < 0.05) of pertussis toxin pretreatment (n = 3; Fig. 6B), indicating effective blockade of Gι by pertussis toxin pretreatment. Pertussis toxin alone had no effect on ERK/MAPK phosphorylation yielding relative band intensities of 92 ± 7.4% of untreated values (P > 0.05; n = 3). Together, these studies demonstrate that a pertussis-sensitive G protein is not an intermediate in the carbachol-mediated activation of Lyn kinase.

To determine whether our findings could be confounded by the expression of functional M3 muscarinic receptors in nontransformed cultured human airway smooth muscle cells, we performed RT-PCR analysis of total RNA isolated from these cells, and functional inositol phosphate assays were performed in the presence of carbachol or bradykinin. Messenger RNA and total inositol phosphate synthesis was also measured in CHO cells stably expressing the human M3 muscarinic recep-

---

**Fig. 3.** Representative image of nontransformed cultured human airway smooth muscle cells subjected to immunoprecipitation (IP) with an anti-Lyn antibody followed by immunoblotting with antibodies directed against Src kinase family members. Two isoforms (short arrows) of Lyn were identified by immunoprecipitation and immunoblotting using a Lyn-specific antibody. Immunoprecipitation with anti-Lyn antibody did not immunoprecipitate other Src kinase family members (Fyn, c-Src, or Yes). Long arrows indicate IgG antibody fragment from immunoprecipitation reactive with secondary antibody used in immunoblotting. WB, Western blot.

**Fig. 4.** Representative image of an immunoblot of empty vector-infected vs. lentivirus-Lyn-infected cultured human airway smooth muscle (ASM) cells. Arrows indicate a severalfold increase in Lyn expression in cells infected with lentivirus-Lyn. GAPDH immunoreactivity is shown for lane loading control.

**Fig. 5.** Top: representative image of immunoprecipitated Lyn kinase phosphorylation of an Src family substrate peptide. Human cultured airway smooth muscle cells were treated with 10 μM carbachol for 5 or 10 min, solubilized, and subjected to immunoprecipitation with a Lyn-specific antibody. Kinase activity was then measured by the incorporation of 32P in a peptide substrate. Carbachol increased Lyn kinase activity. Image is representative of 4 experiments. Bottom: relative band intensities of 32P peptide after in vitro Lyn kinase assay. N = 4; *P < 0.05.
tor (used as a positive control). Carbachol (1–100 μM for 30 min) failed to increase total inositol phosphate synthesis in cultured human airway smooth muscle cells (Fig. 7C) in agreement with the lack of expression of mRNA encoding the M3 muscarinic receptor (Fig. 7A). In contrast, bradykinin increased total inositol phosphate synthesis to 726 ± 57% of basal levels in these cells (n = 3; P < 0.05; Fig. 7C). In contrast, carbachol increased inositol phosphate synthesis in CHO M3 cells in a dose-dependent manner (n = 3, P < 0.05 compared with basal inositol phosphate synthesis; Fig. 7C). These data suggest that carbachol is not activating M3 muscarinic receptors in these cultured human airway smooth muscle cells.

In contrast, we detected both the mRNA expression (Fig. 7D) and functional coupling (Fig. 7D) of the M2 muscarinic receptor in nontransformed cultured human airway smooth muscle cells. Forskolin stimulation resulted in 22.4 ± 2.4 pmol of cAMP per well per 15 min (n = 8). One micromolar carbachol decreased activity to 76.6 ± 2.6% of forskolin-stimulated activity levels (n = 8; P < 0.05) indicating appropriate coupling of the M2 muscarinic receptor to inhibition of adenylyl cyclase in these cells.

We questioned whether these nontransformed cultured human airway smooth muscle cells expressed the Gα protein as an alternative coupling pathway for the M1 muscarinic receptor in these cells. Figure 8 shows that Gα was not detected in these cells by immunoblot analysis.

**DISCUSSION**

The present study shows for the first time that the Src family tyrosine kinase member Lyn kinase is expressed at both the mRNA and protein level in human cultured airway smooth muscle cells. A second major finding is that carbachol, an M2 muscarinic receptor agonist in these cells, activates Lyn kinase by a pertussis toxin-insensitive signaling pathway.

Fyn, c-Src, and Yes have previously been identified in colonic (37) and vascular smooth muscle (9). Our findings for c-Src, Fyn, and Yes in cultured human airway smooth muscle cells also agree with the currently held view that these three Src family tyrosine kinases are nearly ubiquitously expressed (42). Lyn kinase, however, was thought to be preferentially expressed in B lymphocytes (28, 38, 42). Our data demonstrating both mRNA and protein for Lyn kinase in cultured human airway smooth muscle cells and the identification by immunoblotting of Lyn kinase in a vascular smooth muscle cell line derived from human aorta (5, 23) and in smooth muscle cells harvested from explants of human saphenous vein (9) suggest wider expression of this kinase at least in smooth muscle. Dumler and coworkers (5) also identified Hck and Fgr in cultured aortic smooth muscle cells by immunoblot analysis. We did not attempt to identify these Src kinases by immunoblotting in cultured human airway smooth muscle cells because mRNA for FGR and HCK was not found by RT-PCR. It is unlikely that the primers that were used failed to identify these kinases
Src kinases in our airway cells because these primers identified mRNA for FGR in Ramos cells and mRNA for HCK in RNA isolated from human spleen. FRK (also known as RAK) was identified by RT-PCR in airway smooth muscle cells. Frk is expressed in epithelial and some other cell types but has not, to our knowledge, been identified in airway or other types of smooth muscle (2, 35).

To study the role of Lyn kinase in G protein-coupled receptor signaling, wild-type human Lyn was overexpressed in human airway smooth muscle cells using an HIV-1 lentivirus-based transduction system (16). A three-plasmid expression system was used to generate the lentiviral vector particles. This system has three advantages over other systems. It can be used on relatively slowly dividing cells such as airway smooth muscle (25), does not require a cell surface receptor for entry into the target cell but mediates viral entry through lipid binding and plasma membrane fusion (27), and allows higher transduction efficiency than most other methods in smooth muscle cells. Airway smooth muscle cells transduced with wild-type Lyn kinase were used in both the Lyn kinase activation studies and studies using an antibody that recognizes Lyn kinase phosphorylation at Tyr508, although Lyn kinase activation was also detected in nontransformed cultured human airway smooth muscle cells.

Two important tyrosine residues implicated in Lyn kinase regulation are the Tyr397 activation site and the Tyr508 inhibition site (41). Tyr508 is phosphorylated by Csk to inactivate Lyn kinase and dephosphorylated by a phosphatase allowing Lyn to undergo a conformation switch and become activated. The decrease in LynTyr508 phosphorylation after carbachol treatment is evidence for Lyn kinase activation. An α-pLyn-Tyr508 antibody has been used to demonstrate IL-6 activation.

Fig. 7. A: RT-PCR of mRNA encoding the M3 muscarinic receptor. Cultured human airway smooth muscle cells did not express detectable levels of mRNA encoding the M3 muscarinic receptor. Chinese hamster ovary (CHO) cells stably overexpress the M3 muscarinic receptor used as positive control (468-bp product). CHO cells stably overexpressing the M3 muscarinic receptor and reaction tubes with no RNA input are shown as negative controls. B: RT-PCR of mRNA encoding the M3 muscarinic receptor. Cultured human airway smooth muscle cells express mRNA encoding the M3 muscarinic receptor (253-bp product). CHO cells stably overexpressing the M3 muscarinic receptor are used as positive control. CHO cells stably overexpressing the M3 muscarinic receptor and reaction tubes with no RNA input are shown as negative controls. C: Inositol phosphate synthesis in response to carbachol in cultured human airway smooth muscle cells and CHO cells stably expressing the human M3 muscarinic receptor (positive control). Carbachol (1–100 μM, 30 min) failed to increase total inositol phosphate synthesis in human airway smooth muscle cells despite an increase from 10 μM bradykinin. Carbachol effectively stimulated total inositol phosphate synthesis in CHO M3 cells in a dose-dependent manner. N = 3. *P < 0.05 compared with basal inositol phosphate synthesis. D: 1 μM carbachol significantly inhibited 10 μM forskolin-stimulated adenyl cyclase activity in cultured human airway smooth muscle cells indicating classic coupling of expressed M3 muscarinic receptors in these cells. N = 8. *P < 0.05. MW, molecular weight.

Fig. 8. Representative immunoblot of Gα protein. Homogenates of cultured human airway smooth muscle cells (100 μg) were subjected to immunoblot analysis along with rat brain homogenate (positive control). No detectable expression of Gα was identified in airway smooth muscle cells.
of Lyn in human myeloma cells (15, 41). Although it is possible that the antibody used in this study to detect Lyn phosphorylation may cross-react with the inhibitory residue of other Src family kinases, it is largely detecting Lyn kinase in these studies, as Lyn is the dominant Src family member expressed in the cells overexpressing Lyn, and an immunoreactive band was barely detectable using this pLynTyr508 antibody in native human airway smooth muscle cells.

Radioligand binding and Northern blot analysis demonstrate that the predominant subtypes of muscarinic receptors in human airway smooth muscle are M₂ and M₃ (21). No evidence of other muscarinic receptor subtypes has been reported (21). M₂ muscarinic receptors signal to inhibition of adenyl cyclase via the activation of Gₛ, whereas M₁ receptor activation leads to phosphatidylinositol turnover via activation of Gₛ. In contrast to intact airway smooth muscle, cultured human airway smooth muscle cells have detectable levels of mRNA encoding the M₂ muscarinic receptor and functional coupling to Gₛ but no detectable levels of mRNA encoding the M₃ muscarinic receptor and no activation of inositol phosphate by carbachol. These data agree with previously published data in these cells demonstrating expression of M₂ muscarinic receptors that couple to Gₛ protein (7, 39) but not M₃ muscarinic receptors. The loss of M₃ muscarinic receptors in the cultured cells is not a weakness of the model system, but rather a strength, because it allows activation of the M₂ receptors by cholinergic agonists without the confounding activation of M₃ receptors.

There are a number of explanations for the data generated in this study showing that carbachol activated Lyn kinase by a pertussis toxin-insensitive pathway. Wan and coworkers (40) described a signaling pathway involving M₁ muscarinic receptors, Gₛ, and Lyn in DT40 cells (avian B cells). It is unlikely that carbachol signaled to Gₛ in the cultured human airway smooth muscle cells via a small number of M₁ muscarinic receptors since mRNA for the M₁ muscarinic receptor was not detected by RT-PCR in the cultured human airway smooth muscle cells but was detected in CHO cells overexpressing M₃ muscarinic receptors. Moreover, bradykinin increased total inositol phosphate synthesis in the CHO cells, whereas carbachol failed to do so in the human cultured airway smooth muscle cells. Second, it is not possible that ineffective blockade of Gₛ by pertussis toxin pretreatment occurred in our preparation since pertussis toxin attenuated the carbachol-induced increase in ERK/MAPK phosphorylation. Third, it is not possible that carbachol via an M₂ muscarinic receptor signaled to the pertussis toxin-insensitive Gₛ protein Gₛ as Gₛ is not expressed in these cells. It is more likely that Lyn directly interacts with the M₂ muscarinic receptor independently of the Gₛ protein. There is precedence for Src family kinases interacting directly with G protein-coupled receptors, including evidence that the M₄ muscarinic receptor contains an SH3 binding domain (31).

In summary, we show for the first time that the Src family tyrosine kinase member Lyn kinase is expressed in human cultured airway smooth muscle cells and that carbachol, an M₂ muscarinic receptor agonist in these cells, activates Lyn kinase by a pertussis toxin-insensitive signaling pathway. One may speculate that this signaling pathway may play a role in cell proliferation, cell migration, or actin reorganization.

ACKNOWLEDGMENTS

We thank Dr. Jeremy Luban for providing the pFUOW, pCMVΔR8.91, and pMD.G plasmids and Martha Neagu and David Sayah for technical assistance.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-62340.

REFERENCES

23. Mukundan L, Milhorn DM, Matta B, and Suttles J. L500 LYN KINASE IN AIRWAY SMOOTH MUSCLE CELLS