Syntaxin 1A is transiently expressed in fetal lung mesenchymal cells: potential developmental roles

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Lung branching morphogenesis and epithelial cytodifferentiation require that epithelial cells interact with surrounding mesenchymal cells and/or basement membrane components. The expression of genes encoding extracellular matrix, growth factors, and growth factor receptors has been shown to be developmentally regulated in the lungs of rats and mice (53, 56). Many growth factors produced by epithelial cells in the developing lung can promote type II cell differentiation in a mesenchymal cell-dependent fashion (19, 26, 33, 42, 49, 52). These factors include epidermal growth factor (45, 50, 63), parathyroid hormone-related protein (32), basic fibroblast growth factor (FGF) (22), and platelet-derived growth factor (23). Other epithelial cell-derived growth factors such as FGF10, bone morphogenetic protein-4, epidermal growth factor, and bombesin-like peptide (BLP) have also been implicated in the induction of branching morphogenesis (5, 6, 29, 42). Pulmonary mesenchymal cells can signal the developing epithelium through the secretion of extracellular matrix components such as laminin, tenascin, and nidogen (51, 53, 67) or alternatively via secreted growth factors such as acidic FGF and keratinocyte growth factor (15). Recent work (4, 20) has implicated the secreted mesenchymal cell factor sonic hedgehog and members of the Gli gene family in pulmonary embryogenesis. It appears that glucocorticoids can induce type II pneumocyte differentiation and surfactant production via a mesenchymal cell intermediary (54), potentially via a secreted substance derived from developing fibroblasts termed “fibroblast-pneumocyte factor” (48), the identity of which remains elusive.

In eukaryotic cells, regulated secretion occurs as a complex series of steps beginning with exocytic vesicle formation and leading to docking and fusion of the secretory vesicle with the plasma membrane (64). A conserved molecular apparatus, known as the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex, is highly conserved in a wide variety of species including yeast, plants, fruit flies, nematodes, invertebrates, and mammals (16, 27, 38). This complex is involved in the docking and fusion steps of the vesicular transport and membrane trafficking pathways of several cell types. The primary components of this machinery have been identified, and their molecular interactions have been examined. In the process of vesicle docking and fusion, a core complex is formed between the vesicular SNAREs, vesicle-associ-
ated membrane protein (VAMP), and synaptotagmin located on the vesicular membrane surface and the terminal SNAREs, syntaxin, and synapse-associated protein-25 (SNAP-25) located at the plasma membrane. The exocytosis core complex then recruits soluble factors such as NSF and soluble NSF attachment proteins (α-, β-, and γ-SNAPs) to form the mature 20S fusion complex. Disruption of the mature complex occurs through NSF-mediated ATP hydrolysis.

Syntaxins are terminal SNAREs that have been defined primarily in terms of their role in cellular secretion (7). As plasma membrane proteins, syntaxins form part of the secretosomal docking complex involved in exocytosis and were originally described in neurons as regulators of synaptosomal trafficking. In mammals, syntaxin (Stx) 1A was originally identified in a limited analysis as being neural or neuroendocrine specific (7) but has since been reported in malignant colonic epithelial cells as well (8). Other syntaxins have been shown to be widely distributed in a variety of tissues including neurons, endocrine cells, and exocrine cells, in which they are also involved in regulated exocytosis (7, 18, 37, 44).

Syntaxins have been implicated in developmental processes. Stx1 is required for cellularization of Drosophila embryos (11), and a syntaxin homolog in Arabidopsis is required for proper seedling development (38). Hirai et al. (24) found that murine lung buds cultured with an antibody to epimorphin demonstrated impaired morphogenesis, but the mechanism underlying this observation has not been clarified. Subsequently, epimorphin was found to be identical to Stx2 (7).

Here we examine the expression and function of Stx1A during late fetal and early postnatal lung development in the rat. Initially, we anticipated that Stx1A would be localized only to developing neurons and possibly to neuroendocrine cells in view of its known specific immunolocalization to the enteric nervous system in the developing rat gut (10). Consistent with previous observations (7, 18, 44), we did observe some Stx1A expression in pulmonary nerve fibers, epithelial cells, and neuroendocrine cells. Surprisingly, however, most of the Stx1A mRNA and protein is associated with a mesenchymal cell population that is lipid rich and transiently expressed. We demonstrate that BLP promotes the differentiation of isolated fetal mesenchymal cells into lipofibroblasts and that the effect of BLP on isolated type II pneumocytes differentiation requires the presence of these lipofibroblasts. Finally, the anti-Stx1A antibody HPC-1 completely blocks the effect of BLP on type II cell differentiation in fetal lung organ cultures while it promotes cell proliferation. These observations support a role for Stx1A in mediating lipofibroblast secretory processes that contribute to terminal differentiation of the pulmonary epithelium.

METHODS

Materials. Collagenase A (~ 0.85 U/mg) and Protease Inhibitors Complete were obtained from Boehringer Mannheim (Indianapolis, IN). Dulbecco’s modified Eagle’s medium (DMEM) and Hank’s balanced salt solution (HBSS) were purchased from Gibco BRL (Life Technologies, Gaithersburg, MD); filter-sterilized fetal calf serum (FCS) was obtained from Atlanta Biologicals (Norcross, GA), Anti-Stx1A antibody (HPC-1), MOPC21, and rat IgG were obtained from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was supplied by Bio-Rad (Hercules, CA).

Primary fetal cell culture. Pregnant Sprague-Dawley rats were killed on day 19 of gestation. The fetal lungs were obtained, and the surrounding tissues (heart, thymus, extra-pulmonary airways, and large blood vessels) were discarded. The lungs were minced into 0.1-mm cubes with a Mclwaine tissue chopper. The minced explants were placed into a sterile culture flask containing 15 ml of HBSS (~1 ml/lung) and collagenase (0.2 mg/ml). The flask was sealed and gently agitated at 37°C for 30 min, then an equal volume of ice-cold HBSS was added and the entire mixture was filtered through two layers of sterile gauze into sterile glass centrifuge tubes (Kimax). The cells were then sedimented by centrifugation at ~20 g for 4 min, and the cell pellet was washed again in HBSS.

The mixed fetal pulmonary cells were resuspended in warm serum-free DMEM (~1 ml/lung) and plated onto 100-mm Corning tissue culture plates (10 ml/plate) for incubation. Some of the minced lung explants were placed directly into tissue culture plates and grown in DMEM with 10% (vol/vol) FCS.

Isolation of fetal pulmonary fibroblasts. The plates containing a mixture of fetal pulmonary cells were incubated for 20 min at 37°C in humidified 5% CO2. After incubation, the supernatant and nonadherent cells were removed for the preparation of epithelial cells enriched for type II pneumocytes (see Isolation of pulmonary epithelial cells). Fresh DMEM with 10% (vol/vol) FCS was added to the plates containing the adherent pulmonary fibroblasts.

Isolation of pulmonary epithelial cells. Falcon bacteriologic plates were coated with 5 ml of a solution of rat IgG (0.5 mg/ml) suspended in 50 mM Tris (pH 8.8) for 3-6 h at room temperature. The plates were washed three times with HBSS and once with serum-free DMEM.

Plates containing mixed fetal pulmonary cells were incubated for 20 min at 37°C in humidified 5% CO2. After this, the nonadherent cells were harvested by gently swirling the plates and transferred to IgG-coated plates, which were incubated at 37°C for 1 h under the same conditions. The supernatant containing the nonadherent type II cells was then removed and placed on fresh tissue culture plates, with FCS added to a final concentration of 10% (vol/vol).

RT-PCR and Southern hybridization analysis. Total RNA was prepared from whole lungs and cell cultures by homogenization as previously described (13) with TRIzol reagent purchased from Molecular Research Center (Cincinnati, OH). Single-stranded cDNA was prepared with total lung RNA (2 µg), Moloney murine leukemia virus RT and random hexamers as described elsewhere (55). Parallel reactions were carried out on each sample without adding RT, hereafter designated RT−reactions. Five percent of the RT reaction volume was then used for PCR. A 652-bp DNA fragment coding for Stx1A was amplified by PCR with Stx1A.F (5′-GAA AAC GTG GAG GAC CTT CC-3′) and Stx1A.R (5′-ATC TTC TTT CGT GCT GCC TTT-3′) as the reverse primer. In addition, an 825-bp DNA fragment coding for Stx2 (epimorphin (EPI)) was amplified with EPI.F (5′-TGA AAT ACT CTT TGG GAT GCC TCT GCC-3′) as the forward primer and EPI.R (5′-TGA TTT GCC AAC CGA CAA GCC-3′) as the reverse primer. Rat syntaxin sequences were taken from Genbank accession numbers U35039 through U35047. As neural or neuroendocrine markers, rat gastrin-releasing peptide (GRP) and Dopac decarboxylase (DDC) were also amplified from...
certain RT reaction products. A 283-bp DNA fragment coding for GRP was amplified by PCR with RGRP.F (5'-ACT GGG CTG TAG GAC ACT T-3') as the forward primer and RGRP.R (5'-GAG AAC CTG GAG CAG AGA GTC TAC CTT-3') as the reverse primer. A 501-bp DNA fragment coding for DDC was amplified with DDC.F (5'-TTC TTC GCT TAC TTC CCC ACG G-3') as the forward primer and DDC.R (5'-GAG AAC CTG GAG CAG AGA GTC TAC CTT-3') as the reverse primer. All PCRs were carried out with the Hot-Start protocol for 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 120 s.

As an internal control for the amplification, rat 18S RNA was amplified from the same original volume of the RT reaction. A 568-bp DNA product was amplified with 18S.F (5'-TAG CTC TTT CTC GAT TCC GTG G-3') as a forward primer and 18S.R (5'-ATG ATC CTT CCG CAG GTT CAC-3') as a reverse primer. PCR was carried out as above for 21 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 120 s.

To control for possible DNA contamination, 5% of each RT reaction was subjected to PCR under the same conditions as the RT reactions. The primers used for Stx1A and Stx2 (epimorphin) span multiple introns so that genomic DNA clearly would yield a larger band than the predicted size of cDNA amplification. None of our negative control "RT reactions yielded RT-PCR products.

The PCR products were run on an 0.8% agarose gel, transferred by capillary blotting to a nitrocellulose filter, and hybridized to a specific 32P-labeled oligonucleotide probe internal to the amplified sequence as follows: Stx1A, Stx1A.2F (5'-CCC GAT GAG AAG ACC AAG GAG-3'); syntaxin 2 (epimorphin), EPI.2F (5'-TGG ATG CGG CCT TTG CTT CGC-3'); GRP, GRP.2F (5'-TTT CTG GCC GCT TCG GCC-3'); and DDC, DDC.2F (5'-TTT TTG GCT GGA AGA GCT GGG G-3'). All filters were washed several times under high-stringency conditions (5× saline-sodium citrate at 60–65°C) and exposed to autoradiography film with intensifying screens.

Protein analysis. Protein lysates were prepared with a functional protein lysis buffer composed of 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, and 1× Protease Inhibitor Complete as previously described (28). All protein lysates were assayed to determine their protein content with the Bio-Rad assay with a BSA standard.

For Western blotting, 40 µg of protein lysate were subjected to SDS-PAGE on a 12% polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. Transfer was assessed with Ponceau S staining (Bio-Rad). The nitrocellulose filters were reacted first with a monoclonal mouse anti-rat Stx1A antibody (HPC-1) at a 1:4,000 dilution followed by a biotinylated horse anti-mouse IgG at a 1:1,000 dilution. The filters were then treated with avidin and biotin conjugated to horseradish peroxidase (ABC Reagent, Vector Laboratories) and visualized by chemiluminescence with the HRPL kit (National Diagnostics, Atlanta, GA).

Immunohistochemical and histochemical analyses. For immunoperoxidase studies, lungs harvested from ED19 rat
Fetuses were fixed for 4 h in 4% paraformaldehyde and placed in 30% sucrose in PBS overnight before being embedded in optimum cutting temperature compound and frozen on powdered dry ice. Three-micrometer frozen sections were prepared, and immunoperoxidase analyses were carried out with a mouse monoclonal anti-Stx1A antibody (HPC-1) at a 1:50 dilution and the avidin-biotin complex immunoperoxidase technique with diaminobenzidine and biotinylated tyramide (tyramide system amplification) as a substrate as previously described (60). In addition, serial 3-µm sections were used for immunostaining for the neural or neuroendocrine markers calcitonin gene-related peptide and protein gene product (PGP) 9.5, the general epithelial marker keratin, and the mesenchymal markers desmin and vimentin as previously described (21, 58, 59).

For detection of lipids in tissue sections, frozen sections of lungs harvested from ED19 rat fetuses were stained for lipids with 0.5% oil red O in 60% isopropanol for 15 min, with pre- and posttreatment 60% isopropanol washes (14). Serial frozen sections were used for immunoperoxidase analyses for Stx1A as described above.

Lung organ culture. Fetal lung was harvested on ED18 at 4°C in Waymouth medium supplemented with 5% FCS. The tissue was chopped into 0.5-mm cubes and cultured in six-well plates with the same medium with and without added BLP or other agents. Cultures were grown for 72 h at 37°C in 5% CO₂ on a rocking platform at 3 oscillations/min (57). Tissue viability was checked by histological analyses, satisfactory baseline incorporation of [³H]choline and [³H]thymidine, intact 18S and 28S RNA bands on ethidium gels, and good positive control responses with dexamethasone (Dex) and GRP after 72 h.

Determination of [³H]DNA, [³H]disaturated phosphatidylcholine, DNA, and protein content. After 68 h of culture, tissue was incubated with [³H]thymidine (4 µCi/ml) or [³H]choline (16 µCi/ml; NEN-Dupont, Boston, MA) for 4 h at 37°C in 5% CO₂-air on a rocking platform before being harvested and analyzed for [³H]DNA, DNA content, [³H]disaturated phosphatidylcholine, and protein content. [³H]thymidine incorporation into acid-precipitable counts was carried out as previously described (57). DNA was assayed after trichloroacetic acid precipitation with the method of Burton (12). For determination of the rate of surfactant phospholipid synthesis, lipids were extracted from cell homogenates with chloroform-methanol, and [³H]choline incorporation into phosphatidylcholine was determined as previously described (61). Protein was determined with the method of Bradford (9), with BSA as the standard. Experimental values were normalized by defining the mean of the control groups as baseline and expressing the values as percent changes above or below this.

Statistical analyses. Numerical data were analyzed with unpaired Student’s t-test, with values expressed as means ± SE.

Fig. 2. Stx1A expression in primary fetal lung cell cultures. A: RT-PCR analyses for mRNAs encoding Stx1A and Stx2 (epimorphin) in primary fetal lung cell cultures. RT-PCR of 18S rRNA was used as a control to verify integrity of RNA and reverse transcripts. Lane 1, mixture of fetal pulmonary cells; lane 2, fetal pulmonary fibroblasts; lane 3, fetal pulmonary epithelial cells (enriched for type II pneumocytes); lane 4, intact pulmonary explants grown in culture. B: Immunoblot for Stx1A (HPC-1) in same primary cell cultures. No. on right, molecular mass. C: densitometry of RT-PCR analyses was carried out with Hewlett-Packard ScanJet 4c, and area of bands was integrated with Scan Analysis ver. 2.55 software program for Macintosh. Mix, mixture of fetal pulmonary cells; Fibro, fetal pulmonary fibroblasts; Epith, fetal pulmonary epithelial cells (enriched for type II pneumocytes); Explant, intact pulmonary explants grown in culture. Values were normalized for relative amounts of 18S RNA and are means ± SE. P value compared with fetal fibroblasts. D: densitometry of Western chemiluminescence analysis was carried out as in C. Peak protein levels for Stx1A are present in mesenchymal fraction.
**RESULTS**

Syntaxin mRNA and protein analyses. RT-PCR analyses of rat fetal lungs during development demonstrated elevated Stx1A mRNA levels between ED16 and ED19, with lower levels from ED20 through postnatal development. These are semiquantitative RT-PCR analyses comparing the relative levels of mRNAs normalized to the relative levels of 18S RNA. Each lane represents a pool of mRNA from at least 20 fetal lungs (lungs from 2 litters with 10–14 fetuses/litter), so that each blot demonstrates averaged data from numerous fetal lungs. RT-PCR was carried out three times (with two separate pools of RNA), consistently with the same result, i.e., that peak expression occurs at ED19. For comparison, mRNAs encoding additional components of a potential secretosomal complex were also analyzed, including Stx2 (epimorphin), Rab26, and Munc18; all mRNAs were most highly expressed at ED19 (Fig. 1, A and C, and data not shown). Levels of 18S rRNA were near equivalence in all lanes. All RT-PCRs were carried out with conditions such that positive control RNA (normal rat brain in the present study) was in the linear range of detection (65). Western blotting of protein lysates from the same fetal lungs demonstrated elevated Stx1A protein levels between ED17 and ED20, peaking at approximately ED18–19 (Fig. 1, B and D). These Western analyses were carried out twice with two separate pools of protein lysates, with at least 20 fetal lungs at each time point, with the same result, i.e., that peak Stx1A protein levels occur at ED18–19. It should be noted that the Western analyses were developed with chemiluminescence in which detection was semiquantitative rather than absolutely quantitative due to the rapid extinction of the substrate over time.

RT-PCR analysis of primary cell cultures from fetal rat lungs on ED19 (Fig. 2A) demonstrated expression of the Stx1A gene in the mixed cell population before enrichment (lane 1), >95% fetal fibroblasts (lane 2), >95% distal lung epithelial cells enriched for type II pneumocytes (lane 3), and intact whole lung explants (lane 4). Stx1A mRNA levels in the fibroblast cultures were consistently over threefold greater than those in type II cell cultures (Fig. 2C). In contrast, there was no significant difference in Stx2 mRNA expression in fibroblasts versus epithelial cells (Fig. 2, A and C). 18S rRNA was expressed at similar levels in both mesenchymal and epithelial cell populations. Western blotting of protein lysates from the same primary cultures demonstrated detectable Stx1A protein in all of the primary cell cultures, with higher levels of Stx1A in the mesenchymal cell cultures (Fig. 2B, lane 1, and C), consistent with the results of RNA analyses.

We carried out immunostaining of these same cell populations grown on glass coverslips to verify that the
fibroblast cultures were composed of >95% vimentin-positive and <5% Clara cell 10-kDa secretory protein (CC10) and surfactant protein C, keratin-positive cells (200 cells counted for each of 2 experiments). Conversely, the lung epithelial cultures enriched for type II pneumocytes were composed of >95% keratin-positive cells (approximately two-thirds surfactant protein C positive and one-third CC10 positive) and <5% vimentin-positive cells (200 cells counted for each of 2 experiments; data not shown). Over half of the cells in the fibroblast cultures were strongly Stx1A positive, whereas <20% of cells in the type II cell-enriched cultures were weakly Stx1A positive, with additional rare strongly Stx1A-positive cells having multiple dendritic processes, consistent with neuroendocrine cells (data not shown).

To further assess each of the primary cultures for the presence of neuroendocrine or neuronal cells, we carried out RT-PCR for DDC and GRP. The mesenchymal cell cultures demonstrated a complete absence of DDC and GRP mRNAs on ethidium gels, with only trace amounts in the type II cell-enriched populations. Southern blotting of the RT-PCR products demonstrated barely detectable levels of DDC and GRP mRNAs in the fibroblast cultures and low levels in the type II cell cultures relative to the cell mixtures and intact cell explants (data not shown).

Immunolocalization studies. Using the Stx1A-specific antibody HPC-1 (3), we carried out definitive immunolocalization studies in intact ED19 fetal lung frozen sections (Figs. 3–5). In the conducting airways, Stx1A was present at high levels in occasional clusters of neuroendocrine cells, representing <5% of the airway epithelial cells (Fig. 3A, between red arrows). Stx1A was also present at high levels in nerve fibers in the submucosa and muscularis (Fig. 3A, purple arrows). In serial sections, the neuroendocrine cells immunostained for PGP9.5 (Fig. 3B, between red arrows) and calcitonin gene-related peptide (Fig. 3C, between red arrows). The nerve fibers immunostained for both Stx1A (Fig. 3A) and PGP9.5 (Fig. 3B, purple arrows). An additional serial section immunostained in parallel with PGP9.5 antiserum that had been preabsorbed with PGP9.5 (21) is devoid of immunostaining (Fig. 3D). There was weak immunostaining of the nonneuroendocrine epithelial cells for both Stx1A (Fig. 3A) and PGP9.5 (Fig. 3B, left side of left red arrow), consistent with our previous published results in human fetal lungs (21).

Light-microscopic analysis of cross sections of ED19 rat lung demonstrated the most prevalent Stx1A staining in undifferentiated mesenchymal cells, with the highest levels surrounding developing blood vessels (Fig. 4B, blue arrow) and moderate levels occurring around the conducting airways (Fig. 4B, AW) and in the interstitium of the primitive alveoli (Fig. 4B, alv, white arrows). This staining pattern markedly differs from that of keratin immunostaining in an immediately serial section (Fig. 4A) in which the epithelium of the conducting airways was strongly positive and the developing alveolar epithelium was weakly positive, whereas mesenchymal cells were negative. Most of the Stx1A-positive undifferentiated mesenchymal cells are also positive for vimentin (Fig. 4C) and desmin (data not shown).

**Fig. 4.** Immunostaining of E19 fetal rat lung for Stx1A vs. epithelial and mesenchymal markers. A: pattern of keratin immunostaining is quite distinctly apparent in airway epithelium (AW) in a 3-µm section of E19 fetal rat lung. There is only weak keratin immunostaining of primitive alveolar epithelium (alv, white arrows). Blood vessels (v, blue arrow) are negative as are mesenchymal cells (tissue section 30). B: Stx1A immunostaining is most prominent in undifferentiated mesenchymal cells surrounding developing blood vessels and in interstitium of primitive alveoli in an adjacent serial section (tissue section 31). C: most of Stx1A-positive undifferentiated mesenchymal cells are also moderately positive for vimentin in another serial section (tissue section 32). However, endothelial cells and desmin-positive differentiated smooth muscle cells surrounding airways and blood vessels are not Stx1A positive. Original magnification, ×100.
oil red O as described in METHODS (14). Note intracellular red lipid HPC-1 immunostaining (Fig. 5) carried out oil red O staining for lipids in parallel with determine whether the Stx1A-positive cells might in- lipocytes, or lipid fibroblasts, in the developing lung. To determine whether the Stx1A-positive cells might in- lung explants. To determine whether the effect of BLP on type II cell differentiation requires the presence of cocultured fibroblasts, similar to Dex (54), we analyzed [3H]choline uptake into saturated phosphatidylcholine as the rate-limiting step in surfactant phospholipid synthesis. As shown in Fig. 6A, there was no BLP- induced increase in choline uptake in ED18 type II cell cultures in the absence of added mesenchymal cells, whereas there was over a 50% increase in choline uptake in the presence of ED18 fetal fibroblasts (P < 0.001). Furthermore, BLP induced a significant increase in [3H]triglyceride uptake directly into ED18 fetal fibroblasts (62), suggesting that triglyceride uptake by fibroblasts is hormonally inducible by BLP, similar to Dex (Fig. 6B) (46).

We then evaluated the potential role of Stx1A in mediating the effects of optimal concentrations of BLP and Dex on type II cell differentiation and DNA synthesis in ED18 whole lung organ cultures. We carried out five experiments analyzing inhibition of function in fetal lung organ cultures, comparing the well-characterized anti-Stx1A blocking monoclonal antibody (murine IgG1) HPC-1 (7, 66) to the irrelevant murine IgG1 MOPC-21. HPC-1 completely blocked the effect of BLP (1 nM) on [3H]choline uptake and reduced the effect of Dex to less than half of control values as shown in Fig. 6C (both P < 0.001 comparing HPC-1 with MOPC). In parallel cultures, [3H]thymidine incorporation was stimulated by HPC-1 (Fig. 6D), augmenting the small but significant BLP effect on cell proliferation (P < 0.03) and reducing the growth inhibitory effect of Dex (P < 0.005).

HPC-1 also blocks baseline choline incorporation and augments baseline [3H]thymidine incorporation in a dose-dependent fashion (Fig. 6E), indicating a significant role for Stx1A in lung automated and growth cessation.

The HPC-1 antigen was originally determined to be extracellular in location (2, 25). However, a more recent analysis (7) has demonstrated that the HPC-1 antigen may be cytoplasmically oriented. Regardless of its orientation, we do observe highly reproducible effects of HPC-1 on thymidine incorporation and choline incorporation in the fetal samples. The developing cells in fetal lung explants are well recognized to take up antisense oligodeoxynucleotides in culture experiments in the absence of any agent to enhance cellular permeability, and antisense oligodeoxynucleotides can clearly lead to functional and molecular alterations in the explant system (34, 36, 43, 63). The developing cells apparently actively endocytose or pinocytose the culture medium, which we believe to be the case in our experiments. To unequivocally test this hypothesis, we have carried out immunoperoxidase analyses using biotinylated horse anti-mouse IgG (IgG fraction) versus biotinylated normal horse IgG at the same protein concentration as the primary antisera on sections of cultured ED19 rat lungs exposed to medium alone, HPC-1, or the irrel-
Fig. 6.[^3]H-choline (PC) and [^3]H-thymidine uptake in E19 fetal rat lungs are modulated by bombesin-like peptide (BLP) and HPC-1. A: primary isolates of E19 rat type II (TII) cells with and without fibroblasts (Fibro) from same lungs were cultured in absence (Neg) and presence of BLP (gastrin-releasing peptide) for 3 days before determination of choline uptake as described in METHODS. Pooled results presented represent 3 independent experiments. *P < 0.001 compared with untreated control cultures. B: primary E19 fetal rat lung Fibro were cultured in absence and presence of BLP for 24 h before determination of [^3]H-triglyceride uptake as previously described (46). Pooled results represent 3 independent experiments. **P < 0.01 compared with untreated control cultures. C: choline uptake in lungs treated with dexamethasone (Dex) or BLP with 100 µg/ml of HPC-1 or MOPC. Pooled results represent 5 independent experiments. P value compared with MOPC cultures. D: thymidine uptake in lungs treated with Dex or BLP with 100 µg/ml of HPC-1 or MOPC. Pooled results represent 5 independent experiments. P values compared with MOPC control cultures. E: thymidine (Th) and choline (Ch) uptake in lungs incubated for 72 h with HPC-1 or MOPC. [IgG1], IgG1 (HPC-1 or MOPC) concentration. Pooled results represent 3 independent experiments. P value compared with MOPC control cultures.
evant isotype-matched murine IgG1 MOPC as our negative control (which was used in all of our HPC-1 functional experiments). These immunohistochemical analyses clearly demonstrate murine IgG (both HPC-1 and MOPC) within the cytoplasm of numerous mesenchymal cells in the rat fetal lung explants (data not shown), indicating that the monoclonal antibodies are taken up by the cells in culture and retained in sufficient quantities for visualization in tissue sections. There was no immunostaining observed with normal horse IgG instead of horse anti-mouse IgG with the exception of occasional alveolar macrophages. Sections of the same lungs incubated without IgG added to the culture medium did not immunostain with horse anti-mouse IgG.

DISCUSSION

The present study demonstrates the expression of mRNAs encoding multiple synaptosomal proteins in rat fetal lungs, including Stx1A, Stx2 (epimorphin), Rab26, and Munc18, all of which peak at ED19, immediately before type II cell differentiation and the onset of surfactant synthesis. We have explored the distribution and function of Stx1A in depth because this molecule is most highly expressed in mesenchymal cells that did not express detectable markers of neuroendocrine or neuronal differentiation by RT-PCR or immunostaining.

There are at least three potential mechanisms for Stx1A-mediated effects on cell differentiation and growth in fetal lungs both at baseline and induced by Dex and BLP. First, Stx1A could be acting as a critical relay molecule, regulating the secretion of other mesenchymal cell-derived molecules similar to its role in regulated exocytosis from neurons and neuroendocrine cells (30). Second, Stx1A could regulate the topographical translocation of membrane-bound molecules from the interior of vesicles to the external surface of the plasma membrane and thus could alter the availability of receptors on the cell surface. Such a role for exocytosis core complex proteins has been implicated for receptors such as GLUT-4 in pancreatic cells as well as for transporters such as aquaporin in renal collecting duct cells (39, 41). Third, Stx1A, or a fragment of Stx1A, could be secreted by fetal mesenchymal cells to function directly as developmental signaling molecules (31, 47), although it is probably unlikely that it directly interacts with the target cell. We cannot rule out the possibility that HPC-1-mediated inhibition of growth factor secretion by neuroendocrine cells or other epithelial cells might be contributing to the dose-dependent decline in baseline choline uptake in fetal lung organ cultures.

A previous study (17) indicated that Stx1A is not expressed in mature adipocytes. The mesenchyme is known to play an important role in specifying epithelial cell fates. The expression of Stx1A in a transient population of lipid-rich fibroblasts is of particular interest because this syntaxin was previously demonstrated to be expressed only by neurons and neuroendocrine cells in adult mammals, the only exception being one report of two malignant colonic epithelial cells (8). The peak of Stx1A mRNA and protein expression on ED19 coincides with the peak expression of several developmental signaling molecules (53), including the receptor for GRP, the major known pulmonary BLP (1).

A significant role for Stx1A in rat pulmonary development is indicated by the observed effects of HPC-1 on type II cell differentiation and cell proliferation in fetal lung organ cultures. The ability of HPC-1 to block Dex- and BLP-induced type II cell differentiation supports a role for secretory fibroblasts in these processes (35). The dose-dependent increase in new DNA synthesis and the parallel decrease in new surfactant production suggest that Stx1A can indirectly regulate both cell proliferation and type II pneumocyte differentiation. A role for syntaxins in development is not without precedent. Cellular proliferation has been demonstrated to be regulated by syntaxin 1 in Drosophila embryos (11). A syntaxin homolog in Arabidopsis is required for proper seedling development (36). Hirai et al. and Oka and Hirai demonstrated that syntaxin 2 (epimorphin) could play a role in early lung morphogenesis (24) and induce human endothelial cells in culture to form tubular structures resembling capillaries (47).

In summary, the present study demonstrates Stx1A gene expression is developmentally regulated in fetal lung mesenchymal cells and that Stx1A can, in turn, regulate both cell differentiation and proliferation in a dose-dependent fashion, probably as a key component of the secretory complex mediating fibroblast exocytosis in fetal lungs.

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