

The Phosphatidylinositol 3-Kinase/Akt Signaling Pathway Modulates the Endocrine Differentiation of Trophoblast Cells

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Activation of Lyn, a Src-related nonreceptor tyrosine kinase, in trophoblast cells is associated with trophoblast giant cell differentiation. The purpose of the present work was to use Lyn as a tool to identify signaling pathways regulating the endocrine differentiation of trophoblast cells. The Src homology 3 domain of Lyn was shown to display differentiation-dependent associations with other regulatory proteins, including phosphatidylinositol 3-kinase (PI3-K). PI3-K activation was dependent upon trophoblast giant cell differentiation. The downstream mediator of PI3-K, Akt/protein kinase B, also exhibited differentiation-dependent activation. Lyn is a potential regulator of the PI3-K/Akt

signaling pathway, as are receptor tyrosine kinases. Protein tyrosine kinase profiling was used to identify two candidate regulators of the PI3-K/Akt pathway, fibroblast growth factor receptor-1 and Sky. At least part of the activation of Akt in differentiating trophoblast giant cells involves an autocrine growth arrest-specific-6-Sky signaling pathway. Inhibition of PI3-K activities via treatment with LY294002 disrupted Akt activation and interfered with the endocrine differentiation of trophoblast giant cells. In summary, activation of the PI3-K/Akt signaling pathway regulates the development of the differentiated trophoblast giant cell phenotype. (Molecular Endocrinology 16: 1469–1481, 2002)

THE PLACENTA is an extraembryonic tissue that permits the embryo to develop within the female reproductive tract. The parenchymal cells of the placenta are derived from the trophoblast cell lineage. The appearance of trophoblast cells represents the initial differentiation event of embryogenesis (1). Trophoblast cells go on to differentiate along a multilineage pathway (2–7). Each branch of the trophoblast lineage develops specialized functions required for successful pregnancy. Disruptions in trophoblast development can lead to early pregnancy loss, intrauterine growth retardation, and tumorigenesis.

Differentiation of trophoblast cells in the rat or mouse is directed toward at least four recognizable phenotypes (4): 1) trophoblast giant cells, 2) spongiotrophoblast cells, 3) glycogen cells, and 4) syncytial trophoblast cells. Each of these cell types has unique functional and morphological attributes. The trophoblast giant cell is the first differentiated cell type to arise during development of the trophoblast lineage. Giant cells possess invasive and endocrine activities and are critically situated at the maternal interface. Trophoblast giant cell differentiation can be monitored

via morphological (DNA content and nuclear size) and gene activation end points [steroidogenic enzymes, PRL gene family, metalloproteinases, prolactin (PRL), etc.] (4, 8). Spongiotrophoblast cells are morphologically distinct from trophoblast giant cells. However, similar to trophoblast giant cells, spongiotrophoblast cells exhibit endocrine activities, including expression of a subset of members of the PRL gene family. Glycogen cells accumulate glycogen and are a potential energy reserve (9). Syncytial trophoblast cells are multinucleated cells that probably arise from the fusion of trophoblast progenitor cells and have been implicated in bidirectional transport of nutrients and wastes (10, 11).

There have been several routes to dissecting the regulation of trophoblast development. The Rcho-1 trophoblast cell line is a useful *in vitro* model system for studying trophoblast giant cell differentiation. Rcho-1 cells were derived from a rat transplantable choriocarcinoma (8, 12, 13). They represent a trophoblast stem cell population that can proliferate or can be induced to differentiate depending upon the conditions of their culture (8, 13, 14). Rcho-1 trophoblast cell differentiation is incremental, recapitulating the normal ontogeny of giant cell differentiation-specific gene expression (14, 15). These features permit dissection of regulatory mechanisms necessary for progression along the trophoblast giant cell differentiation pathway. Trophoblast stem cells represent an additional model. They have been derived from mouse blastocysts and possess features consistent with a

Abbreviations: AP-1, Activating protein-1; FBS, fetal bovine serum; FGF, fibroblast growth factor; FGFR-1, fibroblast growth factor receptor-1; Gas6, growth arrest-specific-6; GSK3 β , glycogen synthase kinase-3 β ; GST, glutathione-S-transferase; HS, horse serum; PI3-K, phosphatidylinositol 3-kinase; PL-1 or -2, placental lactogen-1 or -2; PLP-A, PRL-like protein-A; PMSF, phenylmethylsulfonylfluoride; PRL, prolactin; SH, Src homology; TS, trophoblast stem.

stem cell phenotype showing the capacity to recapitulate the development of each of the four-trophoblast cell lineages (16).

Trophoblast giant cells arise by a process referred to as endoreduplication. As giant cells differentiate they continue to synthesize DNA, but cease cell division (17). This transition involves marked alterations in regulators of the cell cycle, (18–21), cell-extracellular matrix interactions (13, 22), and the expression of specific sets of genes (4, 8). As trophoblast stem cells differentiate into trophoblast giant cells, there are dramatic changes in the activities of components of signal transduction pathways. Tyrosine kinases have been implicated in trophoblast giant cell differentiation (17, 23). Disruption of tyrosine kinase activities inhibits endoreduplication and expression of the trophoblast giant cell-specific phenotype. Among the tyrosine kinases activated during trophoblast giant cell differentiation are three members of the Src family of nonreceptor tyrosine kinases: Src, Yes, and Lyn (23). Each of the three Src family members exhibits a distinct activation pattern during the transition from proliferation to differentiation in trophoblast cells (23). Src and Yes are active in proliferating and differentiating trophoblast cells (23). Lyn is activated only in differentiating trophoblast giant cells and shows a differentiation-dependent accumulation (23). The impact of Lyn on trophoblast giant cell differentiation is presently not known. Src family members are known to regulate downstream signaling pathways that influence nuclear events (24). Src family activation can stimulate MAPKs and phosphatidylinositol 3-kinases (PI3-K), resulting in changes in gene transcription, cell survival, cell division, and cell differentiation (25).

In the present report we examine the interactions of Lyn with other trophoblast regulatory proteins and demonstrate the association of Lyn with PI3-K. The activation of PI3-K was dependent upon trophoblast giant cell differentiation, and the PI3-K/Akt pathway was shown to regulate the trophoblast giant cell phenotype.

RESULTS

Interactions between the Lyn SH3 Domain and Trophoblast Proteins

Src homology 2 (SH2) and SH3 domains represent sites of protein-protein interaction in signal transduction networks involving tyrosine kinases (26). To begin to identify components of trophoblast cell regulatory pathways involving Lyn kinase, we examined the association of Lyn SH2- and SH3-glutathione-S-transferase (GST) fusion proteins with cellular lysates from proliferating and differentiating trophoblast cells. We reasoned that proteins specifically associating with Lyn in the differentiated state might be involved in the differentiation-dependent activation of Lyn kinase or the downstream effects of Lyn kinase. Pronounced

differentiation-dependent binding of tyrosine-phosphorylated proteins was not observed for trophoblast protein interactions with GST-Lyn SH2 (data not shown). In contrast, trophoblast cellular proteins exhibited differentiation-dependent differences in their interactions with Lyn SH3. The binding of pp45–50 and pp85 phosphotyrosyl-containing proteins to the Lyn SH3 domain was differentiation dependent and was not observed for Src SH3 domain (Fig. 1).

Association of Lyn with PI3-K in Differentiating Trophoblast Cells

The association of Lyn-SH3 with a phosphotyrosyl-containing protein of 85 kDa prompted an investigation of the association of p85, the regulatory subunit of PI3-K, with Lyn. PI3-K was previously shown to associate with the SH3 domain of Src family members (27, 28) and to be part of the downstream signaling path-

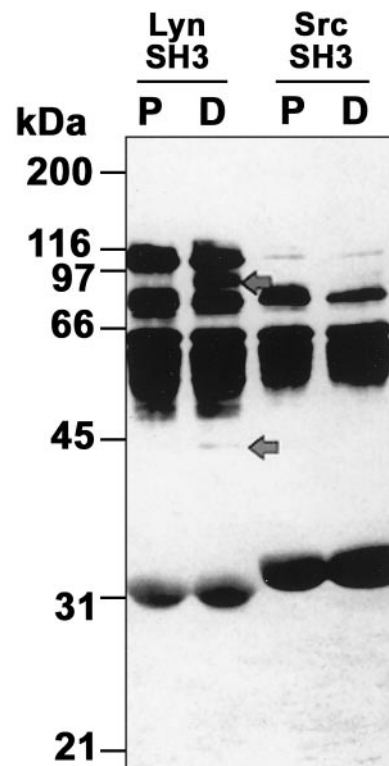


Fig. 1. Analysis of Tyrosine-Phosphorylated SH3-Binding Proteins during Trophoblast Cell Differentiation

Proliferating (P) and differentiating (D) Rcho-1 trophoblast cells were lysed in RIPA buffer and incubated with immobilized GST-Lyn SH3 (lanes 1 and 2) and GST-Src SH3 (lanes 3 and 4). Bound proteins were eluted with electrophoresis sample buffer, separated by SDS-PAGE in 10% gels, transferred to polyvinylidene difluoride membranes, and probed with antiphosphotyrosine monoclonal antibody 4G10. The Lyn SH3 domain bound to several tyrosyl-phosphorylated proteins (pp45–50, pp85; indicated by arrows) that were specific for differentiating trophoblast cells. Molecular mass standards, $\times 10^{-3}$.

way activated by Lyn in B cells (28, 29). Immunoprecipitates were prepared from proliferating and differentiating Rcho-1 trophoblast cells using anti-PI3-K, anti-Lyn, or anti-Src antibodies and were assayed for PI3-K activity. PI3-K activity increased during trophoblast cell differentiation and was specifically associated with Lyn, but not Src (Fig. 2). The expression of the p85 regulatory subunit of PI3-K was not affected by the differentiation state of trophoblast cells (Fig. 3A); however, its phosphotyrosyl content increased during differentiation (Fig. 3B). The p85 regulatory subunit of PI3-K isolated from proliferating and differentiating trophoblast cells was also capable of binding to the Lyn SH3-GST fusion protein (Fig. 3C).

Akt Activation in Proliferating and Differentiating Trophoblast Cells

PI3-K is known to signal through several pleckstrin homology domains containing regulatory proteins, including the Ser/Thr protein kinase, Akt (also called protein kinase B) (30, 31). Akt represents the principal downstream mediator of PI3-K actions within the nucleus (31). Akt protein expression was not impacted by the differentiation state (Fig. 4A). Akt was specifically activated during the differentiation of Rcho-1 trophoblast cells. Akt activation was demonstrated by increases in phospho-specific Akt levels, Akt kinase

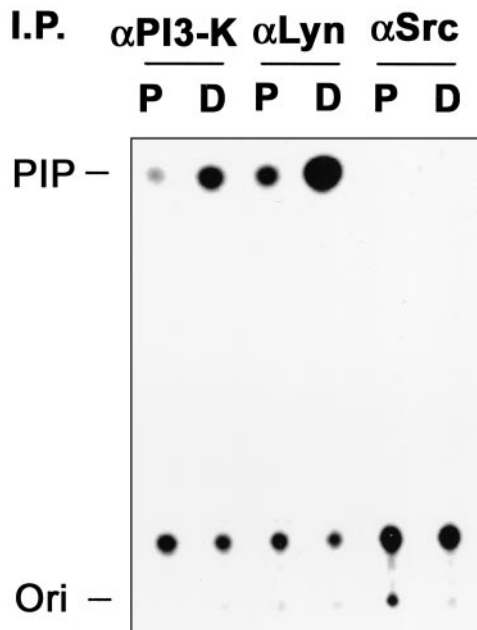


Fig. 2. Coimmunoprecipitation of PI3-K Activity with Lyn in Trophoblast Cells

Immunoprecipitates were prepared from proliferating (P) and differentiating (D) Rcho-1 trophoblast cells using anti-PI3-K (lanes 1 and 2), anti-Lyn (lanes 3 and 4), or anti-Src antibodies (lanes 5 and 6) and were assayed for PI3-K activity. Reaction products were resolved by thin layer chromatography. P1P, Phosphatidylinositol monophosphate.

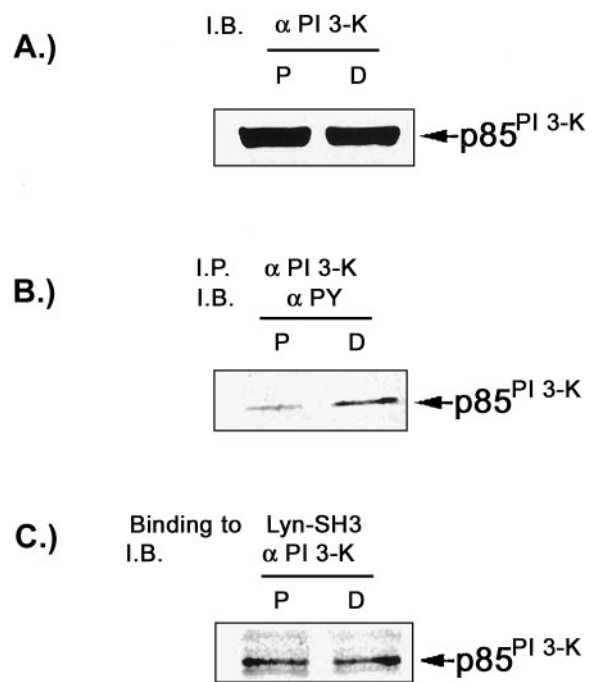


Fig. 3. The p85 Subunit of PI3-K Tyrosyl Phosphorylation and Association with the Lyn SH3 Domain in Proliferating and Differentiating Trophoblast Cells

A, Nonidet P-40 lysates from proliferating (P) and differentiating (D) Rcho-1 trophoblast cells were subjected to SDS-PAGE in 7.5% gels, transferred to polyvinylidene difluoride membranes, and probed with anti-PI3-K antibodies. B, Immunoprecipitates from proliferating (P) and differentiating (D) Rcho-1 trophoblast cells using anti-PI3-K antibodies were subjected to SDS-PAGE in 7.5% gels, transferred to polyvinylidene difluoride membranes, and probed with antiphosphotyrosine monoclonal antibody 4G10. C, Proliferating (P) and differentiating (D) Rcho-1 trophoblast cells were lysed in RIPA buffer and incubated with immobilized GST-Lyn SH3. Bound proteins were eluted with electrophoresis sample buffer, separated by SDS-PAGE in 7.5% gels, transferred to polyvinylidene difluoride membranes, and probed with anti-PI3-K antibodies.

activities, and phospho-specific Akt substrate expression (Fig. 4, A and B). Akt is comprised of at least three closely related proteins, Akt1, Akt2, and Akt3. Using specific antipeptide antibodies for Akt1 or Akt2, we found Akt1 protein to be abundantly expressed in both proliferating and differentiating Rcho-1 trophoblast cells, whereas Akt2 protein was not readily detectable (data not shown). We used phospho-specific antibodies that recognize proteins containing a consensus Akt phosphorylation site (R-X-R-X-X-S/T-X). Unique immunoreactive Akt phosphosubstrate protein species are observed in proliferative and differentiating trophoblast cells (Fig. 4C). Differentiation-specific Akt phospho-substrates included proteins with apparent molecular masses of 36, 47, 107, and 220 kDa. The 36- and 220-kDa Akt phospho-substrate protein species were also present in d 11 rat choriovitelline placenta, an enriched source of trophoblast giant cells develop-

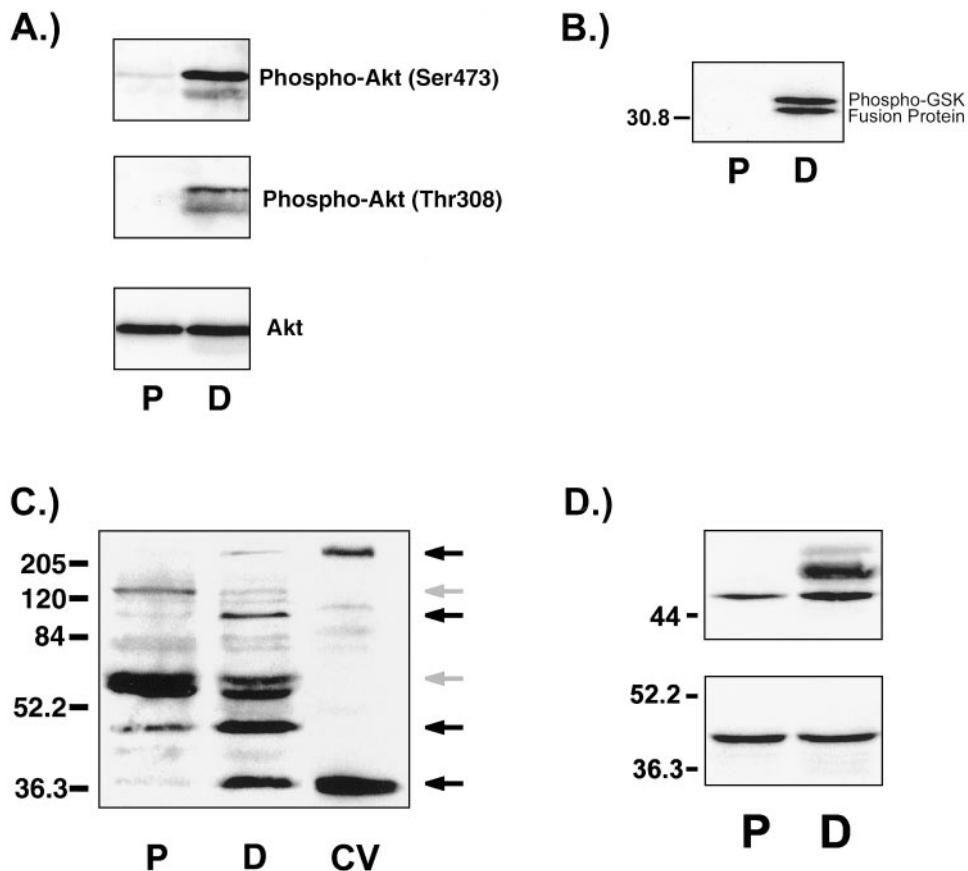


Fig. 4. Activation of the Akt Signaling Pathway in Differentiating Rcho-1 Trophoblast Cells

A, Analysis of phospho-Akt (Ser⁴⁷³ and Thr³⁰⁸) and total Akt expression in lysates from proliferating (P) and differentiating (D) trophoblast cells. B, Analysis of Akt kinase activity in lysates from proliferating (P) and differentiating (D) trophoblast cells. C, Analysis of phospho-Akt substrates in proliferating (P) and differentiating (D) Rcho-1 trophoblast cells and trophoblast giant cells developing within the d 11 gestation choriovitelline placenta (CV). D, Analysis of phospho-GSK-3 β (Ser⁹) and total GSK-3 β in lysates from proliferating (P) and differentiating (D) trophoblast cells.

ing *in situ* (Fig. 4). Glycogen synthase kinase-3 β (GSK3 β), a known target for Akt, also exhibited a differentiation-dependent phosphorylation pattern (Fig. 4D).

To determine the generality of Akt activation during trophoblast giant cell differentiation, we examined a second model system. Mouse trophoblast stem (TS) cells were established from normal mouse blastocysts and are capable of differentiating along the trophoblast giant cell lineage (16). Mouse TS cells induced to differentiate also showed activation of Akt, as demonstrated by Akt phospho-specific antibodies and Akt kinase activity (Fig. 5). Thus, activation of the Akt pathway appears to be a common feature in at least two model systems for trophoblast giant cell development.

Tyrosine Kinase Profiles in Proliferating and Differentiating Trophoblast Cells

The regulation of trophoblast cell proliferation and differentiation involves tyrosine kinase signaling pathways (17, 23). Ligand-tyrosine kinase receptor sys-

tems are established activators of the PI3-K/Akt pathway (32). Consequently, we mapped the tyrosine kinase composition of proliferative vs. differentiating Rcho-1 trophoblast cells using a PCR-based strategy employing degenerate primers to the conserved tyrosine kinase catalytic domain (33). An assortment of receptor and nonreceptor tyrosine kinases was identified by sequence analysis of the amplified cDNAs (Table 1). In proliferating trophoblast cells, 78% of the identified kinase-related clones were nonreceptor tyrosine kinases (Lyn, Src, Fgr, Lck, and Btk), 14% were receptor tyrosine kinases [insulin receptor, fibroblast growth factor receptor-2 (FGFR-2), platelet-derived growth factor receptor- α , and Sky], and the remainder represented previously unreported kinases. In differentiating trophoblast cells, 54% of the kinase-related clones identified were nonreceptor kinases (Lyn, Src, and Fgr) and 45% were receptor tyrosine kinases (FGFR-1, Sky, and insulin receptor), and there was a single isolation of the serine/threonine polo-like kinase. Expression and activation patterns of each kinase provide additional insights into possible roles of

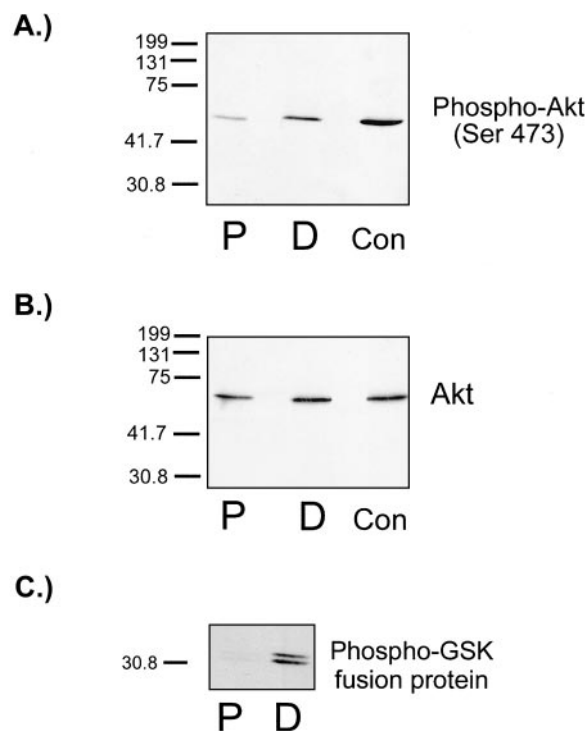


Fig. 5. Activation of the Akt Signaling Pathway in TS Cells Induced to Differentiate

A, Analysis of phospho-Akt (Ser⁴⁷³) in proliferating (P) and differentiating (D) TS cells and Rcho-1 trophoblast cells (Con). B, Measurement of total Akt in proliferating (P) and differentiating (D) TS cells and Rcho-1 trophoblast cells (Con). C, Analysis of Akt kinase activity in lysates from proliferating (P) and differentiating (D) TS cells.

the kinases in trophoblast cells. FGFR-1 and Sky showed dramatic up-regulation during trophoblast differentiation (34) (Fig. 6). Sky, FGFR-1, and Lyn are known upstream regulators of PI3-K. FGF-2, a ligand for FGFR-1, has previously been shown to promote acquisition of the trophoblast giant cell phenotype (34). Sky is a member of the Axl subfamily of receptors activated by the ligand, growth arrest-specific-6 (Gas6) (35). Trophoblast cells expressed Gas6, and addition of Gas6 to trophoblast cells stimulated Akt kinase activity (Fig. 6). Thus, Gas6 and Sky represent components of an autocrine signaling pathway contributing to the promotion of Akt kinase activation during trophoblast giant cell differentiation.

Trophoblast Cell Responses to Inhibition of the PI3-K Signaling Pathway

Given the association of Lyn with PI3-K and the differentiation-dependent activation of PI3-K, we next evaluated the effects of the PI3-K inhibitor, LY294002, on the behavior of trophoblast cells. The aqueous stable, cell-permeable, low molecular weight compound LY 294002 was used to disrupt PI3-K activity. LY294002 inhibits each of the classes of PI3-K capa-

Table 1. Summary of cDNAs Identified by Tyrosine Kinase Profiling in Proliferating vs. Differentiating Trophoblast Cells

Clone Description	Frequency
Proliferating Trophoblast Cells	
Nonreceptor tyrosine kinases	
Lyn	28
Src	15
Fgr	1
Lck	1
Receptor tyrosine kinases	
FGFR1	1
FGFR2	1
Sky	2
Insulin receptor	1
PDGF receptor α	1
Differentiating Trophoblast Cells	
Nonreceptor tyrosine kinases	
Lyn	28
Src	16
Fgr	1
Receptor tyrosine kinases	
FGFR1	18
Sky	11
Insulin receptor	2

FGFR, Fibroblast growth factor receptor; PDGF, platelet-derived growth factor.

ble of activating Akt (32). At concentrations ranging from 5–20 μ M, the inhibition is specific and informative about the involvement of PI3-Ks in biological processes (32). Treatment of trophoblast cells with LY294002 (10 μ M) specifically inhibited Akt kinase activation and phosphorylation of Akt substrates (Fig. 7). A number of the putative Akt substrates shown in Fig. 4 were sensitive to LY294002, further indicating their dependence on PI3-K activation. Most prominent among these proteins is a 36-kDa protein (Fig. 7). Treatment with LY294002 did not adversely impact the formation of trophoblast giant cells. However, LY294002 did affect the trophoblast giant cell differentiated phenotype, as monitored by expression of members of the PRL family (Fig. 8). Placental lactogen I (PL-I), PL-II, and PRL-like protein-A (PLP-A) immunoreactive proteins were diminished in LY294002-treated trophoblast cells, as were mRNA concentrations for several members of the PRL gene family (Fig. 8). The expression of PLP-A was particularly sensitive to the inhibition of PI3-K. Levels of PLP-A mRNA were decreased to less than 5% of control levels. The results indicate that PI3-K participates in regulation of the trophoblast giant cell phenotype.

DISCUSSION

Pregnancy-dependent adjustments in the maternal compartment are orchestrated by the endocrine activities of the trophoblast giant cell (4). We previously showed the importance of tyrosine kinase signaling

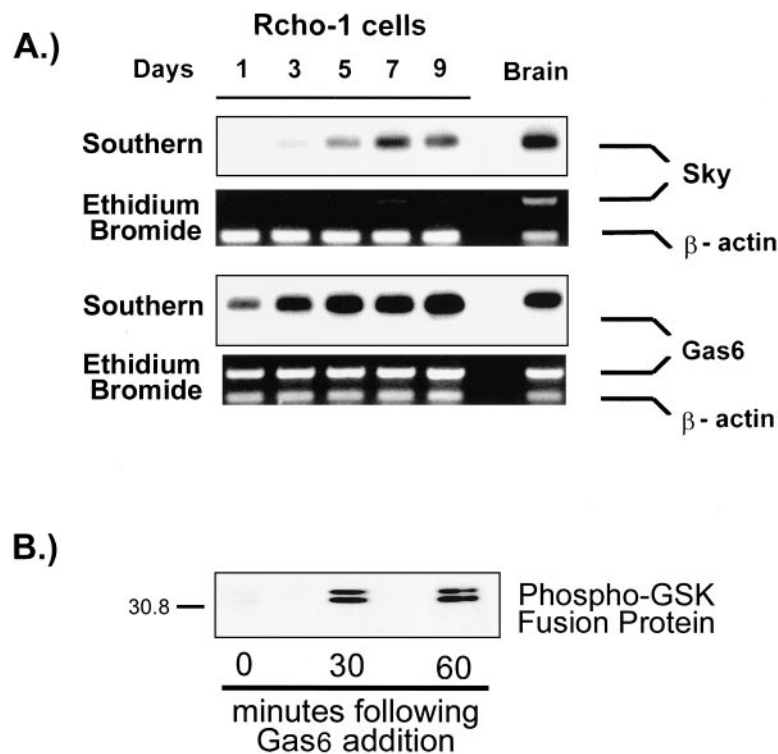


Fig. 6. Autocrine Regulation of Akt by Gas6-Sky Ligand-Receptor System in Differentiating Trophoblast Cells

A, Expression of Sky and Gas-6 in differentiating Rcho-1 trophoblast cells. RT-PCR, agarose electrophoresis and either Southern blotting (*upper*) or ethidium bromide staining (*lower*) were used to monitor Sky and Gas-6 transcripts. Brain tissue served as a positive control for both Sky and Gas-6 expression. β -Actin expression was monitored by ethidium bromide staining and served as a control for the integrity of the samples. B, Gas6 (20 ng/ml) stimulation of Akt kinase activity in differentiating Rcho-1 trophoblast cells.

pathways in the regulation of trophoblast giant cell differentiation (17, 23). More specifically, Lyn, a member of the Src family, is activated during trophoblast giant cell differentiation (23).

Src family tyrosine kinases represent key regulatory proteins in a variety of different signal transduction networks (24, 25, 36). Individual Src family kinases can possess unique roles in the regulation of cellular physiology (37, 38). However, an exclusive role for Lyn in trophoblast development is unlikely. The absence of a notable placental-associated phenotype in Lyn-deficient mice (39, 40) is probably attributable to functional redundancies among the Src family kinases expressed in trophoblast cells (23, 38). Consequently, we used Lyn as a means for discovering other potential regulatory proteins involved in trophoblast cell differentiation. A strategy of identifying binding partners for Lyn led to the elucidation of an additional component of the trophoblast giant cell regulatory pathway. We demonstrate a linkage between Lyn and PI3-K/Akt and acquisition of the trophoblast giant cell phenotype.

Src family tyrosine kinases interact with signal-transducing molecules via specific protein-protein interactions involving two distinct protein-protein recognition sites, SH2 and SH3 domains (26). SH2 domains most typically recognize phosphotyrosine-containing

amino acid regions, and SH3 domains recognize polyproline-containing amino acid regions (26). Lyn-SH2 and Src-SH2 domains exhibited limited recognition of proteins within differentiating trophoblast cells (present study). Such observations may be consistent with a decrease in SH2 domain accessibility after kinase autoactivation (41). In contrast, Lyn-SH3 and Src-SH3 domains recognized specific proteins in trophoblast cells that differed depending upon differentiation state. Unlike Src, Lyn is only activated accompanying trophoblast giant cell differentiation (Ref. 23 and present study). The Lyn-SH3 domain uniquely associates with tyrosyl-phosphorylated proteins present in differentiating trophoblast cells, whereas the Src-SH3 domain does not recognize these differentiation-associated proteins. The recognition specificity of individual Src family SH3 domains has previously been demonstrated (42-47). Among the trophoblast cell proteins recognized by the Lyn-SH3 domain was the p85 regulatory subunit of PI3-K. The association of Src family SH3 domains with the regulatory subunit of PI3-K has previously been shown to activate PI3-K (28, 47). PI3-K is present in proliferating and differentiating trophoblast cells, shows a differentiation dependence in its activation state, and has a preference for Lyn vs. Src (present study). The potential for a physical association between PI3-K and the

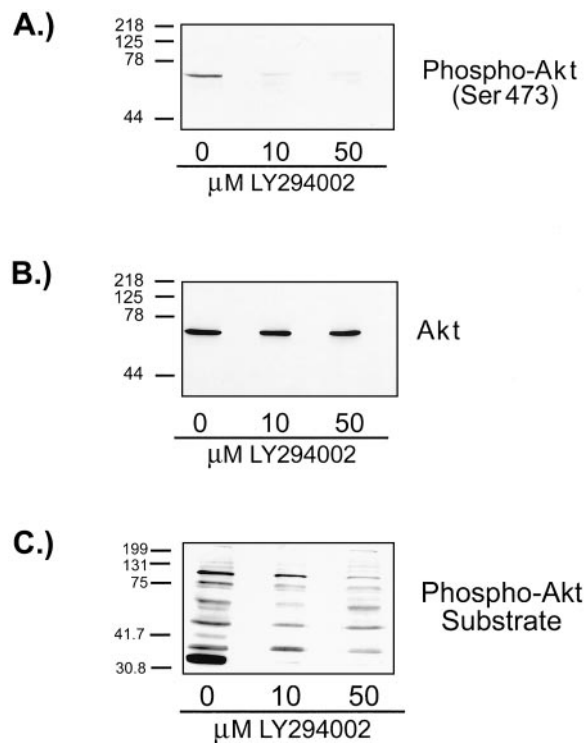


Fig. 7. Effects of Inhibiting PI3-K with LY294002 on Akt Activation and Profiles of Akt Phospho-Substrate Protein Species in Differentiating Trophoblast Cells

Differentiating Rcho-1 trophoblast cells were treated with LY294002 (10 or 50 μM) or dimethylsulfoxide vehicle (0.1% final concentration) for 30 min and then harvested. A, Analysis of phospho-Akt (Ser⁴⁷³) in control and LY294002-treated differentiating trophoblast cells. B, Total Akt protein expression in lysates from control and LY294002 treated cells. C, Akt phospho-substrate protein species in control and LY294002-treated differentiating trophoblast cells. Note the prominent disappearance of the 36-kDa protein species in LY294002-treated trophoblast cells.

SH3 domain of Lyn was observed independently of the state of trophoblast cell differentiation. Thus, the observations suggest that a simple association between the SH3 domain of Lyn and PI3-K either may not exist in proliferative cells or may not represent a sufficient means of activating PI3-K in trophoblast cells. An activated Lyn kinase appears to be a necessary participant in the differentiation-dependent regulation of PI3-K in trophoblast cells.

In addition to Src family kinases, PI3-K is known to interface with other signaling molecules (48–51). These associations may represent upstream activators or downstream mediators of PI3-K signaling. Protein tyrosine kinase profiling identified potential upstream activators of the PI3-K pathway. Two receptor tyrosine kinases, FGFR1 and Sky, showed differentiation-dependent patterns of expression. Both FGFR1 and Sky signal through PI3-K (52–56). FGF2, an FGFR1 ligand, has previously been shown to promote acquisition of the trophoblast giant cell phenotype (34). Gas6, a Sky ligand,

stimulated activation of the PI3-K pathway in trophoblast cells via an autocrine mechanism (present study). Thus, in trophoblast cells, PI3-K is potentially a key mediator of growth factor action.

PI3-K is a known regulator of a variety of cellular processes, including cytoskeletal organization (50, 51), cell cycle (57), cell survival (31), cell differentiation (55, 58–60), and responses to oxygen and oxidative signals (61, 62). Each of these cellular processes is prominently involved in the regulation of trophoblast cell development. Consistent with these known PI3-K actions, inhibition of PI3-K with LY294002 interfered with the acquisition of specific features of the trophoblast giant cell phenotype. As trophoblast cells progress along the trophoblast giant cell lineage, they acquire the capacity to produce a collection of hormones/cytokines, the PRL family (63). These hormones/cytokines possess biological actions critical to the establishment and maintenance of pregnancy (63). Inhibition of PI3-K activity blunted PRL family gene expression. The extent of the inhibition varied and was most dramatic for PLP-A, a known trophoblast cell regulator of uterine natural killer cell function (64). Although members of the PRL family appear to be coordinately expressed by trophoblast cells, it is apparent that there are fundamentally different regulatory mechanisms controlling the expression of some members of the PRL family. The impact of PI3-K on the acquisition of other aspects of the trophoblast giant cell phenotype, including endoreduplication, invasiveness, and steroid hormone biosynthesis, remains to be investigated.

Akt is one of the principal downstream mediators of PI3-K action within the nucleus (31, 65). Accordingly, PI3-K activation during trophoblast giant cell differentiation was accompanied by activation of Akt and phosphorylation of Akt substrates, and disruption of PI3-K inhibited events downstream of Akt (present study). There are intriguing intersections between downstream mediators of Akt action and the control of trophoblast giant cell differentiation. GSK3 β is a known substrate of Akt. Phosphorylation of GSK3 β by Akt inhibits GSK3 β kinase activity (57). GSK3 β has a variety of targets, including two of special interest to the differentiation of trophoblast giant cells: cyclin D1 and the Jun family of activating protein-1 (AP-1) transcription factors. In fibroblasts, GSK3 β directs cyclin to the cytoplasm and stimulates the proteolysis of cyclin D1 (57). Inhibition of GSK3 β activity via the PI3-K/Akt pathway stabilizes cyclin D1, similar to that seen in the trophoblast giant cell (18, 19, 21). GSK3 β also directly regulates members of the Jun family via phosphorylation (66, 67). GSK3 β -dependent phosphorylation inhibits the ability of Jun family members to bind to DNA and thus decreases AP-1 transcriptional activity. Trophoblast giant cell-specific gene expression is dependent upon AP-1 transcriptional activity (34, 68–71). Akt activation in differentiated trophoblast cells leads to phosphorylation of GSK3 β and thus probably a decrease in GSK3 β kinase activity, and removal of the inhibition of AP-1 transcrip-

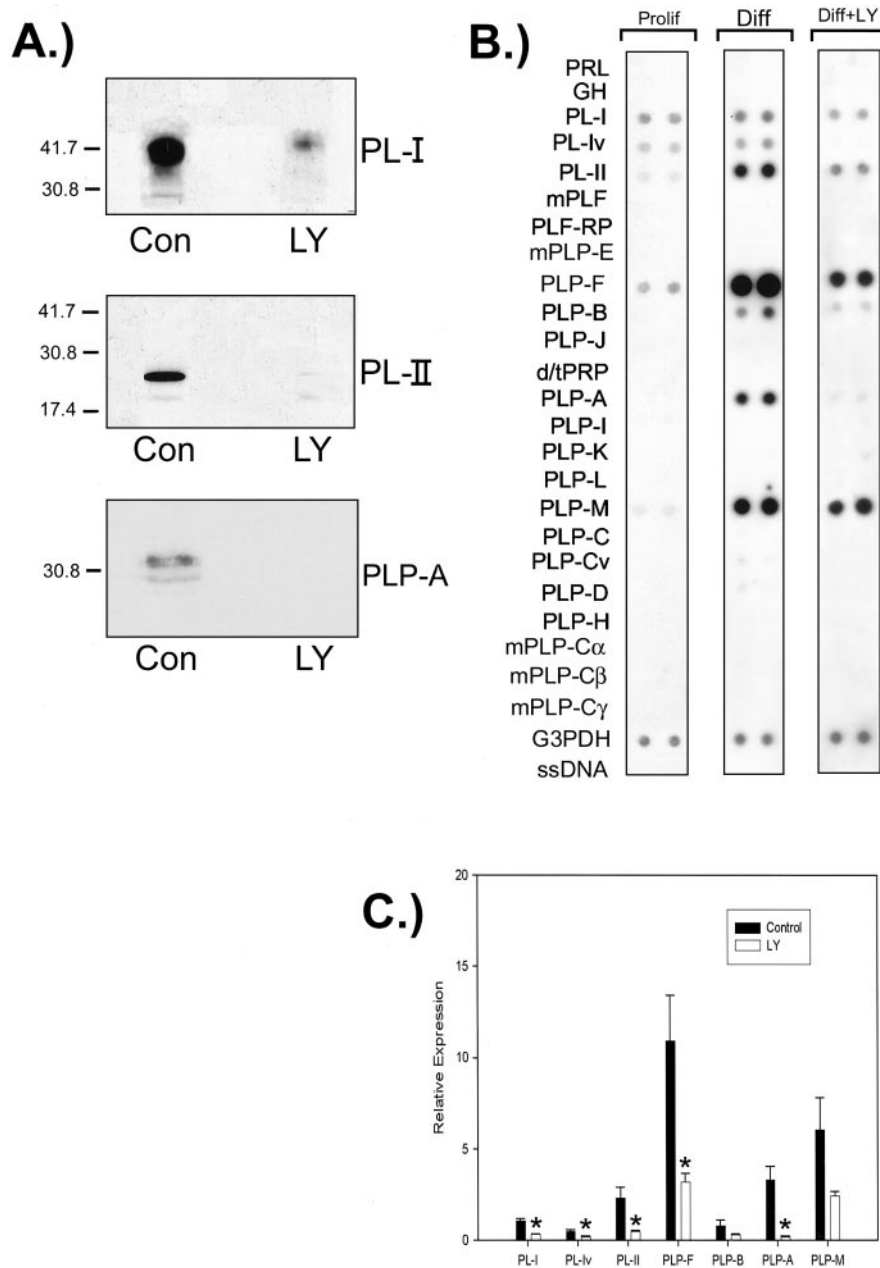


Fig. 8. Effects of Inhibiting PI3-K with LY294002 (LY) on Rcho-1 Trophoblast Cell Differentiation
 Rcho-1 trophoblast cells were maintained under proliferating conditions or grown to near confluence and transferred to NCTC culture medium containing 1% HS. Cells were allowed to differentiate in the presence of LY294002 (10 μ M) or dimethylsulfoxide vehicle (0.05% final concentration). Culture medium was changed daily. After 8 d of treatment, conditioned medium and cells were harvested for estimation of PRL family expression. A, Western blot analysis of PL-I (upper), PL-II (middle), and PLP-A (lower) production by control and LY294002-treated trophoblast cells. B, Representative PRL family miniarray analysis in proliferating, differentiating, and differentiating trophoblast cells treated with LY294002. C, Relative PRL family mRNA expression levels (mean \pm SEM) for control (■; n = 4) and LY294002-treated cultures (□; n = 4). PL-Iv, PL-I variant; PLF-RP, proliferin-related protein; PLP, PRL-like protein; mPLP-E, mouse PLP-E; PLP-Cv, PLP-C variant; d/tPRP, decidual/trophoblast PRL-related protein; mPLF, mouse proliferin; mPLP-C α , β , or γ , mouse PRL-like protein-C α , β , or γ ; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ssDNA, single-strand DNA. Note that LY294002 treatment significantly inhibited the expression of PL-I, PL-Iv, PL-II, PLP-F, and PLP-A expression (*, $P < 0.02$, by t test). LY294002 treatment did not significantly affect G3PDH expression.

tional activity and thus an increase in trophoblast giant cell-specific gene expression. This hypothesis is currently being tested. Other Akt substrates within differ-

entiating trophoblast cells were identified and represent additional candidate downstream mediators of the PI3-K/Akt pathway.

In conclusion, Lyn and PI3-K/Akt are linked and specifically activated in differentiating trophoblast cells. Activation of this pathway is probably driven by several redundant ligand-receptor systems, including the Gas6-Sky ligand-receptor system, culminating in the development of the trophoblast giant cell phenotype.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) and donor horse serum (HS) were purchased from JRH Bioscience (Lenexa, KS). Antiphosphotyrosine mAb 4G10, antirat PI3-K, and antipeptide Akt1 and Akt2 antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-GSK3 β antibodies were acquired from BD Biosciences (San Diego, CA). Dr. Joseph Bolen (DNAX, Stanford, CA) and Dr. Paolo Bellosta (New York University, New York, NY) provided the Src family kinase antibodies (72, 73) and Gas6, respectively. GST fusion protein expression vectors were obtained from Dr. John C. Cambier (University of Colorado, Denver, CO). Antibodies to Akt, phospho-Akt, Akt phosphosubstrates, and phospho-GSK3 β were obtained from Cell Signaling Technology (Beverly, MA). Reagents for SDS-PAGE were obtained from Bio-Rad Laboratories, Inc. (Richmond, CA). Immobilon-P was purchased from Millipore Corp. (Bedford, MA). Silica gel 60 was acquired from Merck & Co., Inc. (Darmstadt, Germany). Pansorbin and LY294002 were acquired from Calbiochem (La Jolla, CA). Protein A-Sepharose CL 4B and glutathione-Sepharose 4B were purchased from Pharmacia Biotech (Piscataway, NJ). ³²P-Radiolabeled nucleotides were obtained from NEN Life Science Products (Boston, MA). TOPO TA cloning kits were obtained from Invitrogen (Carlsbad, CA). Reagents for enhanced chemiluminescence were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). X-OMAT AR film was obtained from Eastman Kodak Co. (Rochester, NY). Unless otherwise stated, all other reagents were acquired from Sigma (St. Louis, MO).

Animals and Tissue Dissections

Holtzman rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The animals were housed in an environmentally controlled facility, with lights on from 0600–2000 h, and were allowed free access to food and water. Timed pregnancies were generated by cohabitation of female and male rats. The presence of a copulatory plug or sperm in the vaginal smear was designated d 0 of pregnancy. Rat placental tissues were dissected as previously described (74). The University of Kansas animal care and use committee approved protocols for the care and use of animals.

Maintenance of the Rcho-1 Trophoblast Cell Line

Elucidation of regulatory networks controlling trophoblast giant cells has been facilitated by the availability of the Rcho-1 trophoblast cell line (8, 13). Rcho-1 trophoblast cells can be manipulated to proliferate or to differentiate along the trophoblast giant cell lineage (8, 14). The Rcho-1 trophoblast cell line was routinely maintained in subconfluent conditions with either RPMI 1640 or NCTC-135 culture medium supplemented with 10–20% FBS as previously reported (8). Differentiation was induced by growing cells to near confluence in FBS-supplemented culture medium and then replacing the serum supplementation with HS (1% or 10%). A high cell density and the absence of sufficient growth stimulatory fac-

tors (removal of FBS) facilitate trophoblast giant cell formation (8).

TS Cells

TS cells represent a population of cells derived from the mouse blastocyst with the capacity to differentiate into all trophoblast cell lineages (16). TS cells have been obtained from Dr. Janet Rossant (Mount Sinai Hospital, Toronto, Canada). Dr. Rossant and co-workers (16) previously demonstrated that heparin and FGF-4 stimulate the proliferation of TS cells and inhibit TS cell differentiation. TS cells were maintained in heparin/FGF-4-supplemented culture medium comprised of 30% TS medium (RPMI 1640 supplemented with 20% FBS, 1 mM sodium pyruvate, and 50 μ M 2-mercaptoethanol) and 70% mouse embryonic fibroblast-conditioned medium as previously described (16). Heparin and FGF-4 were added to final concentrations of 1 μ g/ml and 25 ng/ml, respectively. These culture conditions promote the optimal proliferation of TS cells (16). Culturing the cells in culture medium devoid of FGF-4, heparin, and embryonic fibroblast-conditioned culture medium induces trophoblast differentiation (16).

GST Fusion Proteins

The expression and purification of GST, GST-Lyn SH3, and GST-Src SH3 fusion proteins using the expression vector pGEX-2T was performed as previously described (42, 75). Proliferating and differentiating Rcho-1 trophoblast cells were harvested and then lysed in RIPA buffer [10 mM Tris-HCl (pH 7.2), 1% Triton X-100 or 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μ g/ml aprotinin]. After clarification at 12,000 \times g for 10 min at 4 C, supernatants were incubated with 100 μ g GST, GST-Lyn SH3, or GST-Src SH3 fusion proteins and 50 μ l of a 1:1 slurry of glutathione-Sepharose 4B beads for 4 h at 4 C. The beads were washed with RIPA buffer five times, and binding proteins were eluted by boiling in electrophoresis sample buffer. Proteins were resolved by SDS-PAGE in 7.5% gels, then were electrophoretically transferred and immunoblotted with the indicated antibodies.

Immunoprecipitation and Immunoblotting

Immunoprecipitations were performed on trophoblast cells lysed in Nonidet P-40 lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μ g/ml aprotinin, and 1 mM PMSF] for 15 min on ice. Lysates were precleared by incubation with *Staphylococcus aureus* Cowan I strain (Pansorbin) for 1 h at 4 C. Precleared lysates were then incubated with antibodies for 3–4 h on ice, followed by a 45-min incubation with a slurry of protein A-Sepharose CL 4B at 4 C. The beads were washed four times in lysis buffer, and bound proteins were solubilized in electrophoresis sample buffer. In some instances double immunoprecipitation procedures were performed. Beads were boiled in elution buffer [20 mM Tris-HCl (pH 8.0), 0.5% sodium dodecyl sulfate, and 1 mM dithiothreitol], and samples were diluted to a final sodium dodecyl sulfate concentration of 0.1% with lysis buffer. Secondary immunoprecipitations were then performed as described above. Western analyses of Akt, phospho-Akt, and Akt phosphosubstrates were performed on trophoblast cells lysed in RIPA buffer. Samples were resolved by SDS-PAGE. Separated proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon-P) or nitrocellulose, and filters were probed with the designated antibodies. Blots were incubated with horseradish peroxidase-conju-

gated antibodies to mouse or rabbit IgG for 45 min at room temperature. Reaction products were visualized by incubation with enhanced chemiluminescence substrate and developed with X-OMAT AR x-ray film.

Measurement of PI3-K Activity

Immunoprecipitates from proliferating and differentiating cells using anti-PI3-K, anti-Lyn, and anti-Src antibodies were washed three times with Nonidet P-40 lysis buffer, followed by three washes with TNE buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 0.2 mM sodium orthovanadate]. Kinase reaction mixture [30 mM HEPES (pH 7.4), 30 mM MgCl₂, 50 μM ATP, 20 μCi [³²P]ATP, and 0.2 mg/ml sonicated crude brain phosphoinositides] was added to the immunoprecipitates and incubated for 5 min at room temperature with vigorous shaking. Adding 1 N HCl stopped the reaction. Lipids were extracted with a chloroform/methanol mixture (1:1, vol/vol) and resolved on oxalate-coated thin layer chromatography plates (Silica Gel 60), developed in 1-propanol/2 M acetic acid (65:35 mixture), and visualized by autoradiography. Phospholipid standards were run on the same plate and visualized by exposure to I₂.

Analysis of Akt Kinase Activity in Trophoblast Cells

Akt kinase activity was monitored in trophoblast cells lysed with a 20 mM Tris (pH 7.5) buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM PMSF. Lysates were immunoprecipitated with Akt 1G1 monoclonal antibody-agarose and washed twice with cell lysis buffer and twice with kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM MgCl₂]. Pellets were then resuspended in kinase buffer supplemented with 200 μM ATP and GSK3β fusion protein. After incubation for 30 min at 30 C, the reactions were terminated by the addition of sodium dodecyl sulfate sample buffer and heating. Samples were then analyzed by immunoblotting with antibodies to phosphorylated GSK3β (Ser⁹).

Tyrosine Kinase Profiles in Proliferating and Differentiating Trophoblast Cells

PCR-based tyrosine kinase profiling was performed on proliferating and differentiating Rcho-1 trophoblast cells according to the method of Wilks (33). Total RNA was isolated from both proliferative Rcho-1 trophoblast cells (d 2 of culture) and from differentiating Rcho-1 trophoblast cells (d 9 of culture) using TRIzol (76). Reverse transcription was performed using oligo(deoxythymidine) primers. PCR was then performed with a Perkin-Elmer Corp. model 2400 thermocycler using PTK I (5'-ATCCACA/CGNGAC/TC/TT-3') and PTK II (CTG/ACAG/CACCAGGAA/TACC-3') primers as previously described (33). PCR products were size fractionated by electrophoresis. cDNAs of approximately 210 bp (predicted size of tyrosine kinase-amplified products) were extracted from the gels and cloned into the TA cloning vector. DNA sequencing was performed using a PE Applied Biosystems model 310 sequencer and Dye Terminator Cycle Sequencing kits (PE Applied Biosystems, Foster City, CA). Sequences were analyzed with the BLAST database search program (77). Expression of the receptor tyrosine kinase, Sky (upstream primer: sense (nucleotides 297–316), 5'-TACTTCAGCCTAAAGTCAGCG-3'; downstream primer: antisense (nucleotides 1091–1110), 5'-AGCTCATCCTGGGTTCCATT-3') and its ligand, Gas6 (upstream primer: sense (nucleotides 1134–1153), 5'-CCATCAACCACGGCATGTGG-3'; downstream primer: antisense (nucleotides 1706–1725), 5'-TCGCACACCTTGATTTCCAT-

3') were confirmed by RT-PCR (78, 79). Primers for the amplification of β-actin were included as an internal control as previously described (80). Amplifications were performed within the linear range for each gene product (30 cycles). The amplified products (Sky, 815 bp; Gas6, 593 bp) for each RT-PCR reaction were then subjected to 2% agarose gel electrophoresis and processed for Southern blot hybridization using ³²P end-labeled oligonucleotides corresponding to sequences within the amplified products. Sequence analysis was used to verify the authenticity of each product.

Analysis of Trophoblast Cell Responses to an Inhibitor of PI3-K

The effects of LY294002, a stable PI3-K inhibitor (81), on trophoblast cell development were examined. Rcho-1 trophoblast cells were grown to near confluence and then shifted to differentiation medium (NCTC-135, including 1% HS) containing vehicle (dimethylsulfoxide, 0.5% final concentration) or differentiation medium supplemented with LY294002 (10 μM). Conditioned medium was collected, and cells were harvested after various intervals of culture in differentiation conditions. Akt activation was determined as described above. Total RNA was isolated from the cultured cells as described above and analyzed for the expression of members of the rat PRL gene family. Concentrations of PL-I, PL-II, and PLP-A immunoreactivities in conditioned medium were monitored by immunoblotting (14, 23, 82).

PRL Family Miniarray Analysis

Trophoblast cell differentiation was assessed by measurement of PRL family gene expression with the PRL gene family miniarray assay (83). Oligonucleotide primers were designed to amplify cDNAs corresponding to the nucleotide sequences encoding the mature proteins for each of the known members of the rat PRL family. Nucleotide regions corresponding to 5'- and 3'-untranslated regions and signal peptides were excluded. Amplified cDNAs were denatured and spotted onto nylon membranes (20 ng) using a vacuum-driven dot-blot apparatus (Bio-Rad Laboratories, Inc.). Additional DNAs, including salmon sperm DNA and glyceraldehyde-3-phosphate dehydrogenase, were spotted and used as negative and positive controls, respectively.

At the termination of the culture, cells were harvested, and total RNA was extracted as described above. cDNAs were generated and radiolabeled from total RNA (10 μg) by reverse transcription with Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). Radiolabeled cDNAs were used to probe nylon membranes spotted with PRL family cDNAs.

Spotted nylon membranes were prehybridized in 50% formamide/6× SSPE buffer (0.18 M NaCl; 10 mM NaH₂PO₄; 10 mM EDTA, pH 7.4)/5× Denhardt's reagent/0.5% sodium dodecyl sulfate/100 μg/ml salmon sperm DNA at 42 C for 2 h. Arrays were hybridized in the same buffer solution containing the radiolabeled cDNA probe at 42 C overnight. Washing was performed in 2× SSPE/0.5% sodium dodecyl sulfate at room temperature for 30 min, followed by a second wash in 0.1× SSPE/0.5% sodium dodecyl sulfate at 68 C for 30 min. Membranes were dried and exposed to x-ray film at –80 C or placed in PhosphorImager cassettes and quantified using ImageQuant software (version 4.2A, Molecular Dynamics, Inc., Sunnyvale, CA).

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