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Exogenous Amino Acids Regulate Trophectoderm Differentiation in the Mouse Blastocyst through an mTOR-Dependent Pathway

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At the late blastocyst stage, the epithelial trophectoderm cells of the mammalian embryo undergo a phenotypic change that allows them to invade into the uterine stroma and make contact with the maternal circulation. This step can be regulated *in vitro* by the availability of amino acids. Embryos cultured in defined medium lacking amino acids cannot form trophoblast cell outgrowths on fibronectin, an *in vitro* model of implantation, but remain viable for up to 3 days in culture and will form outgrowths when transferred into complete medium. The amino acid requirement is a developmentally regulated permissive event that occurs during a 4- to 8-h period at the early blastocyst stage. Amino acids affect spreading competence specifically by regulating the onset of protrusive activity and not the onset of integrin activation. Rapamycin, a specific inhibitor of the kinase mTOR/FRAP/RAFT1, blocks amino acid stimulation of embryo outgrowth, demonstrating that mTOR is required for the initiation of trophectoderm protrusive activity. Inhibition of global protein translation with cycloheximide also inhibits amino acid-dependent signals, suggesting that mTOR regulates the translation of proteins required for trophoblast differentiation. Our data suggest that mTOR activity has a developmental regulatory function in trophectoderm differentiation that may serve to coordinate embryo and uterus at the time of implantation.

Key Words: **mouse; trophectoderm; trophoblast; amino acids; mTOR; rapamycin; differentiation; p70S6 kinase.**

INTRODUCTION

Mammalian embryos must make contact with an external source of nutrients to continue development after cleavage and blastocyst formation. The mouse embryo accomplishes this by implanting into the stroma of the uterus and forming direct contacts with the maternal blood supply that lead to the establishment of a hemochorial placenta (Schlafke and Enders, 1975; Enders, 1976). Implantation and placentation are mediated by trophoblast cells, a specialized population of cells. Trophoblast cells first adhere to, then displace and phagocytose the uterine epithelium, gaining access to the underlying decidualized stroma and maternal capillaries. The trophoblast are the first cells to differentiate during mammalian development; they form the outer epithelial layer of the preimplantation blastocyst, then undergo a further differentiation to become invasive at the time of implantation (4.5 days of gestation, or E4.5). This differentiative step comprises a change in both adhesive and motile behavior, as well as other phenotypic changes; trophoblast cells cease to undergo cytokinesis, become multinucleate, polyploid, and enlarged, and are thus known as trophoblast giant cells (Chavez *et al.*, 1984; Bevilacqua and Abrahamsohn, 1988; Rinkenberger and Werb, 2000).

The transformation from epithelial trophectoderm to invasive trophoblast cells can be modeled *in vitro* by providing blastocysts with a substrate to which the trophoblast can attach and spread (Jenkinson, 1973; Armant *et al.*, 1986; Sutherland *et al.*, 1988). Trophoblast cells acquire the ability to adhere to extracellular matrix substrates as they differentiate from early to late blastocyst stages as a result of changes in receptor activation and localization (Schultz and Armant, 1995; Schultz *et al.*, 1997). Trophoblast motility is concomitantly regulated, as time-lapse recordings of the differentiation of newly hatched to late blastocysts show that the previously quiescent trophectoderm cells exhibit an abrupt onset of protrusive activity at the late blastocyst stage and subsequently begin to spread on the substrate (Sutherland *et al.*, 1988).

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The molecular events underlying the transition from epithelial to invasive trophoblast are not known, but several studies have suggested a role for amino acids in regulating the onset of trophoblast differentiation. Gwatkin (1966a,b) found that omitting two amino acids (leucine and arginine) from the culture medium prevented subsequent trophoblast outgrowth. Later studies found lesser effects of omitting leucine and arginine by themselves but a complete effect if glucose was also omitted (Spindle and Pedersen, 1973; Naeslund, 1979). Other studies support an *in vivo* role for amino acids in regulating trophoblast differentiation; embryos in diapause, or delayed implantation, show very low rates of amino acid uptake which may be regulated by low sodium levels in the uterine environment (Weitlauf, 1973; Van Winkle, 1981, 1983).

Work from several laboratories has shown in cultured cell systems that amino acids, particularly leucine, activate intracellular signaling pathways that regulate insulin receptor signaling, protein translation, cell-cycle progression, and gene expression (Brooks, 1977; Brunn *et al.*, 1997; Lawrence and Abraham, 1997; Thomas and Hall, 1997). These effects are accomplished through regulation of the activity of mTOR (mammalian target of rapamycin, also known as FRAP or RAFT1) (Lawrence and Abraham, 1997; Thomas and Hall, 1997; Fox *et al.*, 1998; Hara *et al.*, 1998; Wang *et al.*, 1998; Xu *et al.*, 1998; Kimball *et al.*, 1999; Pham *et al.*, 2000; Gingras *et al.*, 2001), a serine-threonine kinase in the family of phosphoinositide kinase-related kinases (PIKK; Gingras *et al.*, 2001). The primary result of mTOR activity is a change in the initiation of mRNA translation, through phosphorylation of two key proteins: p70S6 kinase and PHAS-I (also known as 4EBP-1) (Lin *et al.*, 1994; Pause *et al.*, 1994). Phosphorylation of p70S6K leads to preferential translation of the 5′ TOP class of mRNAs, that contain a characteristic polypyrimidine tract in their 5′-untranslated region (reviewed in Meyuhas and Hornstein, 2000). Phosphorylation of PHAS-I leads to an increase in the unbound proportion of the cap-binding factor eIF4E, which can then contribute to the formation of eIF4F initiation complexes (Pause *et al.*, 1994; Brunn *et al.*, 1997). This mechanism leads to a general increase in cap-dependent translation, but also to a disproportionate increase in the translation of mRNAs that have extensive secondary structure in the 5′UTR (De Benedetti and Rhoads, 1990; Lazaris-Karatzas *et al.*, 1990; Koromilas *et al.*, 1992). Insulin and the insulin-like growth factors regulate mTOR activity through phosphoinositol 3-kinase (PI3K) and protein kinase B (PKB, also known as Akt), while amino acids are thought not to require either of these kinases for their activation of mTOR (Hara *et al.*, 1998; Patti *et al.*, 1998; Wang *et al.*, 1998; Kimball *et al.*, 1999).

In this study, we show that amino acids regulate trophoblast spreading behavior by regulating the onset of protrusive activity. Amino acid-dependent signals are required for a 4- to 8-h period at the early blastocyst stage, and depend on mTOR, suggesting that mTOR plays an important role in regulating blastocyst activation at the time of implantation.

MATERIALS AND METHODS

Materials

ICR mice were obtained from Hilltop (Scottsdale, PA). Essential amino acids, salts, embryo-tested bovine serum albumin (BSA), hormones, embryo-tested mineral oil, and cycloheximide were purchased from Sigma Chemicals (St. Louis MO). Nonessential amino acids, bovine fibronectin, and molecular weight markers were purchased from Life Technologies, Inc. (Grand Island, NY). Rapamycin and FK506 were purchased from Calbiochem (San Diego, CA). Recombinant fibronectin fragment 9–11 was the gift of Dr. Douglas DeSimone (University of Virginia) (Ramos and DeSimone, 1996). Antibodies to the Thr389 and Thr421/Ser424 phosphorylated forms of p70S6 kinase were obtained from Cell Signaling Technology (Beverly, MA). Antibodies to nonphosphorylated p70S6 kinase were the kind gift of Dr. John C. Lawrence, Jr. (University of Virginia) (Azpiazu *et al.*, 1996).

Embryo Culture

Embryos were flushed at the two-cell stage from superovulated and mated ICR female mice and cultured to 120 h posthCG as described previously (Sutherland *et al.*, 1993). At 120 h posthCG, unhatched blastocysts were switched to serum free Eagle Plus (EP) medium (Spindle and Pedersen, 1973; Spindle, 1980; Stephens *et al.*, 1995) supplemented only with 4 mg/ml BSA, and cultured for a further 48–72 h, depending on the experimental protocol. Hereafter, we will refer to this medium as EP+ medium, designating that it contains amino acids. Amino acid-free EP medium (EP−) was made by leaving out all essential and nonessential amino acids from the recipe. For inhibition of protein synthesis, embryos were incubated in cycloheximide (50 μg/ml). Rapamycin (25 nM) was used to inhibit mTOR activity and FK506 (20 μM) was used as a competitive inhibitor of rapamycin. Stock concentrations of cycloheximide (5 mg/ml), rapamycin (1 mM), and FK506 (200 mM) were prepared by using sterile filtered DMSO and stored protected from light at −20°C. Stock concentrations were diluted to final working concentrations in either EP+ or EP− medium as dictated by experimental design.

Regulation of Trophoblast Cell Spreading

Substrates of bovine fibronectin were prepared as described previously (Sutherland *et al.*, 1993). Embryos collected and cultured as described above were placed onto prepared substrates at designated times and analyzed at specific intervals for the presence of spreading trophoblast cells. For treatment with inhibitors, embryos were first incubated in EP− medium containing the specific inhibitor for 1 h, then placed into EP+ medium containing the same inhibitor. After 8 h of culture in EP+ medium containing inhibitor, embryos were washed four times in 550 μl per wash of either EP− or EP+ medium, depending on protocol, and cultured for an additional 16 h. Control embryos were treated for 8 h in EP+ medium and transferred to EP− medium. At 144 h posthCG, all embryos were placed onto substrates of bovine fibronectin in either EP− or EP+ medium, cultured for an additional 48 h, and trophoblast outgrowth was analyzed as described previously (Sutherland *et al.*, 1993).

Time-Lapse Videomicroscopy

Embryos collected and cultured as described above were placed in microdrops of medium under oil in 35-mm Greiner tissue culture dishes in a PDMI stage incubator (Harvard Apparatus, Boston, MA) on an Olympus IX 70 microscope equipped with Hoffman Modulation Contrast optics. Time-lapse images were captured at 60-s intervals by using Openlab 2.0 software driving a Hamamatsu Orca camera. Time-lapse recordings were made over a period of 48–72 h at 1-min intervals with 2-min interruptions every 3 h for file saving. Protrusive activity was measured manually by counting the number of extensions made from the apical surface of the trophoblast cells in the plane of focus.

Regulation of Trophoblast Cell Adhesivity

Blastocysts collected at 120 and 168 h phCG were potentiated to bind fibronectin by a 2-h incubation in soluble FN120 at 37°C, and then washed twice in EP− medium. Fluorescent microspheres (Molecular Probes, Inc; 0.1 μm) were prepared by incubating the beads with a recombinant fibronectin fragment that contains the central cell binding regions (FN9–11; Ramos and DeSimone, 1996) on ice for 2 h. Beads were then washed with 1× PBS and placed into culture with the potentiated blastocysts in EP+ medium as described previously (Schultz and Armant, 1995). Fibronectin-binding activity was measured by quantifying the intensity of bound beads to each blastocyst by using Openlab 2.0.

Reactivation of Trophoblast Cell Spreading

Embryos were incubated in EP− medium from 120 h posthCG to 192 h posthCG. At 144 h phCG, embryos were placed onto substrates of bovine fibronectin as described above. After 72 h of incubation on fibronectin in the absence of amino acids, embryos were placed into EP+ medium on fibronectin and were analyzed for trophoblast cell spreading at various intervals.

Statistical Analysis

For the experiments examining protrusive activity, cell spreading, and bead binding, the data from individual experiments were averaged, and the averages and standard deviations from three experiments were analyzed for statistical significance using a one-tailed ANOVA and the Student–Newman–Keuls test (Glantz, 1992).

Developmental Regulation of Molecular Marker Expression

Embryos at designated developmental stages were collected into 3–5 μl of medium in a 1.5-ml microcentrifuge tube and frozen at −80°C. Poly(A)+ RNA was isolated by using the Micro Poly(A)-Pure Kit (Ambion, Inc., Austin, TX), from which single-stranded cDNA was synthesized by using random hexamer primers and Superscript II RNase H-reverse transcriptase at 48°C. The cDNA was extracted with phenol/chloroform, ethanol precipitated, and resuspended in 30 μl of sterile water. For amplification, 1–3 μl of cDNA was used in a 50-μl reaction volume, with *Taq* polymerase (Life Technologies Inc., Grand Island, NY) and 25 pmol of each forward and reverse primer. The primers used were: integrin _1 (FNRβ) forward 5′-gtgacccattgcaaggagaagga-3′ and reverse 5′-gtcatgaattatcattaaaagtttcca-3′; eomesodermin forward 5′-cttcaacataaacggactcaaccc-3′ and reverse 5′-agcgatgaactgtgtctctgagaag-3′; and placental lactogen I (mPL-I) forward 5′-cttcccctgtgtcatactgcttcc-3′ and reverse 5′-ctgcagttcttcgagtccagacca-3′. All primers were obtained from Genset Inc. (La Jolla, CA). Amplification was performed by using a three-step process as described previously (Sutherland *et al.*, 1993) with a final annealing temperature of 60°C. Amplified products were separated on 2% agarose gels by using the 100-bp DNA ladder (Life Technologies, Inc.) for size comparison.

Immunoblotting of p70S6 Kinase and Its Phosphorylated Forms

Phosphospecific antibodies to Thr389 and Thr421/Ser424 of p70S6 kinase were used to probe Western blots of blastocyst lysates for active forms of p70S6 kinase. Nonphosphorylated p70S6 kinase was detected by using a polyclonal antibody produced against p70S6K (R802) (Azpiazu *et al.*, 1996). Blastocysts were collected over a 6-week period and stored at −80°C until needed, then prepared for immunoblotting by lysing in homogenization buffer (50 mM sodium β-glycerophosphate, pH 7.4, 1 mM sodium orthovanadate, 10 mM magnesium chloride, 5 mM EGTA, 10 mM potassium phosphate, 2 mM DTT, 1.0 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM PMSF and 0.1 μM Microcystin-LR). After embryo lysis, 16 μg of total protein was loaded onto a 10% SDS–PAGE gel. The gel was transferred to nitrocellulose paper (Stratagene, La Jolla, CA), which was incubated overnight at 4°C in a 0.5% BSA primary antibody solution. After washing, the blot was incubated with HRP-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA) for 1 h, washed again, and then chemiluminescence (Cell Signaling Technology, Beverly, MA) was used to detect the presence of p70S6 kinase and its phosphorylated forms.

RESULTS

Regulation of Trophoblast Cell Spreading by Amino Acids

Earlier studies describing the *in vitro* culture conditions of mouse embryos demonstrated that culture medium deficient in certain amino acids prevents blastocyst outgrowth (Gwatkin, 1966a,b; Spindle and Pedersen, 1973; Naeslund, 1979). We replicated these previous studies and found that the deficiency in certain essential amino acids has a very specific effect on trophectoderm cell behavior. Blastocysts cultured in suspension in a modified Eagle's medium (Spindle and Pedersen, 1973; Spindle, 1980) lacking amino acids (EP− medium) retain an expanded blastocoele cavity as late as 168 h posthCG, and they are unable to attach and spread on fibronectin substrates (Figs. 1A and 1B). In contrast, blastocysts cultured in suspension in the presence of amino acids (EP+ medium) have a collapsed blastocoele cavity by 168 h posthCG, and can attach and spread by 192 h posthCG when cultured on fibronectin substrates (Figs. 1A and 1B). To determine whether culture in EP− medium has an irreversible, detrimental effect on development, we cultured embryos from 120 to 192 h posthCG in EP− medium, transferred them at various times to EP+ medium on fibronectin substrates, and exam-

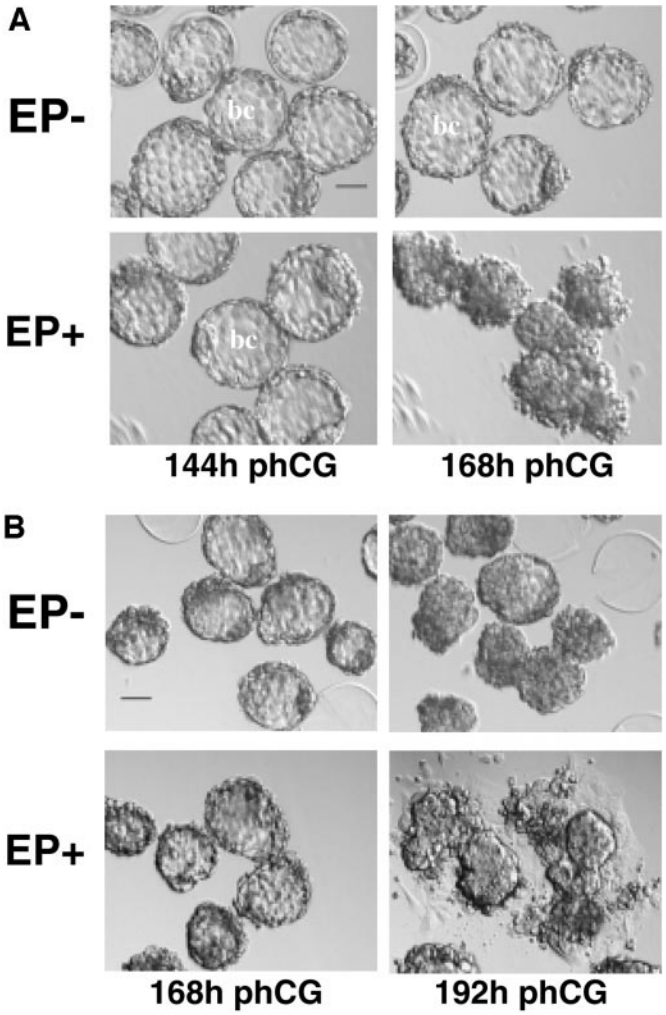

FIG. 1. (A) Blastocysts cultured in the presence or absence of amino acids. Blastocysts cultured in Eagle's medium without amino acids (EP−) maintain an expanded blastocoele cavity, while those cultured in Eagle's medium plus amino acids (EP+) exhibit a collapsed blastocoele cavity by 168 h posthCG. (B) EP− and EP+ medium also affect trophoblast cell spreading. Culture in EP− medium prevents formation of trophoblast cell outgrowths. bc, blastocele cavity; scale bar, 50 μm

ined their ability to form outgrowths. Embryos cultured in EP− medium remained viable after 3 days, and were able to form outgrowths on fibronectin when transferred into EP+ medium (Fig. 2).

We observed that the onset of spreading occurs within a defined time period following exposure to amino acids. Embryos cultured for 24, 48, 72, or 96 h in EP− and then transferred to EP+ medium began to spread between 24 and 48 h after exposure to amino acids (data not shown). This time course is similar to that seen when embryos are placed directly into EP+ medium at 120 h posthCG, suggesting that amino acid availability initiates a cascade of events that culminate in spreading competence after 24–48 h. Placing embryos into EP+ medium earlier than 120 h posthCG does not result in earlier spreading (data not shown). Thus, amino acids appear to facilitate or permit a developmental event that normally begins at 120 h posthCG. The fact that the embryos remain viable and maintain consistent timing between placement into EP+ medium and the onset of blastocoele collapse and spreading, suggests that culture in EP− medium causes the trophectoderm to remain at the stage of differentiation characteristic of the early blastocyst stage.

Amino Acid Availability Specifically Regulates the Onset of Trophectoderm Protrusive Activity

The above results show that lack of amino acids leads to an apparent arrest in trophectoderm differentiation from an epithelial phenotype to a motile, adhesive phenotype. To determine whether this represents a general arrest of trophectoderm differentiation, we examined the expression of two trophoblast markers by reverse-transcription of blastocyst mRNA followed by polymerase chain reaction amplification (RT-PCR): eomesodermin, which is a marker of trophoblast stem cells, and placental lactogen-I (mPL-I), which is a marker of giant-cell differentiation (Nieder, 1990; Nieder and Nagy, 1991; Tanaka *et al.*, 1998). Eomesodermin is expressed both in the early blastocyst (120 h posthCG) and in the late blastocyst (168 h posthCG), and its expression is maintained in the 168-h blastocysts cultured in EP− medium. mPL-I is not expressed at 120 h posthCG, but is expressed by 168 h both in embryos cultured in EP+ medium and in embryos cultured in EP− medium (Fig. 3). Thus, lack of amino acids does not result in a global inhibition of trophoblast differentiation.

Previous studies have shown that blastocysts become progressively able to respond to fibronectin during the

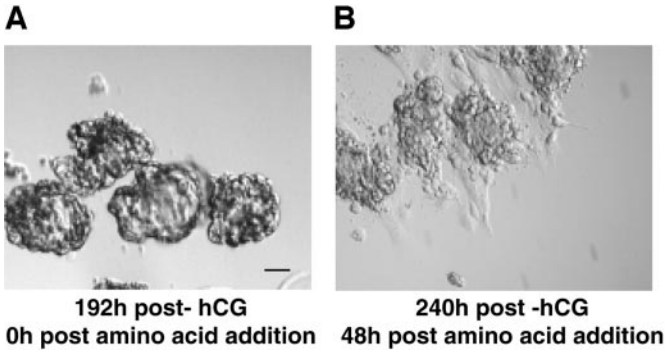

FIG. 2. Blastocysts cultured in EP− medium can be reactivated to form trophoblast outgrowths by exposure to EP+. (A) Blastocysts cultured on bovine fibronectin in EP− medium from 120 to 192 h posthCG are not able to form trophoblast cell outgrowths. (B) Embryos transferred to EP+ medium at 192 h posthCG formed trophoblast cell outgrowths within 48 h. Scale bar, 50 μm.

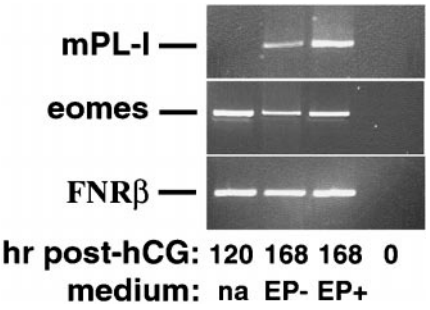

FIG. 3. RT-PCR of trophoblast cell differentiation markers. mRNA from blastocysts at 120 h posthCG and from blastocysts at 168 h posthCG cultured in EP+ or EP− medium for 48 h (120 to 168 h posthCG) was isolated, reverse-transcribed, and amplified with primers to eomesodermin (eomes, a stem cell marker) or placental lactogen I (mPL-I, a giant cell differentiation marker). Primers for the integrin β1 subunit (FNRβ) were used as positive controls for the cDNA. na, not applicable.

period between 120 and 168 h posthCG (Schultz and Armant, 1995). During this same period of time, the trophectoderm cells begin to extend apical protrusions and become motile (Sutherland *et al.*, 1988; McRae and Church, 1990; P.M.M. and A.E.S., unpublished observations). Thus, either adhesivity or motility, or both, could be affected by amino acid availability. To determine which aspect of spreading is being affected by amino acid availability, we compared the adhesivity and motility of blastocysts cultured in EP− and EP+ medium.

To test adhesivity, we used fluorescent beads coated with a recombinant fragment of fibronectin containing the tripeptide RGD sequence and the synergy cell-binding domains (FN9–11; Ramos and DeSimone, 1996). In agreement with previous data (Schultz and Armant, 1995), we found that, 120 h posthCG, blastocysts show only low adhesivity for fibronectin and are not induced to upregulate fibronectin binding by incubation with soluble FN-120, while, 168 h posthCG, blastocysts can be induced (Figs. 4A and 4B; Schultz and Armant, 1995). Somewhat surprisingly, blastocysts at 168 h posthCG that have been cultured in EP− medium were induced by FN-120 to bind even more FN9–11-coated beads than those cultured in EP+ medium (Figs. 4A and 4B). The inability of embryos cultured in EP− medium to spread on fibronectin is therefore not due to a lack of adhesivity for the substrate.

We then examined the motility of the trophectoderm cells in EP+ and EP− medium. To determine the amount of trophoblast cell protrusive activity under these conditions, we cultured blastocysts in EP− medium or EP+ medium (without an extracellular matrix substrate) from 120 to 192 h posthCG and recorded time-lapse images of their behavior from 144 to 192 h posthCG. There is significantly less protrusive activity in blastocysts cultured in EP− medium at all stages than in blastocysts cultured in EP+ medium (Fig. 5). These data demonstrate that the inability of embryos to form outgrowths in EP− medium correlates with a lack of the normal onset of protrusive activity by the trophectoderm cells.

Amino Acid Availability Regulates Trophoblast Spreading during a Narrow Window of Time

To determine whether the requirement for amino acids is continuous from 120 to 168 h posthCG, or whether a shorter time will suffice, we exposed embryos to EP+ medium for defined periods of time. We placed embryos at 120 h posthCG into EP+ medium for 4, 8, or 24 h, washed them three times in EP− medium, and then cultured them for the remainder of the 48-h period in EP− medium. As a positive control, embryos were cultured in EP+ medium, washed, and replaced in EP+ medium for the duration of the 48-h period. The results demonstrate (Fig. 6A) that the requirement for amino acids is very discrete, as embryos cultured for only 8 h in EP+ medium and then placed in EP− medium will form outgrowths at 168 h posthCG nearly as well as those cultured continuously in EP+ medium.

To determine whether the requirement for amino acids occurs specifically at 120 h posthCG, we performed a similar window culture experiment on younger embryos. We cultured embryos from 96 to 120 h posthCG in EP+ medium, then placed them on fibronectin substrates in EP− medium and examined their ability to form outgrowths during the subsequent 48 h. Only a very few of these embryos were able to form outgrowths on fibronectin (Fig. 6B), similar to embryos cultured only in EP− medium. These results are consistent with the notion that the requirement for amino acids is not continuous, but occurs at a very specific point in trophectoderm differentiation.

mTOR Activity Is Required for Amino Acid Signaling in Embryos

Previous data in cultured cells have shown that amino acid availability regulates the activity of the kinase mTOR (Hara *et al.*, 1998; Wang *et al.*, 1998). To test whether mTOR activity is required for amino acid signaling in embryos, we treated embryos with rapamycin, a specific inhibitor of mTOR (reviewed in Gingras *et al.*, 2001). Early blastocysts (120 h posthCG) were incubated for 8 h in EP+ medium containing 20 nM rapamycin, then washed and placed into either EP+ or EP− medium for an additional 40 h, to 168 h posthCG. Embryos placed into EP− medium were unable to spread, demonstrating that inhibition of mTOR by rapamycin inhibits the effect of the 8-h amino acid incubation (Fig. 7). In contrast, embryos placed into EP+ medium after washing out the rapamycin were able to spread similarly to diluent controls (not shown), ruling out any toxic effects of the 8-h rapamycin treatment. The specificity of the rapamycin effect was further tested by addition of the competitive inhibitor FK506 to rapamycin-containing EP+ medium during the 8-h incubation. Addi-

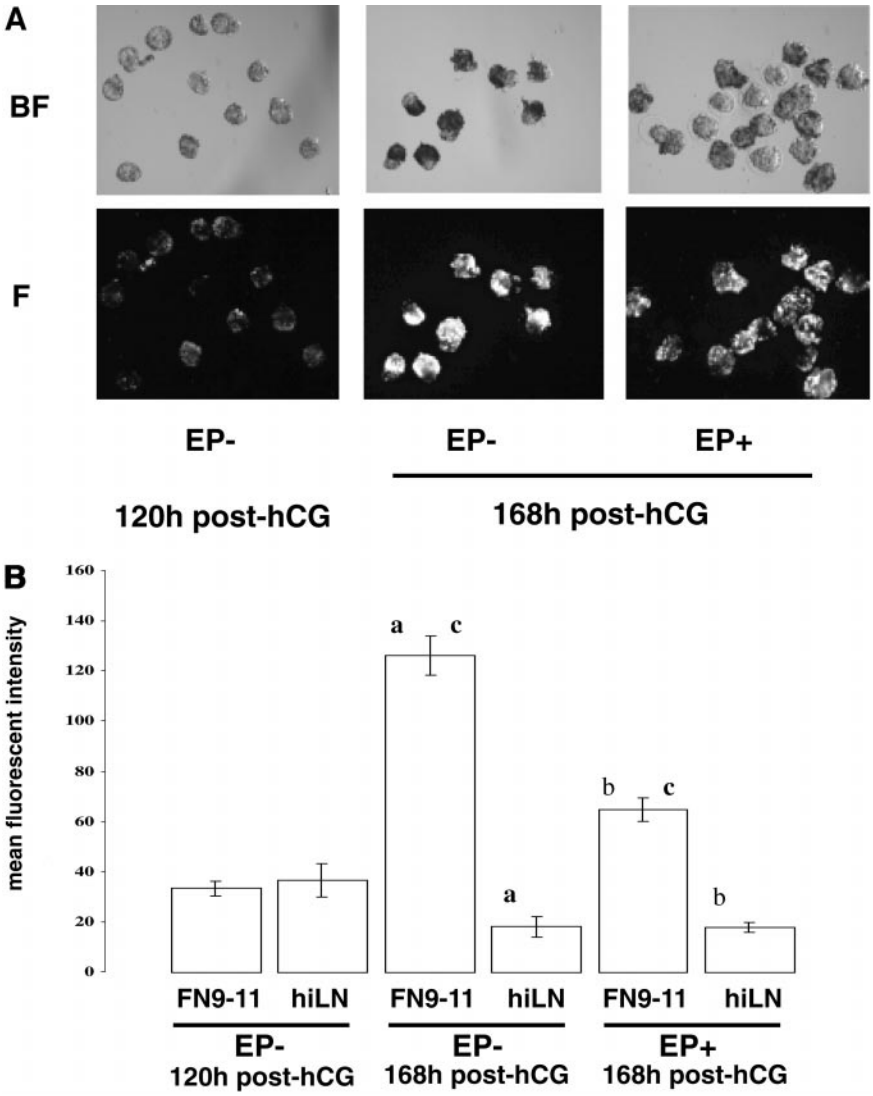

FIG. 4. Bead binding analysis of blastocyst adhesion competence induction. Blastocysts were incubated in medium containing the 120-kDa fragment of fibronectin for 3 h, and induction of adhesion competence was measured by binding of fluorescent microspheres coated with the recombinant FN9–11 fragment containing the fibronectin cell binding domain. (A) Micrographs of embryo bead binding (BF, brightfield; F, epifluorescence). (B) Digital quantitation of average fluorescence intensity per embryo in each treatment. Heat-inactivated laminin (hiLN) was used to coat microspheres as a control for nonspecific binding of beads to embryos. The data shown are the mean ± SEM for three independent experiments. Bars labeled with the same letter are significantly different from one another; a, $P < 0.01$; b, $P < 0.01$; c, $P < 0.01$.

tion of FK506 reversed the effect of rapamycin and allowed the onset of spreading (Fig. 7).

Amino Acid Signals Induce mTOR-Dependent Phosphorylation of p70S6K

mTOR is a serine-threonine kinase known to phosphorylate at least two other proteins. It phosphorylates the p70S6 kinase at Thr389, leading to its activation, and phosphorylates the eIF4E binding protein PHAS-I, which leads to its dissociation from eIF4E. We examined p70S6K activation in embryos cultured for 8 h (120–128 h posthCG) in the presence or absence of amino acids by Western blotting with phosphospecific antibodies (Fig. 8). In the absence of amino acids, p70S6K was phosphorylated at Thr421/Ser424, but not at Thr389. An 8-h exposure to amino acids induced phosphorylation at Thr389, while treatment with rapamycin during the 8-h exposure to amino acids inhibited phosphorylation at all three sites (Fig. 8). These results show that amino acids induce mTOR-dependent phosphorylation of p70S6K at Thr389 at the early blastocyst stage.

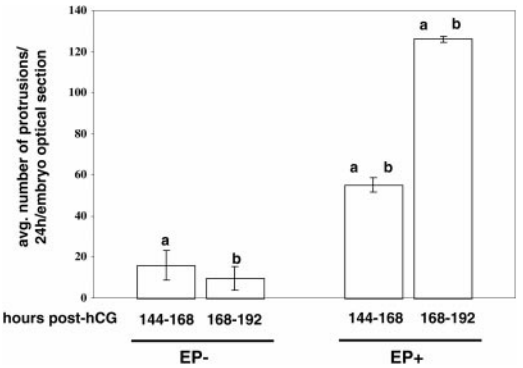

FIG. 5. Regulation of trophoblast cell protrusive activity is affected by amino acid availability. Time-lapse digital images were recorded for embryos cultured either in EP− medium or EP+ medium, and the number of protrusions extended from the external surface of the trophectoderm cells in a single optical section were counted manually. The graph shows the total number of protrusions extended in two subsequent 24-h periods, from 144 to 168 h posthCG, and from 168 to 192 h posthCG. The values shown are the mean ± SEM for three experiments. Bars labeled with the same letter are all significantly different from one another. a, $P < 0.01$; b, $P < 0.01$.

Regulation of Translation Accounts for the Amino Acid Effect

Phosphorylation of p70S6K and PHAS-1 affects translation initiation. Phosphorylation of p70S6K leads to its activation and an increase in translation of 5′TOP messages (reviewed in Meyuhas and Hornstein, 2000), while phosphorylation of PHAS-1 decreases its binding to the cap-binding protein eIF4E, and leads to an increase in cap-dependent translation (reviewed in Kimball and Jefferson, 2000). To test whether translation is required for the amino acid effect on trophectoderm differentiation, we inhibited translation in a general way, by treating blastocysts with cycloheximide during the 8-h exposure to amino acids at 120 h posthCG (Fig. 9). This treatment inhibited subsequent outgrowth, demonstrating that protein translation is required during this period for the later onset of motility.

DISCUSSION

It has long been known that mouse embryo development *in vitro* depends on the appropriate culture environment, and several studies have explored the amino acid requirements for normal development (reviewed in Van Winkle, 2001). In the current study, we have extended these observations to show that amino acids, in addition to being essential nutrients for growth of the embryo, play a fundamental role in the regulation of trophoblast differentiation. This effect is stage-specific, activates intracellular signaling pathways that involve mTOR, and leads to the onset of trophoblast protrusive activity and the ability to implant. We hypothesize, based on these results, that this signaling pathway may serve at least two functions. First, by regulating trophoblast differentiation in response to environmental conditions in the uterus, amino acid signals can provide for optimal coordination between the onset of invasive behavior of the blastocyst and the state of receptivity of the uterus. Second, amino acid signaling may be a regulatory factor of diapause, which is a special case of implantation delay that occurs as a normal part of pregnancy in many species (for review see Renfree and Shaw, 2000).

Amino Acid Availability at the Blastocyst Stage Specifically Affects the Onset of Trophoblast Motility

We find that amino acid signaling specifically affects the onset of motility in trophectoderm cells of the blastocyst.

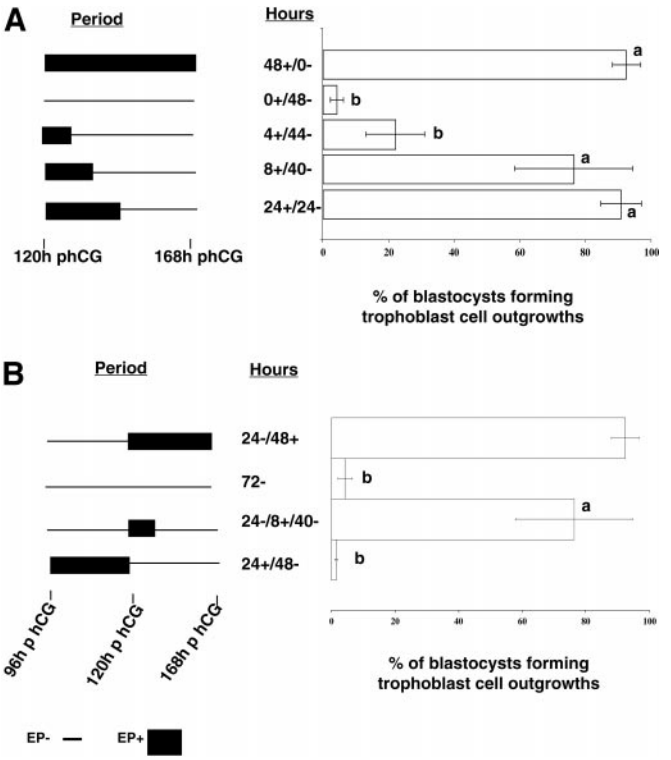

FIG. 6. Determination of the time requirement for amino acid exposure. Early blastocysts (120 h posthCG) were cultured in EP+ medium for varying amounts of time before being transferred to EP− medium. (A) Early blastocysts cultured in EP+ medium for a minimum of 8 h are able to form trophoblast cell outgrowths by 192 h posthCG. (B) Early blastocysts cultured in EP+ medium between 96 and 120 h posthCG, are not able to form trophoblast cell outgrowths. The data are graphed as the mean percentages ± SEM for three independent experiments. Bars labeled with different letters are significantly different from one another ($P < 0.01$). Bars labeled with the same letter are not significantly different from one another ($P > 0.05$).

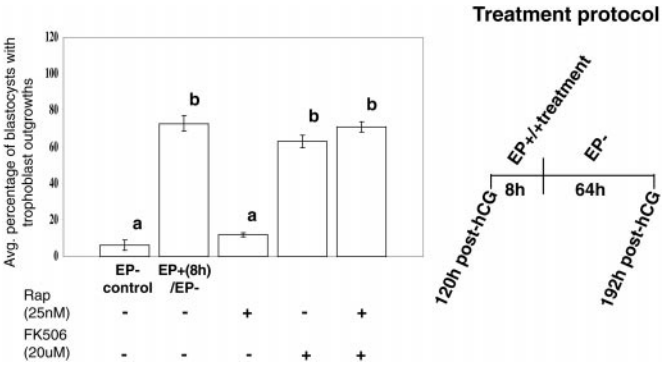

FIG. 7. Inhibition of mTOR activation prevents trophoblast cell outgrowth formation. Blastocysts at 120 h posthCG were cultured for 8 h in EP+ medium either without additional treatment, or containing either 25 nM rapamycin, 20 μm FK506 (a competitive inhibitor of rapamycin), or a combination of rapamycin and FK506. Rapamycin treatment prevents trophoblast cell outgrowth formation. Addition of FK506 has no effect on trophoblast outgrowth, but in combination with rapamycin it blocks the effect of rapamycin and allows subsequent trophoblast cell outgrowth. Bars labeled with different letters are significantly different from one another ($P < 0.01$). Bars labeled with the same letter are not significantly different from one another ($P > 0.05$).

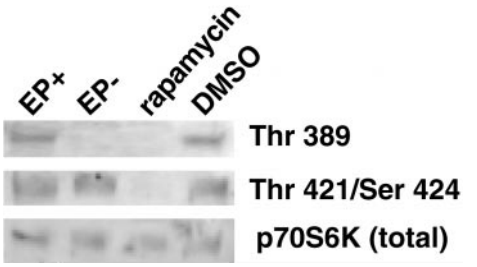

FIG. 8. Western blot of p70S6K and its phosphorylated forms (Thr389; Thr421/Ser424) in blastocysts cultured in EP+, EP−, EP+ with rapamycin, or EP+ with DMSO (diluent control), for 8 h (from 120 to 128 h posthCG).

When the embryo forms an outgrowth *in vitro*, or implants into the uterus *in vivo*, the trophectoderm cells undergo changes in adhesivity (Schultz and Armant, 1995; Schultz *et al.*, 1997) and in protrusive activity (Sutherland *et al.*, 1988; McRae and Church, 1990) to become invasive. Simultaneously, the trophectoderm cells undergo differentiation and begin to express new proteins, one of which is placental lactogen I (mPL-I) (Nieder, 1990). When embryos are cultured in medium that lacks amino acids, the trophectoderm cells proceed to change the activation properties of their surface integrins and to express mPL-I in the same way as embryos cultured in EP+ medium. In contrast, they do not show the normal onset of protrusive activity. Thus, amino acid deprivation does not inhibit differentiation in general, but specifically prevents the onset of implantation behavior by limiting trophoblast motility. These observations are important in terms of defining the mechanism by which amino acids regulate trophoblast outgrowth, but also provide a method for manipulating and investigating the normal changes in trophoblast cell motility.

The observation that amino acids regulate cell motility is unique to this system, and is particularly intriguing in light of the function of TOR2 in regulating actin dynamics in yeast (Helliwell *et al.*, 1998). To date, no homologue of TOR2 nor homologous function for mTOR in regulating actin has been identified in mammalian cells. Further investigation of mTOR regulation of cell motility will clearly be important for our understanding not only of the regulation of trophoblast invasion, but also of the cellular functions of mTOR.

Amino Acid Regulation of Outgrowth Is Stage-Specific

The timing of the amino acid requirement is specific to the early blastocyst stage. Embryos treated with amino acids prior to 120 h posthCG do not form outgrowths, while an 8-h treatment at 120 h posthCG is sufficient to trigger protrusive activity and trophoblast outgrowth in most of the embryos. This suggests that the blastocyst becomes

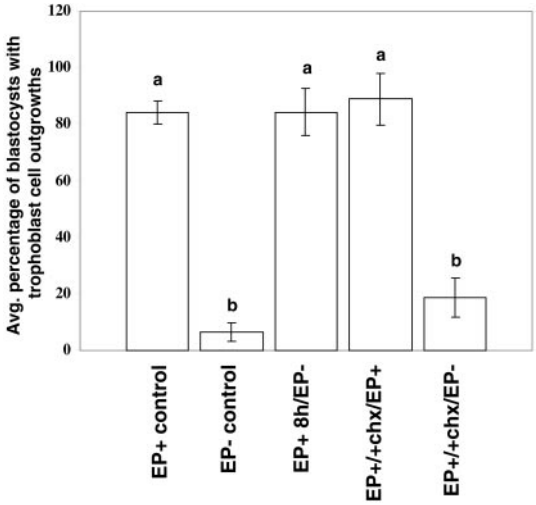

FIG. 9. Cycloheximide treatment of blastocysts during an 8-h exposure to amino acids. Embryos were pretreated with cycloheximide (50 μg/ml) for 1 h in EP− medium, then transferred to either EP− or EP+ medium containing cycloheximide (chx) for an additional 8-h period. They were then washed and transferred either to EP− or EP+ medium for 16 h, and then transferred to fibronectin substrates for outgrowth assays. Trophoblast cell spreading was analyzed 48 h later, as described in Fig. 5. The data are shown as the mean percentages of embryos with trophoblast outgrowth ± SEM for three independent experiments. Bars labeled with different letters are significantly different from one another ($P < 0.01$). Bars labeled with the same letter are not significantly different from one another ($P > 0.05$).

competent either to transmit or to respond to amino acid-dependent signals at the time of attachment to the uterine epithelium. Our results were obtained with embryos cultured *in vitro* from the two-cell stage onwards, but previous results show that embryos cultured *in vivo* exhibit the same characteristics. Blake *et al.* (1982) found that embryos flushed from the uterus at 96 h posthCG did not respond to pulse treatments of complete medium (with FBS) before 120 h posthCG, but 12 h of culture in complete medium after 120 h posthCG was sufficient to induce outgrowth in all embryos. Van Winkle and colleagues (Van Winkle and Campione, 1987; Van Winkle *et al.*, 1990a) have shown that amino acid uptake is inhibited in the mouse embryo in the period just before implantation, which may correspond to the refractory period between 96 and 120 h posthCG that we observe. Together, these results suggest that the requirement for an amino acid-dependent signal occurs around 120 h posthCG, and that a very short exposure (4–8 h) is required to stimulate the embryo to become outgrowth-competent. In fact, given that mouse embryos exhibit substantial asynchrony in preimplantation development, the required exposure time for an individual embryo may actually be much shorter.

The fact that embryos cultured *in vitro* and *in vivo* show a similar timing in their response to amino acids suggests that this signaling mechanism operates *in vivo* as well as *in vitro*. The "window" of time for successful implantation into the mouse uterus during both normal and delayed states requires a strict temporal coordination between the differentiation of the blastocyst and receptivity of the uterus (reviewed in (Paria *et al.*, 2000, 2001). Other studies have documented that uterine factors are required to "activate" the blastocyst for implantation (Yoshinaga and Adams, 1966; Stewart *et al.*, 1992; Paria *et al.*, 1993, 2001). In addition, Lee *et al.* (2000) showed that preventing embryos from entering the uterus by ligating the oviduct resulted in the embryos going into a state of quiescence, similar to what we see in embryos cultured in EP− medium. We show here that blastocyst activation depends on mTOR, and that amino acid-dependent signals may be a regulating factor.

Potential Role for Amino Acid Signaling in the Regulation of Diapause

Interestingly, embryos cultured in the absence of amino acids also resemble those in the state of diapause, or delayed implantation. Diapause is a naturally occurring phenomenon in many mammalian species in which the embryo develops normally from fertilization to the blastocyst stage, and then enters a state of dormancy which can last from weeks to months (Renfree and Shaw, 2000). Implantation is triggered by seasonal or physiological factors (e.g., weaning) that cause changes in maternal hormonal levels. Embryos in diapause are unable to form outgrowths in the absence of amino acids, although they have been in the uterus for up to a week (Naeslund, 1979). When removed from the uterus and cultured in complete medium, they become activated and will form outgrowths similar to normal embryos (Naeslund, 1979; Blake *et al.*, 1982). There is substantial evidence that amino acid transport is inhibited during diapause (for review see Van Winkle, 2001), though probably not directly by decreases in amino acid concentration in uterine fluid (Gwatkin, 1969). One alternative that has been proposed previously is that the uterus regulates the concentration of sodium in the uterine fluid, which then influences the activity of the sodium-dependent B^{0+} amino acid transporter (Van Winkle and Campione, 1987). However, although regulation of amino acid signaling may contribute to the regulation of diapause, it is likely not the only factor, as DNA synthesis is upregulated in embryos isolated from mice in diapause even when they are cultured in medium lacking amino acids (Nieder and Weitlauf, 1985).

Amino Acids Act as Activators of Intracellular Signaling Pathways That Regulate Translation Initiation

The mechanism by which amino acids exert their effect on the trophectoderm is through regulation of intracellular signaling pathways analogous to those described in many other cell and tissue types, from yeast to mammals (reviewed in Kimball and Jefferson, 2000). Amino acids, and in particular branched-chain amino acids such as leucine, act as activators of intracellular signaling pathways that regulate protein synthesis and catabolism (Xu *et al.*, 1998). In addition, amino acids have been shown to function as permissive elements in signaling pathways downstream of insulin and insulin-like growth factors (Patti *et al.*, 1998). Amino acid signaling leads to phosphorylation of at least two proteins involved in translation initiation, p70S6K and PHAS-I, through activation of the serine–threonine kinase mTOR (Patti *et al.*, 1998; Kimball and Jefferson, 2000).

Phosphorylation of p70S6K causes it to be activated, and results in increased phosphorylation of the ribosomal protein S6. This leads to an increase in translation, particularly of a group of mRNAs known as 5′TOP mRNAs. These messages have a characteristic polypyrimidine tract at their 5′ ends, and typically encode proteins such as elongation factors and ribosomal proteins, that are important components of the translational machinery (reviewed in Meyuhas and Hornstein, 2000). In contrast, PHAS-I is inactivated by phosphorylation. When dephosphorylated, it forms a complex with eIF4E, which is the cap-binding protein in the eIF4F initiation complex. Phosphorylation of PHAS-I leads to dissociation of the complex and an increase in free eIF4E, which can then participate in forming active initiation complexes (reviewed in Lawrence and Abraham, 1997; Raught *et al.*, 2000). The role of eIF4E in regulating cell behavior is of particular interest in this context. eIF4E is involved in mediating Ras transformation of cells, and its overexpression in cultured cells leads to malignant transformation (De Benedetti and Rhoads, 1990; Lazaris-Karatzas *et al.*, 1990, 1992). In addition, eIF4E has been found to play

a role in cellular transformation during development, as overexpression in *Xenopus* animal caps promotes mesoderm induction (Klein and Melton, 1994). The amount and phosphorylation state of eIF4E have been found to be less important for overall rates of protein synthesis than for translation of specific messages (Kleijn *et al.*, 1998; Kimball *et al.*, 1999). The activity of eIF4E is particularly important for efficient translation of messages with complex 5′-untranslated regions (De Benedetti and Rhoads, 1990; Lazaris-Karatzas *et al.*, 1990; Koromilas *et al.*, 1992), which are often genes associated with growth control and differentiation (Kozak, 1987; Lazaris-Karatzas *et al.*, 1992).

Based on consideration of these findings, we hypothesize that amino acid regulation of mTOR activity in trophectoderm cells is promoting the translation of a protein (or proteins) important in regulating the onset of motility. The cellular events triggered by amino acid signaling are likely to be downstream either of p70S6K phosphorylation, PHAS-I phosphorylation, or both. One candidate is translation of insulin-like growth factor II (IGFII), which has been shown to be translationally regulated by mTOR (Nielsen *et al.*, 1995), and is expressed specifically in trophectoderm cells at the time of implantation (Lee *et al.*, 1990). Inactivation of the IGFII gene leads to a phenotype that resembles that of the p70S6K gene knockout (DeChiara *et al.*, 1991; Shima *et al.*, 1998). Alternatively, amino acid availability may regulate IGFII signaling in the trophectoderm through effects on mTOR. Another candidate is translation of ornithine decarboxylase (ODC), whose translation is strongly affected by eIF4E activity (Kimball *et al.*, 1999), and which has previously been shown to be important for embryonic development in the mouse (Van Winkle and Campione, 1983). One final candidate is translation of the T-box transcription factor eomesodermin. Inactivation of the eomesodermin gene by homologous recombination leads to failure of the embryo to implant, and the null blastocysts very closely resemble those cultured in EP− medium in morphology and behavior (Russ *et al.*, 2000).

Potential Role for ATB^{0+} Activity in Amino Acid Signaling

Previous studies have shown that the lack of leucine alone is sufficient to substantially inhibit blastocyst outgrowth (Gwatkin, 1966a,b; Naeslund, 1979), which is consistent with the leucine requirements for activation of mTOR and phosphorylation of p70S6K observed in cultured cell models. Leucine is transported into embryos by the sodium-dependent B0,+ amino acid transport system, which is fairly broad in its specificity, but has a very high affinity for leucine and tryptophan (Van Winkle, 2001). The activity of ATB^{0+} is specifically upregulated at the blastocyst stage, whereas at earlier stages of development leucine transport is mediated by the the sodium-independent b0,+ transporter (Borland and Tasca, 1974; Van Winkle *et al.*, 1988, 1990b). This suggests a role for ATB^{0+} in mediating the amino acid signal in embryos. Studies in cultured cells have not provided a clear model for the signaling system activated by amino acids, and a recent study suggests that the mechanism for transmission of leucine-dependent signals may depend on the cell type (Pham *et al.*, 2000; Lynch, 2001). The potential role for ATB^{0+} may thus be specific to trophectoderm cells. It is clearly important to determine precisely how trophoblast cells sense the presence of amino acids, and how this information is transmitted.

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