Preimplantation mouse embryos express a cell surface receptor for tissue-plasminogen activator

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SUMMARY

The serine protease tissue-plasminogen activator (t-PA) has previously been shown to be intracellular in mouse secondary oocytes and extracellular in fertilized eggs. Here we demonstrate that extracellular t-PA activity is bound to the surface of the fertilized egg. The level of t-PA activity associated with preimplantation mouse embryos decreases in the 2-cell stage embryo, then increases in 4-cell and morula stage embryos. However, morulae grown in culture from fertilized eggs lack t-PA activity but are able to bind exogenously added mouse t-PA. Additionally, northern analysis indicates that preimplantation embryos do not contain detectable levels of t-PA mRNA. Therefore, the enzyme activity associated with 4-cell and morula stage embryos in vivo is derived from t-PA present in the oviduct lumen that binds the embryo, and not from protein produced from translation of embryonic mRNA. The binding activity is species and protein specific in that neither mouse urokinase-type plasminogen activator (u-PA) nor human t-PA bind to cultured morulae. Furthermore, binding activity is dose-dependent and saturable, and does not require the active site of t-PA. These data indicate that a cell surface-specific t-PA-binding activity exists in the preimplantation mouse embryo and may localize function and concentrate the proteolytic activity of t-PA in early mouse development.

Key words: tissue-plasminogen activator, preimplantation mouse embryo, cell surface receptor, serine protease

INTRODUCTION

Proteases have been implicated in many important regulatory, migratory and invasive processes in development (for review see Pittman, 1990). In some cases, these proteases are linked together in a sequential pathway of activation that can dramatically amplify the proteolytic activity generated. An example of such a proteolytic cascade is the plasminogen activator/plasmin system.

Plasminogen activators (PAs) are highly specific serine proteinases that convert the abundant serum zymogen plasminogen to plasmin. Plasmin is a serine protease with a broad substrate specificity capable of degrading most extracellular matrix components and also activating certain latent proteases and growth factors. Due to the abundance of plasminogen and the biological potential of plasmin, PA activity must be tightly controlled (for review see Vassalli et al., 1991). The PA/plasmin system is regulated not only by gene expression and activation of PAs, but also by modulating the localization of specific PAs and plasmin as well as their inhibitors.

Two PAs are known to exist in mammals, tissue-plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA). Although these two enzymes have structural similarities and catalyze the same reaction, they are products of distinct genes. u-PA has a well-characterized cell surface receptor (Vassalli et al., 1985; Stoppelli et al., 1986). Although definitive evidence for a t-PA cell surface receptor has been more elusive, initial characterizations of apparently different binding proteins have recently been reported (Hajjar, 1991; Nguyen et al., 1992; Bu et al., 1992a).

Plasminogen activators are associated with a variety of events during mouse development such as ovulation (Canipari et al., 1987), embryo implantation (Strickland et al., 1976; Sappino et al., 1989) and neurogenesis (Sumi et al., 1992). Huarte et al. (1985) showed that secondary oocytes and fertilized eggs both exhibit t-PA activity. In secondary oocytes, the activity was found to be intracellular, whereas in fertilized eggs, it was extracellular. Results from these experiments also indicated that in the fertilized egg the majority of t-PA activity is not associated with the zona pellucida.

Since the amount of t-PA activity detected in the fertilized egg is similar to that seen in the secondary oocyte, it is likely that the enzyme is either present within the peri-
vitelline space or bound to the surface of the egg. Therefore, we have investigated the localization of t-PA activity in embryos throughout early development. High levels of t-PA activity are associated with 4-cell and morula stage embryos in vivo. This activity is not due to production of the enzyme by the embryo. Rather, t-PA specifically binds to the cell surface of the preimplantation mouse embryo and this binding can account for the embryonic t-PA activity detected. Our results, combined with the recent evidence of a plasminogen receptor on the fertilized egg (Huarte et al., 1993), suggest that localizing the PA/plasmin system to the embryo surface is of biological importance during early mouse development.

**MATERIALS AND METHODS**

**Isolation and culture of mouse oocytes and embryos**

Mouse secondary oocytes and preimplantation embryos were collected from superovulated 3-week-old C57BL/6NTacFBR × DBA/2NTacFBR (BDF-1) females (Taconic Laboratories, Germantown, NY). Mice were superovulated by peritoneal injection of 5 IU of pregnant mare’s serum gonadotropin (Diosynth Inc., Chicago, IL) followed approximately 46 hours later by injection of 5 IU of human chorionic gonadotropin (hCG) (Organon Inc., West Orange, NJ). Secondary oocytes were recovered 18-20 hours after hCG injection. Embryos were obtained by mating superovulated mice with BDF-1 males. Mating was confirmed by the presence of a vaginal plug and fertilization was assumed to have occurred at midnight. Secondary oocytes and preimplantation embryos through the morula stage were collected from the oviduct. Collection of early 1-cell embryos employed bovine testis hyaluronidase (Sigma, St. Louis, MO) to disperse cumulus mass cells (Hogan et al., 1986). For binding studies, embryos were collected between 3 and 6 p.m. day 1 postcoitum (day 1 p.c.) to avoid the use of hyaluronidase. Primary oocytes were collected from the ovary (Huarte et al., 1985) and blastocysts were obtained by flushing the uterus. Embryos were cultured in organ tissue culture dishes (Falcon 3037, Lincoln Park, NJ) containing TE culture medium (Spindle, 1980) at 37°C in 5% CO₂. Fluka (Ronkonkoma, NY) chemicals were used in preparation of TE culture medium.

**Collection of oviduct and uterus samples**

Oviduct and uterus fluids were collected from mice superovulated and mated as above. The oviduct and uterus were isolated and flushed with PBS. Embryos were removed from the fluid and the fluid was centrifuged for 10 minutes at 4°C to remove debris. The oviduct tissues were then homogenized in 2.5% Triton X-100. Protein concentrations were quantitated using the BCA protein assay (Pierce, Rockford, IL).

**Plasminogen zymographic analysis**

Zymographic analysis was performed as described by Vassalli et al. (1984). Briefly, samples were separated by electrophoresis on 8% SDS-PAGE using a Biorad minigel apparatus. After electrophoresis, gels were placed in 2.5% Triton X-100 for 20 minutes followed by two washes, 15 minutes each, in water. The gel was then overlaid on a semi-solid substrate matrix containing 50 µg/ml human plasminogen, 2.5% Carnation nonfat dry milk, 1% LMP agarose (FMC Bioproducts, Rockland, ME) and 0.1% sodium azide. Gels were incubated for 24-48 hours at 37°C in a humidified chamber. A zone of lysis appeared where the plasminogen activators migrated in the SDS-PAGE gel, diffused into the substrate matrix and activated the underlying plasminogen. Biorad-labelled protein markers were included to confirm the molecular weight of the PA. Plasminogen was treated with AEBSF [4-(2-aminoethyl)benzenesulfonylfluoride] (Calbiochem, San Diego, CA) to inactivate residual contaminating plasmin. AEBSF is a more stable and less toxic irreversible serine protease inhibitor than diisopropylfluorophosphate (DFP). Purified plasminogen was resuspended in 2× PBS at 1 mg/ml. 1 mM AEBSF was added and then incubated at 37°C for 30 minutes. AEBSF addition and incubation were repeated twice. AEBSF-treated plasminogen was extensively dialyzed against 1× PBS at 4°C.

**Enzyme localization assay**

Pronase and acidified tyrode solutions were prepared and cells treated according to Bradley (1987). Zona pellucida disappearance was used as a gauge of pronase (5-8 minutes) and acid tyrode (1-2 minutes) solution effectiveness. The embryos were washed twice in 1 ml of TE culture medium. Except where indicated, the embryos were placed directly in 2× sample buffer (4% SDS, 60 mM Tris-HCl, pH 6.8, 0.001% bromophenol blue) and were used directly for zymographic analysis or stored at −70°C. Pronase and pronase-agarose were obtained from Sigma.

**Binding studies**

Mouse t-PA and u-PA were expressed in army worm ovary (Sf9) cells using a baculovirus protein expression system. Briefly, the cDNA of mouse t-PA (Rickles et al., 1988) or mouse u-PA (Belin et al., 1985) were cloned into the Neh1 site of the pJW Neh1 vector (gift of Dr Christopher Richardson) (Viallard et al., 1990) and recombinant baculovirus clones were generated as described by Piwnica-Worms (1990). Proteins were expressed in spinner cultures in complete Graces medium supplemented with 2% fetal bovine serum (Hyclone, Logan, Utah). Expression of both t-PA and u-PA was verified by zymography, then further analyzed by immunoprecipitation with anti-human t-PA antibodies (American Diagnostica, Greenwich, CT), or western blot analysis with anti-mouse u-PA antibodies (a gift of Dr Dominique Belin). Mouse t-PA was partially purified on an Erythrina trypsin inhibitor (donated by Dr E. Dowdle) column (Heussen et al., 1984) and mouse u-PA was partially purified via heparin agarose (Sigma) column chromatography. Mouse t-PA concentration was estimated by comparison to human t-PA activity (provided by Genentech Inc.). Except where indicated, cultured embryos were incubated for 2 hours in approximately 1 nM mouse t-PA, rinsed twice for 5 minutes in 1 ml culture medium and then mixed with electrophoresis sample buffer.

Mouse t-PA was inactivated with 1 mM AEBSF for 1 hour at 37°C. AEBSF was removed by resuspending in 1 ml cold PBS and then concentrated using a Centricron 30 microconcentrator (Amicon, Beverly, MA) that will remove free AEBSF. The t-PA was resuspended and concentrated three times. By zymographic analysis, AEBSF-treated t-PA was completely inactive.

**Northern analysis**

For the construction of a riboprobe template, an AvaI/SpeI fragment derived from the mouse t-PA cDNA (base pair positions 1890-2401) (Rickles et al., 1988) was cloned into an AvaI/SpeI cut pKs vector (Stratagene, La Jolla, CA). To synthesize the RNA probe, the plasmid was cut with AvaI and transcribed with T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) in the presence of 32P-UTP.

RNA was isolated from oocytes and preimplantation embryos using the method of Chomczynski and Sacchi (1987). RNAs were resuspended in northern tracking dye (50% glycerol, 50% formamide, containing 0.15% bromophenol blue and 0.05% xylene cyanol) and fractionated on a 0.8% agarose gel containing 6% formaldehyde and 1× northern buffer (50 mM boric acid, 5 mM Na₂HPO₄; 10 mM NaEDTA, 10 mM Na₂SO₄). Gels were washed twice for 20 minutes in 500 ml dH₂O and once in 250 ml.
t-PA gene is not transcribed at detectable levels in the early through preimplantation development. In these cultured embryos, t-PA activity was not detected in 4-cell and morula stages (Fig. 1B), in contrast to its presence when the same stages were collected from the oviduct (Fig. 1A). In addition, northern analysis of early mouse embryo RNA detects high levels of t-PA mRNA in primary oocytes, low levels of t-PA mRNA in secondary oocytes (Huart et al., 1987b and Fig. 2), and undetectable levels after fertilization. These results demonstrate that the maternal t-PA message is degraded soon after meiotic maturation and suggest that the t-PA gene is not transcribed at detectable levels in the early embryo.

RESULTS

PA activity in the preimplantation mouse embryo

Mouse oocytes and preimplantation embryos were collected and individually analyzed for plasminogen activator activity by zymographic analysis in the presence of plasminogen (Fig. 1A). As previously demonstrated, PA activity was not present in the primary oocyte, but abundant t-PA activity could be detected in the secondary oocyte (Huart et al., 1985). Preimplantation embryos were collected at 9 a.m. (E) and 9 p.m. (L) during the first 4 days postfertilization. The level of t-PA activity in day 1E stage embryos was similar to secondary oocytes; however, by 9 p.m., embryos had drastically decreased t-PA activity. This reduction was sustained through the early time point of day 2, by which time the embryos had undergone the first embryonic cleavage. That evening, significantly increased levels of t-PA could be detected in 4-cell stage embryos. t-PA associated with the early and late morula stage embryos (d3) is similar to 4-cell stage embryos. t-PA levels were barely detected in blastocysts collected from the uterus on day 4 stage embryos. The zone of lysis represents t-PA activity based on its similar electrophoretic migration when compared to the 72×10^3 Mr human t-PA (Fig. 1A,B) (Activase, Genentech), the requirement of plasminogen for activity (data not shown), and previous immunological (Huart et al., 1985) and molecular genetic analysis (Huart et al., 1987b; Strickland et al., 1988).

Two likely possibilities exist for the striking increase in t-PA activity associated with 4-cell and morula stage embryos. (1) The t-PA gene is transcribed in early embryos or (2) the embryos have the capacity to bind t-PA available in the lumen of the oviduct. To examine these possibilities, mouse embryos were cultured in vitro from the fertilized egg through preimplantation development. In these cultured embryos, t-PA activity was not detected in 4-cell and morula stages (Fig. 1B), in contrast to its presence when the same stages were collected from the oviduct (Fig. 1A). In addition, northern analysis of early mouse embryo RNA detects high levels of t-PA mRNA in primary oocytes, low levels of t-PA mRNA in secondary oocytes (Huart et al., 1987b and Fig. 2), and undetectable levels after fertilization. These results demonstrate that the maternal t-PA message is degraded soon after meiotic maturation and suggest that the t-PA gene is not transcribed at detectable levels in the early embryo.

The possibility that the embryo is binding t-PA during its transit through the reproductive tract would require that the oviduct provides a source of t-PA protein for binding. The oviduct is a rich source of t-PA mRNA (Rickles and Strickland, 1988) and analysis of mouse oviduct tissue and fluid during preimplantation embryogenesis demonstrates that oviduct tissue expresses both t-PA and u-PA (Fig. 3 lanes 1,
tocysts, uterine fluid was subjected to zymography (Fig. 3). The oviduct is abolished by pronase (Huarte et al., 1985). To localize t-PA in the early embryo, we used the ability of pronase to degrade extracellular proteins including the zona pellucida (ZP) and acid tyrode solution to degrade the ZP (Bornslaeger and Schultz, 1985). These reagents enabled us to differentiate between embryos isolated from the oviduct (Fig. 4) and those subjected to pronase (P) or acid tyrode (A) treatment. Each lane represents the lysate of five secondary oocytes or embryos subjected to zymographic analysis. Pronase degrades most extracellular proteins and both procedures remove the ZP.

2 and 3) while fluid from the oviduct lumen contains mostly soluble t-PA (Fig. 3 lanes 4, 5 and 6). Therefore, the t-PA activity associated with the 4-cell and morula stage embryos in vivo is most likely due to the capability of the embryo to bind t-PA produced by the oviduct. In order to understand better the low levels of t-PA activity associated with blastocysts, uterine fluid was subjected to zymography (Fig. 3 lanes 7, 8 and 9). Lower enzyme activity was present in the uterine fluid when compared to parallel samples of oviduct fluid. This lower plasminogen activity may account for the lower enzyme activity associated with blastocysts. Alternatively, the PA activity may be concentrated in specific regions within the uterine lumen or there may exist a specific t-PA binding competitor in the uterus but not the oviduct fluid. Our assay is unable to distinguish between these possibilities.

**Extracellular surface localization of t-PA in the early embryo**

These data and previous experiments (Huarte et al., 1985; Zhang et al., 1992) demonstrate that t-PA is extracellular in the fertilized eggs. To localize t-PA in the early embryo more precisely, we utilized the ability of pronase to degrade extracellular proteins including the zona pellucida (ZP) and acid tyrode solution to degrade the ZP (Bornslaeger and Schultz, 1985). These reagents enabled us to differentiate between t-PA bound to the extracellular surface or the ZP (Fig. 4). Oocytes or embryos were exposed to pronase or acid tyrode solution for the length of time needed to degrade the ZP. In the secondary oocyte, t-PA activity is not significantly affected by pronase, while the t-PA activity normally present in fertilized eggs and morulae isolated from the oviduct is abolished by pronase (Huarte et al., 1985, Fig. 4). Since pronase degrades the ZP and most extracellular proteins, this experiment demonstrates the redistribution of the enzyme from an intracellular to an extracellular location. When this experiment is performed with insoluble pronase-agarose, similar results were obtained (data not shown) suggesting that the pronase is not entering the cell. Conversely, t-PA activity in embryos was unaffected by acid tyrode treatment. Since acid treatment also removes the ZP, the majority of t-PA in the early embryo is not bound to the ZP. Furthermore, mechanical isolation of the ZP from the oocyte, fertilized egg and morula reveals little t-PA activity associated with the ZP (Huarte et al., 1985; data not shown). These results are consistent with t-PA being intracellular in secondary oocytes and localized to the cell surface of fertilized eggs and morulae.

**Binding of exogenous mouse t-PA to early mouse embryos**

To characterize t-PA binding to embryos, we examined whether cultured embryos, which do not possess t-PA activity, might retain t-PA-binding properties and serve as an in vitro model. As shown above, t-PA is present in embryos isolated from the oviduct (Fig. 5, lanes a) and absent in cultured embryos (lanes b). When cultured embryos were incubated with saturating amounts of baculovirus-expressed mouse t-PA, the embryos bound t-PA to a level approximately equivalent to that seen in vivo (lanes c). Since a saturating amount of t-PA was used for the incubation (see Fig. 6), this equivalence (Fig. 5, compare lanes a and c) indicates that binding in vivo is also saturated. Furthermore, since the activity associated with the 4-cell stage embryo is approximately 10 times that of 2-cell stage embryos, it is possible that the binding activity is being regulated. Primary oocytes (data not shown) and blastocysts (Fig. 5), although exhibiting undetectable or low levels of t-PA activity when isolated from the reproductive system, are capable of binding t-PA. This demonstrates that the binding activity exists at developmental stages in which the environment contains little or no t-PA. Also no morphological difference is observed between embryos cultured in the presence or absence of t-PA and embryos isolated in vivo (data not shown).

In order to determine whether the binding was saturable, which would indicate a limited number of binding sites, fertilized eggs were cultured to morulae and then incubated in increasing concentrations of mouse t-PA (Fig. 6). The associated activity increased until 1 nM and then remained relatively constant, indicating that t-PA binding to embryos is dose-dependent and saturable.

**Characterization of morula binding**

Morulae cultured from fertilized eggs were used to characterize further t-PA binding in preimplantation embryos. First, cultured morulae that lack t-PA (Fig. 7, lane 2) were allowed to bind mouse t-PA (lane 3) and then treated with either pronase (lane 4) or acid tyrode (lane 5). The results
Five morulae derived from cultured fertilized eggs were incubated with mouse t-PA and then treated with pronase (lane 8) and incubated with mouse t-PA (lane 9). Each lane represents 10 embryo lysates.

To determine whether t-PA binding exhibits a dependence upon a surface protein or the ZP, cultured morula were treated with pronase or acid tyrode solution before incubation with t-PA, and then examined for their ability to bind exogenous enzyme. Pronase treatment destroyed the ability of morula to bind t-PA (lane 6), whereas acid tyrode treatment had no effect (lane 7). The pronase result demonstrates a protein dependence for t-PA binding, whereas the acid treatment shows that t-PA binding is not dependent upon the presence of the zona pellucida.

To test the possibility that t-PA-binding sites are actively being synthesized during preimplantation development, fertilized eggs were treated with pronase to destroy existing binding sites, cultured to morulae, then incubated in exogenous mouse t-PA (Fig. 7, lanes 8 and 9). These morulae bind mouse t-PA suggesting that the cell surface-binding sites are expressed during in vitro culture.

### Binding specificity

To investigate the specificity of t-PA binding, we determined whether inactive t-PA would compete with active t-PA. Embryos were incubated in saturating amounts of mouse t-PA (1 nM) and an increasing molar excess of AEBSF-inactivated t-PA. At a ratio of active:inactive t-PA of 1:50, binding of active enzyme was not detectable (Fig. 8); whereas, incubation with a high concentration of BSA did not affect binding (data not shown). This result demonstrates that inactive t-PA can compete specifically with active t-PA for binding and that the catalytic activity of t-PA is not required for binding. It further indicates that there are a limited number of t-PA-binding sites per cell.

The mouse u-PA receptor binds mouse u-PA, but not mouse t-PA or human u-PA (Estreicher et al., 1989). Likewise, the binding that we have observed with the mouse embryo is highly specific for mouse t-PA (Fig. 9). Cultured morula bind mouse t-PA ($72 \times 10^3 M_r$), but not mouse u-PA ($48 \times 10^3 M_r$) or human t-PA ($72 \times 10^3 M_r$) indicating that the binding is both protein and species specific. Also, this result excludes a plasminogen activator inhibitor 1 (PAI-1)-t-PA complex being responsible for the binding since u-PA and t-PA both bind to PAI-1 with similar affinities (Hekman and Loskutoff, 1987). Additionally, it seems unlikely that the u-PA receptor is present in the preimplantation mouse embryo because morula do not bind u-PA. Others have shown that the u-PA receptor is linked to the cell membrane by a...
specific glycosyl phosphatidylinositol (GPI)-linkage that is cleaved by phospholipase C (PLC) (Ploug et al., 1991). When morula are subjected to PLC, the bound t-PA activity is maintained (data not shown). This result does not exclude all such receptors, since some members of the GPI-receptor family are not cleaved by PLC.

Finally, in order to understand better the t-PA binding, we took several approaches to disrupt and compete for t-PA binding in early embryos (data not shown). (1) The t-PA protein contains a region similar to that used by plasminogen in binding to its receptor. 40 mM ε-amino-n-caproic acid will dissociate plasminogen from its receptor (Plow et al., 1986), but does not disrupt t-PA binding to morula suggesting that a similar interaction is not involved in t-PA binding. (2) Although t-PA is known to bind fibrinogen (van Zonneveld et al., 1986), treatment of the t-PA or embryos with fibrinogen fragments had no effect on binding. These results suggest the fibrin-binding domain of t-PA is not responsible for t-PA binding in morula. (3) t-PA also binds heparin (Andrade-Gordon and Strickland, 1986). However, high salt treatment, which disrupts proteoglycan binding, did not decrease t-PA activity associated with cultured morula, and treatment of embryos with heparinase A and C also had no effect. This indicates that heparin is not likely to be involved in t-PA binding to mouse embryos. (4) t-PA alone or when complexed to PAI-1 binds the LDL-related receptor protein (LRP) and binding is dissociated by the Ca\(^{2+}\) chelator EDTA (Bu et al., 1992b; Orth et al., 1992); conversely, 10 mM EDTA actually enhanced binding of t-PA to morula. Furthermore, t-PA binding to LRP can be inhibited by the receptor-associated protein (RAP) (Williams et al., 1992; Orth et al., 1992) (a gift of Dr Dudley Strickland); however, high concentrations of purified RAP had no effect on t-PA binding in mouse embryos indicating that LRP is not involved with t-PA binding in this system. (5) We also investigated the possibility that t-PA binding in the embryo is mediated by a Ca\(^{2+}\)-dependent mannose receptor, which has been shown to occur in certain systems (Otter et al., 1991). However, as mentioned above, culturing embryos with high EDTA concentrations does not disrupt t-PA binding in the preimplantation embryo. Additionally, t-PA binding in our system is not inhibited by high concentrations of mannose 6-phosphate (100 µM) (Dennis and Rifkin, 1991). Therefore, mannose receptors are unlikely to be involved in t-PA binding to embryos.

**DISCUSSION**

**Evidence for a t-PA receptor**

The data presented here demonstrate that preimplantation mouse embryos bind t-PA. This binding has the characteristics of a specific and novel interaction of t-PA with the cell surface based on the following criteria. (1) The binding is highly specific, since neither mouse u-PA nor human t-PA is capable of binding. This result also indicates that binding is not mediated through plasminogen activator inhibitor-1 (PAI-1), which has been shown to bind both t-PA and u-PA with high affinity. (2) The interaction is dose-dependent and saturable, with an apparent \( K_d \) in the range of 1 nM. (3) The binding does not require the active site of t-PA. (4) The binding is abolished by treatment of the embryos with pronase, suggesting a direct or indirect requirement for an extracellular protein. (5) The interaction is not affected by low pH or phospholipase C treatment, in contrast to the GPI-linked u-PA receptor that is sensitive to both. (6) The binding is not affected by fibrinogen fragments or heparin, although both of these molecules can bind t-PA and binding does not appear to involve LRP or a mannose receptor. Taken together, the results suggest that a component of the embryo membrane is functioning as a previously uncharacterized cell surface receptor for t-PA.

**Regulation of t-PA**

Two levels at which t-PA binding to preimplantation embryos may be regulated are the availability of the t-PA ligand and/or modulation of the number of binding sites. Our results suggest both levels of regulation may be operating in mouse embryos. First, blastocysts are capable of binding t-PA (Fig. 5), but have a low level of associated t-PA that may be due to low levels of t-PA present in the mouse uterus (Fig. 3). Second, 4-cell stage embryos are capable of binding approximately 10-fold more t-PA than 2-cell stage embryos when incubated in an equal concentration of the enzyme, indicating an increase in the number of binding sites in the transition to 4-cell stage embryos. Also, we found that fertilized eggs treated with pronase and cultured to morulae are capable of binding t-PA (Fig. 7, lane 9), suggesting that the embryo is actively synthesizing new binding sites.

**The role of t-PA in the preimplantation mouse embryo**

The present study confirms and extends earlier evidence of t-PA activity in preimplantation mouse development (Sherman, 1980; Huarte et al., 1985), and presents a novel observation of t-PA-binding activity associated with the mouse embryo. The majority of t-PA associated with fertilized eggs probably originates from maternal gene expression in oocytes. After fertilization, the enzyme would be secreted and bound to the embryo surface (Fig. 4). The oocyte store of t-PA does not appear to be essential for in vitro or in vivo development (Strickland et al., 1988; W. G. R. and S. S., unpublished results); however, oviduct lumen t-PA may compensate for the loss of maternal t-PA.

The biological benefits of localizing t-PA activity to the surface of the preimplantation murine embryo are not established. The embryo-bound t-PA could activate soluble plas-
minogen (which could easily pass through the zona pellucida) and the generated plasmin could then diffuse back through the zona and function extracellularly. In this manner, the embryo could control spatially the generation of plasmin activity. The functional advantage of localized t-PA may be not only to localize plasmin action, but also to enhance the efficiency of plasminogen activation and/or protect t-PA from inhibitors. For example, cells with bound u-PA have enhanced invasiveness when compared to cells lacking bound u-PA (Ossowski, 1988; Ossowski et al., 1991). Also, plasmin when bound to its receptor, is protected from its inhibitors (Miles et al., 1988). Interestingly, recent evidence suggests plasminogen binds to the cell surface of secondary oocytes (Haurte et al., 1993). Although plasminogen binding has not been tested during the remainder of preimplantation development, it seems logical that bound t-PA may efficiently activate bound plasminogen, localizing the biological effect of plasmin to the microenvironment of the embryo.

t-PA produced and secreted into the lumen of the oviduct appears to be the sole source of associated PA activity in later stage preimplantation embryos, such as morula, as evidenced by the lack of t-PA mRNA in early embryos (Fig. 1), and the lack of PA activity in cultured embryos (Fig. 1B). This is reminiscent of u-PA and sperm binding during spermatogenesis (Haurte et al., 1997). In this case, spermatozao do not produce u-PA but bind it as they travel through the male genital tract. In 4-cell embryos and morulae, cell-associated proteolytic activity may be important in maintaining the fluidity surrounding the embryo and enhance the embryo’s ability to migrate through the oviduct that is known to contain fibrin deposits (Liedholm and Asted, 1975). Alternatively, t-PA may participate in localizing growth factor or hormone activation. For example, it is known that plasmin activates the latent form of TGF-β1 in certain cell types (Lyons et al.; 1988; Sato and Rifkin, 1989). Specific t-PA binding may act to localize spatially plasmin’s activation of TGF-β1 or similar molecules. It is apparent in Xenopus and Drosophila that TGF-β-like molecules are critical for inductive events in pattern formation (Whitman and Melton, 1989). Therefore, bound t-PA might act as a modulator of growth and differentiation in murine development.

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