Expression of Fertilin and CD9 in Bovine Trophoblast and Endometrium During Implantation

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ABSTRACT

The superficial placentation of cattle involves the development of fetal binucleate cells that arise from the chorion and migrate between adjacent cell tight junctions to fuse with maternal epithelium. Thus, the temporal and spatial patterns of expression of the cell migration, adhesion, and fusion molecules fertilin and CD9 were investigated in bovine trophoblast and endometrium. Bovine fertilin α and fertilin β messenger RNA sequences were amplified by reverse transcriptase-polymerase chain reaction in tests (positive control), peri-implantation (Days 18, 19, and 21), and postimplantation (Days 35–40) trophoblast RNA, but not in caruncular endometrium (Day 40). Northern blot analysis indicated that the transcript hybridizing to fertilin α in trophoblast RNA was approximately 4.0 kilobases (kb), whereas in tests, 2 transcripts of approximately 3.3 and 3.8 kb were indicated. The transcript hybridizing to the fertilin β probe was also larger in trophoblast than in tests (3.8 vs. 2.4 kb, respectively). In situ hybridization revealed that fertilin β mRNA was expressed by trophoblast cells, including binucleate cells. Immunohistochemical study of CD9, a member of the transmembrane-4-superfamily which is thought to be involved in sperm-egg fusion, showed that CD9 was present on the apical surface of uterine epithelium and in a subpopulation of binucleate cells of the trophoblast. Immunoprecipitation followed by Western blot analysis showed association between CD9 and integrin α1 in endometrium. The results support the hypothesis that fertilin and CD9 are involved in bovine binucleate cell migration and fusion.

ABSTRACT

implantation, placenta, trophoblast, uterus

INTRODUCTION

The superficial implantation of cattle initiates by apposition between trophoblast and uterine epithelium at approximately Day 19 [1, 2]. Adhesion between these epithelial layers begins at approximately Days 21–22 by interdigitation of the microvilli of uterine luminal epithelial cells and trophoblastic cells [1, 2]. This process begins in the region near the embryo and spreads outward over both the caruncular and intercaruncular areas. Beginning at approximately Day 18 of pregnancy, binucleate cells arise from the uninnucleate cells of the fetal trophoectoderm [2]. As trophoblast adhesion proceeds, the binucleate cells become mobile and migrate through adjacent cell tight junctions and across the microvillar junction to reach the maternal luminal epithelium, where they fuse with maternal epithelial cells to form trinucleate or giant cells [2, 3]. Binucleate cell migration is maximal at approximately Day 24 of pregnancy, but it continues throughout the bovine pregnancy in both caruncular and intercaruncular regions [3, 4]. Binucleate cells are thought to deliver conceptus-derived molecules to the maternal endometrium after giant cell exocytosis [4]. Placental lactogen, progesterone, and several pregnancy-specific proteins have been identified in mature binucleate cells and in the giant cells of the fetomaternal hybrid epithelium [4].

Binucleate cell migration, adhesion, and fusion presumably require specific adhesion and signaling molecules in interactions. All three of these physiological events (i.e., epithelial cell migration, apical surface adhesion, and cell fusion) are relatively rare. However, sperm migration, egg-sperm binding, and fusion during fertilization represent a relatively well-characterized migration, adhesion, and fusion cascade that may share some characteristics with this system. Two key candidates in the regulation of fertilization are fertilin [5–9] and CD9 [10–12]. Fertilin α and β are members of the “a disintegrin and metalloprotease” (ADAM) family, which are characterized by having a signal domain, prodomain, metalloprotease-like region, disintegrin-like sequence, cysteine-rich domain, epidermal growth factor-like repeat, and transmembrane and cytoplasmic domains [13–15]. Fertilin α and β have been found on the postacrosomal region of the mature spermatozoa of several species, always as a heterodimer [14–16]. Given that fertilin α and β genes are located on different chromosomes [17], and that spermatozoa of fertilin β knockout mice do not express either fertilin β or mature fertilin α [5], the dimer structure is important to fertilin activation. The β subunit is thought to interact with an integrin receptor through its disintegrin loop [6–8] and to be essential for sperm migration and adhesion to the egg [6, 9]. Fertilin α possesses a hydrophobic face in the cysteine-rich domain formed by secondary structures (α helix or β strand) [7, 16]. This characteristic may contribute to a role in fusion, although this function remains unclear [5, 8]. Fertilin β transcription has only been detected in tests, whereas fertilin α mRNA has been identified in many tissues [15].

The glycoprotein CD9 is a widely expressed member of the transmembrane-4-superfamily (TM4SF; also called tetraspans) [18], with a relative molecular mass of 25 000 in cattle [19]. Several TM4SF members, including CD9, CD81, CD82, and CD63, are involved in cell proliferation and differentiation, adhesion, motility, and cancer [18]. CD9 is considered to be critical in fertilization, because CD9 null mice showed complete sterility due to a deficiency in egg-sperm fusion [10–12]. Ectopic expression of CD9 promoted human B-cell motility on fibronectin and laminin substrates [20]. Expression of this molecule in extravillous trophoblast and its involvement in the invasion of a trophoblast-like cell line suggest that it is active in human placentaion [21, 22]. Most functions of CD9 are linked to
its cooperation with integrins. Recent studies indicate that CD9 and several other TM4SF proteins can mediate integrin function and alter tyrosine phosphorylation [20, 23, 24].

Little is known regarding the molecular regulation of binucleate cell migration and fusion in cattle. The purpose of the present study was to determine whether CD9 and fertilin or fertilin-like molecules were present at the fetomaternal interface in cattle in a distribution pattern consistent with a role in binucleate cell migration and fusion.

MATERIALS AND METHODS

Animals and Tissue Collection

All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the Nova Scotia Agricultural College Animal Care and Use Committee. Healthy, mixed-breed beef heifers were observed for at least one natural estrous cycle before breeding by artificial insemination. Reproductive tracts were collected on Day 18 of the estrous cycle and on Days 18, 21, 24, and 30 of gestation (n ≥ 3 per day). Pregnancy was confirmed by examination of the ovaries and the presence of embryonic tissue. The uteri were cut into 1.5-cm blocks, snap-frozen in liquid nitrogen, and then transferred for long-term storage at −80°C. Cotyledary and intercotyledary allantoic membranes, totall-extraembryonic membranes, or caruncular endometrium were dissected on Days 35–40 of gestation from pregnant animals randomly collected at the slaughterhouse (n = 3). Gestational stage was estimated by measuring crown-rump length and pregnant horn diameter [25]. Cattle testis was similarly processed to be used as a positive control tissue for fertilin expression. For RNA analysis of peri-implantation trophoblast (Days 18, 19, and 21; n = 1 per day), embryos were flushed from pregnant dairy cows that were bred by artificial insemination. Embryos were immediately washed in cold, serum-free PBS, and then the embryonic disc was removed by dissection before the trophoblast was frozen in liquid nitrogen for subsequent RNA extraction.

To provide an enriched population of trophoblast binucleate cells for immunohistochemistry, bovine placentomes were isolated from an animal that was approximately 70 days pregnant (determined by measuring crown-rump length). Fetal and maternal tissues were manually separated, immunohistochemistry, bovine placentomes were isolated from an animal (gift of Dr. Alan Wildeman, University of Guelph, Guelph, ON, Canada) using T7 or SP6 RNA polymerase (Roche Diagnostics, Laval, QC, Canada) and incorporating digoxigenin (DIG)-labeled UTP (Roche Diagnostics) following the manufacturer’s instructions. The synthesized riboprobes were used in Northern blot analysis and in situ hybridization.

Northern Blot Analysis

Ten micrograms of each total RNA were electrophoresed in 1% (w/v) agarose5%/v/v formaldehyde gels in morpholinopropanesulfonic acid buffer, then transferred overnight onto a positively charged nylon membrane (Roche Diagnostics). Following cross-linking, the membranes were hybridized at 68°C with 100 ng/ml of fertilin α, fertilin β, or bovine actin antisense riboprobe in hybridization buffer containing 50% (v/v) deionized formamide, 5× SSC (1× SSC: 0.15 M sodium chloride and 0.015 M sodium citrate), 0.1% (w/v) X-lauroylsarcosine, 0.02% (w/v) SDS, and 2% (w/v) blocking reagent. After overnight hybridization, low-stringency washes in 2× SSC and 0.1% SDS at room temperature, followed by high-stringency washes in 0.5× SSC and 0.1% SDS at 68°C, were employed to eliminate nonspecific binding. Riboprobe binding was detected by reaction with anti-DIG conjugated to alkaline phosphatase (anti-DIG-AP; Roche Diagnostics), followed by application of disodium 3-(4-methoxyxipiro{1,2-dioxetane-3,2-[5^-chloro] tricyclo [3.3.1.1dec-4-y]} phenyl phosphate (Roche Diagnostics). The chemiluminescent signal was visualized on x-ray film (SciMed, Truro, NS, Canada). Expressions of fertilin mRNAs were normalized to β-actin mRNA expression and compared using Syngene II densitometry (Fisher Scientific, Whitby, ON, Canada).

In Situ Hybridization

In situ hybridization to localize fertilin β expression was performed on 10-µm cryostat serial cross-sections collected on Superfrost Plus slides (Fisher Scientific). The sections were used immediately or stored at −80°C with desiccant. Before hybridization, sections were dried on a heating block at 55°C for 5 min and fixed in 4% (w/v) paraformaldehyde in PBS at 4°C for 10 min. Following permeabilization with 2 µg/ml of proteinase K for 10 min, sections were acetylated twice with 0.5% acetic acid in 0.1 M phosphate buffer (pH 8.0) for 10 min. Prehybridization and hybridization buffer (4× SSC, 10% [w/v] dextran sulfate, 1× Denhard solution, 2 mM EDTA, 50% formamide, and 250 µg/ml of herring sperm DNA) at 42°C for 2 h. Sections were hybridized overnight using 20 ng of antisense or sense riboprobe diluted in 100 µl of hybridization buffer at 42°C in a humid chamber. Following hybridization, two washes each with prewarmed, serial strength SSC buffers (2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC) were performed at 42°C to remove nonspecific binding. After probing with anti-DIG-AP positive signals were detected by colorimetric reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics).

Immunohistochemistry

Acetone-fixed cross-sections from pregnant uteri were blocked in 10% normal goat serum (Vector Laboratories, Burlington, ON, Canada) in PBS for 1 h, then incubated with 1.3 µg/ml of polyclonal rabbit anti-human CD9 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in 7% normal goat serum in PBS for 2 h. Adjacent sections were incubated with rabbit immunoglobulin (Ig) G (Chemicon, Temecula, CA) as a negative control. Primary antibody binding was detected with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and color development with metal-encrusted diaminobenidine substrate (Pierce, Rockford, IL), and then slides were counterstained with Mayer hematoxylin (Electron Microscopy Sciences, Fort Washington, PA) and viewed by light microscopy. Relative CD9 expression was scored on a 0–6 point scale for intensity (0, negative; 1, very weak; 2, weak; 3, moderate; 4, strong; and 5, very strong), and the proportion of trophoblast cells showing a pericellular distribution of CD9 expression (score, >2) was estimated in each section. At least 3 cryosections from each animal were scored, and at least 3 animals were used for each day (Day 18 of the estrous cycle and Days 18, 21, 24, and 30 of pregnancy). One-way ANOVA (Minitab, Inc., State College, PA) was used to analyze the effect of day on the percentage of total binucleate cells showing CD9 pericellular reactivity. Additional immunohistochemistry experiments were carried out to localize CD9 antibody binding using Alexa 594 goat anti-rabbit IgG conjugate (Molecular Probes, Eugene, OR), followed by mounting in Fluoromount-G (Cedarlane Laboratories Limited, Hornby, ON, Canada) and viewing on a DMLB microscope equipped for fluorescence (Leica Microsystems, Wetzlar, Germany). For colocalization of CD9 and the integrin α1 subunit, acetone-fixed uterine sections and slides of isolated binucleate cells were incubated with
eluted and transferred after 12% SDS-PAGE under reduced conditions to a nitrocellulose membrane (Amersham Pharmacia Biotech, Montreal, QC, Canada). The immunoprecipitated proteins were probed with rabbit polyclonal antisera to the cytoplasmic domain of the human integrin α, subunit (Chemicon) or normal rabbit serum diluted 1:100 in PBS containing 3% dry milk. Thereafter, the membranes were incubated in donkey anti-rabbit IgG HRP conjugate (Pierce) diluted 1:1000 in PBS containing 0.05% Tween 20 for 1 h. Antibody binding was detected using a chemiluminescent substrate (enhanced chemiluminescence; Amersham Pharma- cia Biotech) and x-ray film.

RESULTS

Expression of Fertilin mRNAs

Bovine fertilin α-specific primers amplified reverse-transcriptase products from testis (positive control), peri-implantation trophoblast (Days 18, 19, and 21 of gestation), and postimplantation allantochorion (≥35 days of gesta- tion) but not from caruncular endometrium. Amplified frag- ments comigrated with 900-base pair (bp) DNA marker, which was the expected amplicon size (877 bp) (Fig. 1A). Sequence identity of the trophoblast product was 97% with the published bovine testis cDNA for fertilin α [16].

Amplification with bovine fertilin β-specific primers produced a fragment of 692 bp from bovine testis, tropho- blast, and chorioallantois but, as with fertilin α, not from caruncular endometrium (Fig. 1A). The trophoblast PCR product shared 96% nucleotide identity with the published sequence of bovine fertilin β cDNA [16].

Northern blot analysis suggested that trophoblast from peri-implantation animals and testis expressed similar levels of fertilin α- and β-like mRNAs (Fig. 1B). The tran- script hybridizing with fertilin α probe in trophoblast was approximately 4.0 kilobases (kb) in length, whereas in testis, 2 fertilin α transcripts of approximately 3.3 and 3.8 kb were detected. The riboprobe generated from fertilin β cDNA recognized a single transcript of approximately 3.8 kb in trophoblast and allantochorion, versus a single band of 2.4 kb in testis. Neither fertilin α nor β riboprobe yielded a detectable hybridization signal with Day 40 extraembry- onic membranes (including amnion) or caruncular endo- metrium. The lack of hybridization signal in Day 40 total extraembryonic membranes likely reflects a lower propor- tion of chorionic cells compared to earlier stages, because the amnion and allantois were included in this tissue. Day 40 cotyledon did exhibit hybridization to fertilin probes, and fertilin mRNA was amplified by RT-PCR at this stage.

In situ hybridization localized fertilin β-like mRNA ex- pression to trophoblast in all pregnant animals studied (Fig. 2). Positive staining was detected not only in the principal and binucleate cells of the trophoblast but also in the cells of the mesoderm aligning with trophoblast. Occasional gi- ant cells of the fetomaternal hybrid epithelium also were reactive. Limited but consistent expression of fertilin β-like mRNA was observed in isolated subepithelial stromal cells. No specific hybridization was observed in allantois.

Expression of CD9 During the Peri-Implantation Period

Strong expression of CD9 protein was observed on the apical surface of uterine glandular and luminal epithelium of Day 18 nonpregnant animals and of all pregnant animals examined with the exception of regions of luminal epithe- lium, where extensive modification of the epithelium had occurred in Day 24 and Day 30 sections (Fig. 3). Intensity scores did not vary with stage of pregnancy. In the tropho- blast, a diffuse, relatively weak signal was observed in Day 18 sections, and occasional apical expression was detected.
FIG. 2. Detection of fertilin \( \beta \) mRNA by in situ hybridization in sections of bovine endometrium at Day 18 (A), Day 21 (B), Day 24 (C), and Day 30 (D) of pregnancy. Sections hybridized with fertilin \( \beta \) antisense riboprobe (large panels) showed reactivity in trophoblast, including binucleate cells (closed arrows), the luminal epithelium where modified by binucleate cell migration, and limited signal in the subepithelial stroma (arrowheads). Sense riboprobe did not generate a signal (small inset panels). cLE, Columnar luminal epithelium; mLE, modified fetomaternal hybrid epithelium; S, stroma; T, trophoblast. Bars = 100 \( \mu \)m.

only in samples from 1 animal (and not from 3 others). By Day 21, concentrations of cells displaying apical staining were present in selected areas, and a pericellular expression pattern was observed on a few large oval cells, some of which were obviously binucleate. Accordingly, the mean proportion of trophoblast cells displaying this pericellular expression pattern was lower (\( P < 0.01 \)) in sections from Day 18 (mean, 0.78%) than in sections from Day 21, 24, and 30 (means, 6.7%, 7.8%, and 10.6%, respectively) animals. Immunoreactivity to CD9 antibody also occurred on the surface of the developing allantois.

Immunoprecipitation with anti-CD9 followed by Western blot analysis with antibody to the integrin \( \alpha_3 \) subunit resulted in a single band with a relative molecular mass of 26–27 \( \times 10^3 \), which is the reported size of integrin \( \alpha_3 \) cytoplasmic domain against which the antibody was raised. These results indicate that the \( \alpha_3 \) integrin subunit was associated with CD9 in bovine endometrium (Fig. 4A). Immunofluorescent colocalization of CD9 and integrin \( \alpha_3 \) revealed that only a few trophoblast binucleate cells coexpressed these 2 antigens (Fig. 4B).

**DISCUSSION**

In the present study, fertilin \( \alpha \) and \( \beta \) mRNAs were expressed in bovine trophoblast during the peri-implantation period. To our knowledge, this is the first report of fertilin \( \beta \) mRNA expression outside the testis, although fertilin \( \beta \) protein has been detected in the central nervous system [27]. Unfortunately, because of the lack of suitable antibodies, functional protein expression could not be determined in trophoblast. However, the coincident expression of fertilin \( \alpha \) and \( \beta \) transcripts suggests that they may dimerize in trophoblast, as has been found in mature sperm [16]. The transcripts hybridizing to the fertilin probes in trophoblast were larger than those in testis. We also detected 2 different transcripts of fertilin \( \alpha \) in testis in the present study. The prospective functional regions of the fertilin \( \alpha \) and \( \beta \) cDNAs amplified from trophoblast showed high sequence identities (\( \geq 96\% \)) to their counterparts from testis tissue. Variation in transcript length has been reported for monkey fertilin \( \alpha \) [28] and may reflect alternative splicing. The different transcripts also may represent different genomic segments, as identified for murine fertilin \( \alpha \) [29].

Fertilin \( \beta \) transcription was detected in trophoblast principal and binucleate cells as well as in some multinucleate cells in the fetomaternal hybrid luminal epithelium. This localization suggests that fertilin, or a fertilin-like protein, may participate in binucleate cell adhesion, migration, and/or fusion. These processes likely involve integrins, which can interact with fertilin \( \beta \) via the TDE sequence at the tip of its disintegrin loop [16, 30]. In the guinea pig, this sequence was shown to be critical for integrin recognition
FIG. 3. Immunofluorescent localization of CD9 showing consistent apical expression of CD9 in uterine epithelium in nonpregnant endometrium (NP) at Day 18 of the estrous cycle and Days 18, 21, 24, and 30 of pregnancy. Pericellular CD9 staining of binucleate cells (arrows) was detected from Days 21 to 30 of pregnancy, and allantois also expressed CD9 (arrowheads). Note that luminal epithelium is extensively modified at Days 24 and 30. Rabbit IgG was substituted for polyclonal rabbit anti-CD9 in adjacent sections in each experiment as a negative control. GE, Glandular uterine epithelium; LE, luminal uterine epithelium; S, stroma; T, trophoblast. Bars = 100 μm.

FIG. 4. Coimmunoprecipitation and colocalization of CD9 and integrin α3 subunit. A) Proteins extracted from endometrial tissue were immunoprecipitated with anti-CD9 antibody, then Western blotted with antibody to the integrin α3 subunit, resulting in a single specific band of molecular mass $26-27 \times 10^3$ (lane 1). Lanes 1 and 3 were proteins precipitated with anti-CD9; lanes 2 and 4 were proteins precipitated with rabbit IgG (negative control). Lanes 1 and 2 were probed with anti-integrin α3; lanes 3 and 4 were probed with normal rabbit serum (negative control). MW, Molecular mass ($\times 10^3$) ladder. B) Antibody to the integrin α3 subunit colocalized (closed arrows) with only a small proportion of the CD9-positive binucleate cells in cross-sections of bovine uterus at Day 30 and in isolated binucleate cells. Some cells stained only with antibody to the integrin α3 subunit (open arrows). GE, Glandular epithelium; LE, luminal epithelium; S, stroma. Bars = 100 μm.
during fertilization [8, 16]. In the mouse, the disintegrin domain recognizes and binds to an integrin to mediate sperm-egg binding [6]. Evidence also suggests that \( \alpha_6 \beta_1 \) is the integrin involved [6, 8, 9, 30, 31]. However, oocytes from integrin \( \alpha_6 \) null mice can undergo normal fertilization [32], and some concern exists regarding the in vivo relevance of peptide-blocking studies [33]. The integrin \( \alpha_6 \) subunit is expressed along both basal and lateral surfaces of attaching bovine trophoblast and moderately in uterine epithelium [34]. Integrin \( \alpha_6 \beta_1 \) has also been implicated in fertilin \( \beta \)-dependent migration of murine sperm through the uterotubal junction [9]. The role of fertilin in cell fusion is unclear: Fertilin \( \beta \) null spermatozoa can fuse with normal oocytes, but at 50% the normal rate [35]. Other ADAM proteins are implicated in cell fusion events such as myotube formation, macrophage-derived giant cell formation, and multinucleated osteoclast-like cell differentiation, although again, their mechanism of action is not well defined [36, 37].

The tetrapsanin CD9 was expressed constitutively on the apical surface of uterine epithelium. This pattern of apical expression has been reported in several cell models, including human uterine epithelium [9, 23, 38]. The pericellular distribution of CD9 on binucleate cells of the trophoblast is interesting, because it coincides with the onset of binucleate cell migration. In cows, binucleate cells comprise approximately 20% of the trophoblast cell population, and approximately 25% of them are migrating by Day 30 of pregnancy [3]. Thus, the population of CD9-positive cells (7%–10%) is consistent with expression during migration. It may be that expression of CD9 on the cell surface is a prerequisite for migration. Because CD9 is a multifunctional cell surface molecule, it would not be surprising if it is involved in bovine implantation. Recent evidence suggests that tetrapsanin-integrin association regulates integrin function [23]. CD9 has been reported to associate with several integrins in a number of migration, adhesion, and fusion systems, including those containing the \( \alpha_3 \), \( \alpha_5 \), \( \alpha_6 \), or \( \beta_1 \) subunits [9, 21, 23, 24, 38, 39]. This tetrapsanin may act as a linker between the extracellular domain of the integrin alpha chain and the intracellular signaling molecules [24, 40]. Tyrosine phosphorylation of focal adhesion kinase is an established signaling response to integrin activation that appears to be affected by expression of CD9 [24]. Furthermore, several tetrapsanin proteins, including CD9, can associate with a phosphoinositide 4-kinase [41]. Taken together, it is reasonable to hypothesize that CD9 is involved in the regulation in integrin signaling to facilitate bovine binucleate cell migration. Bovine binucleate cells do not express the \( \alpha_6 \) integrin subunit [42]. However, expression of both the \( \alpha_3 \) and \( \alpha_6 \) subunits has been detected in trophoblast and uterine epithelium, although apical uterine epithelial expression is relatively weak [34]. The integrin \( \beta_1 \) subunit is also present in these tissues [34]. In the present study, the \( \alpha_3 \) integrin subunit coprecipitated with CD9, suggesting that they interact in bovine endometrium. Immunofluorescent colocalization indicated that a small proportion of binucleate cells express both antigens, but it remains to be demonstrated whether these proteins associate in the binucleate cells themselves.

Interactions among fertilin \( \beta \), CD9, and \( \alpha_6 \beta_1 \) were hypothesized in a recently developed model of murine sperm migration. The sperm are thought to “walk” on the epithelium, switching on and off adherence between sperm fertilin \( \beta \) and oviductal integrin \( \alpha_6 \beta_1 \), which is associated with CD9 and the actin cytoskeleton [9]. The variable affinity of \( \alpha_6 \beta_1 \) for fertilin depends on the activation state [43], which, in turn, can be influenced by CD9 [9]. Our studies show that integrins, CD9, and fertilin are distributed in such a way that a similar interplay could mediate the binucleate cell migration, adhesion, and/or fusion with uterine epithelium that characterizes ruminant placentalization.

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