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Rac-1 and IQGAP are potential regulators of E-cadherin–catenin interactions during murine preimplantation development

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Abstract

Adherens junction formation is fundamental for compaction and trophectoderm differentiation during mammalian preimplantation development. We recently isolated an IQGAP-2 cDNA from a differential display-polymerase chain reaction screen of bovine preimplantation developmental stages. IQGAP-1 and -2 proteins mediate E-cadherin-based cell-to-cell adhesion through interactions with β-catenin and the Rho GTPases, rac1 and cdc42. Our study demonstrates IQGAP-1,-2, rac-1 and cdc42 mRNAs are present throughout murine preimplantation development. IQGAP-1 and rac-1 protein distribution changes from predominantly plasma membrane associated to predominantly cytoplasmic as the embryo progresses through cleavage divisions and compaction to the blastocyst stage.

Keywords: IQGAP; Cell adhesion; Cell polarity; Embryo; Oocyte; Blastocyst; Trophectoderm

1. Results and discussion

The mechanisms regulating adheren junction (AJ) mediated cell-to-cell adhesion during preimplantation development are currently being elucidated (Clayton et al., 1999; Fleming et al., 2001; Pauken and Capco, 1999, 2000). IQGAP-1 (Weissbach et al., 1994) and -2 (Brill et al., 1996) are members of the GTPase activating protein (GAP) family of proteins that interact with members of the small Rho GTPase family of proteins, rac-1 and cdc42 (reviewed by Kaibuchi et al., 1999a; Kuroda et al., 1999). GAPs, as a family, interact with GTPases to regulate the inherent GTPase activity of these proteins, thereby mediating their switching from a GTP-bound and active conformation to a GDP-bound and inactive conformation. As such, IQGAPs do not have GAP activity but they are implicated in controlling intercellular adhesion in epithelial cells through interactions with the cadherin/catenin complex and the Rho GTPases, rac-1/cdc42 (reviewed by Kaibuchi et al., 1999b; Kuroda et al., 1999). Kaibuchi et al. (1999b), using epithelial cell lines, has proposed a model that predicts that cell-to-cell adhesion mediated by E-cadherin/catenin interactions may be regulated by IQGAP/β-catenin interactions that are influenced by activation and inactivation 'switching' of the small Rho GTPases, cdc42/rac1. We have isolated an IQGAP-2 cDNA by applying a differential display reverse transcriptase polymerase chain reaction (DD)-(RT-PCR) screen to preimplantation embryonic mRNA pools (Natale et al., 2000). The present study characterized the expression patterns of gene products representing the IQGAP family (IQGAP-1 and -2) and rac1 and cdc42, the small Rho GTPases during murine preimplantation development.

mRNAs encoding IQGAP-1 (expected size 786 bp amplicon) were detected at the oocyte, 4-, 8-cell, morula and blastocyst stages of development but were not consistently detectable at the two-cell stage by RT-PCR (Fig. 1A). In contrast, IQGAP-2 mRNAs were detected by the presence of an expected 486 bp polymerase chain reaction (PCR) product at all stages of murine preimplantation development in each replicate developmental series (Fig. 1B). A PCR amplicon of 370 bp representing rac-1 mRNA was consistently observed in murine oocytes and in embryos at the 2-, 4-, 8-cell, morula and blastocyst stages (Fig. 1D) in all replicates. mRNAs encoding cdc42 were undetectable at the 4-cell stage in all replicates but were consistently observed in oocytes, 2-, 8-cell, morula and blastocyst staged embryos as demonstrated by the presence of an expected 326 bp size PCR product in these samples. This pattern may indicate a delay in onset of cdc42

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embryonic transcription until the 4-cell stage. Prior to amplification using embryo cDNA, positive controls were conducted to evaluate the efficacy of each primer set (data not shown). In order to confirm the sequence identity of each amplicon, PCR products for each gene were cloned, sequenced, and compared to gene bank sequence databases as described. In all cases, the sequence of each product was 100% identical to known murine cDNA sequences.

IQGAP-1 protein was detected in murine embryos at all stages of preimplantation development examined. In oocytes, IQGAP-1 fluorescence was present in the cytoplasm in general, but also localized to regions adjacent to the cell membrane and was particularly evident around the maternal chromosomes (Fig. 2a) localized to the meiotic spindle apparatus. As the embryo progressed through the two- and four-cell stages, IQGAP-1 fluorescence was cytoplasmic with increased intensity encircling the cell borders and margins. Fluorescence intensity was greater in the free, unopposed regions of the cell membranes where the pattern was more diffuse. IQGAP-1 fluorescence at the cell margins near adjacent cells was more tightly localized but was reduced in intensity (Fig. 2b, c). This pattern was maintained until the 8-cell stage when beyond this developmental stage, the fluorescence at the cell margins was further reduced and merged with the cytoplasmic fluorescence. This transition continued until the compacted 8-cell stage where the fluorescence pattern was predominantly cytoplasmic with some apical staining in membrane-associated regions (Fig. 2d). As the embryos transitioned to the morula stage, IQGAP-1 fluorescence intensified and was observed in the cytoplasm as in earlier stages and also in the apical regions of the outer cells. In these embryos, the apical signal was diffuse in membrane-associated regions with additional punctate patterns suggestive of vesicular localization (Fig. 2e). At the morula and blastocyst stages, IQGAP-1 fluorescence was present in the cytoplasm of all cells in a diffuse pattern (Fig. 2f, g). Trophectoderm cells displayed the punctate, vesicular pattern observed in previous stages while inner cell mass cells displayed an apolar fluorescence distribution encircling each cell (Fig. 2h).

Rac-1 protein was detectable by immunofluorescence in murine preimplantation staged embryos. Signals above background levels were detected in oocytes and in all embryo stages examined (2-, 4-, 8-, compacted 8-cell, morula and blastocyst stage embryos). Rac-1 protein expression in oocytes was consistently cytoplasmic with some punctate staining in close approximation to cell membrane regions. In addition, rac-1 localized to the cell margins encircling the oocyte at this stage (Fig. 3a). At the 2- and 4-cell stages, rac-1 was also observed localized adjacent to the cell membrane encircling each blastomere (Fig. 3b, c). While fluorescence was evident in the plasma membrane regions of the blastomeres, punctate vesicular staining was also observed in the cytoplasm near the cell margins, towards the outer edges of each blastomere (Fig. 3b, c). Faint nuclear staining was also observed in embryos at these stages. At the 8-cell stage of development, a shift in rac-1 localization was observed. At this stage and in subsequent stages of development, rac-1 fluorescence became consistently punctate in the cytoplasm. In 8-cell and compacting 8-cell stages, the fluorescence was primarily present around the cell margins of each blastomere, however, as embryos compacted, this pattern became less evident (Fig. 3d, e). By the morula stage, rac-1 was completely absent from the cell margins and was observed entirely as punctate staining in the cytoplasm in close approximation to the nucleus (Fig. 3f). In the blastocyst, rac-1 was observed as punctate or vesicular fluorescence in the cytoplasm. This pattern was more prevalent in trophectoderm cells than inner cell mass cells and exhibited a more apical localization in polar trophectoderm cells when compared to mural trophectoderm cells (Fig. 3g).

In summary, we have demonstrated for the first time that IQGAP-1, IQGAP-2, cdc42, and rac-1 are expressed at the mRNA level during murine preimplantation development and that IQGAP-1 and rac-1 are expressed at the protein level. In addition, the localization of these proteins varies throughout preimplantation development and these variations in protein distribution coincide with the events of compaction.

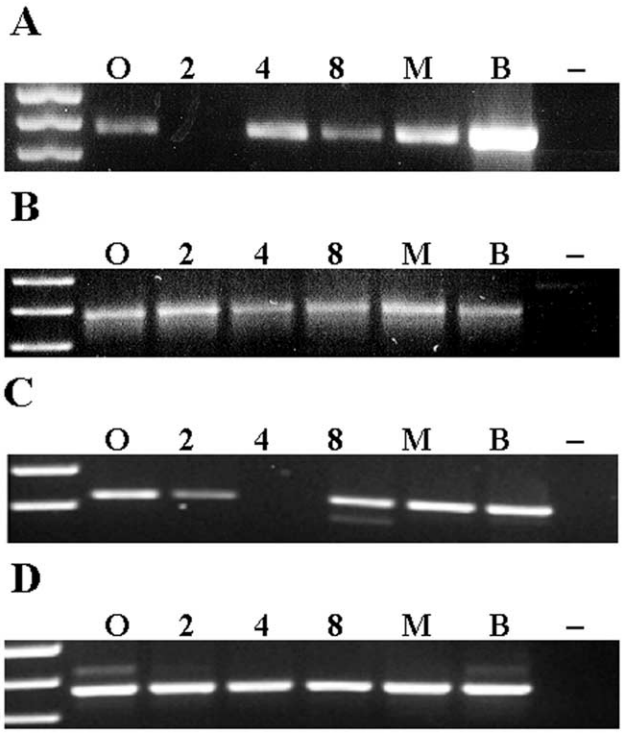

Fig. 1. Detection of IQGAP-1 (A), -2 (B), cdc42 (C) and rac1 (D), mRNA transcripts during murine preimplantation development. Each lane contains the specific RT-PCR gene products derived from total RNA representing the equivalent of two embryos. Embryo stages shown include oocyte, 2-, 4-, 8-cell, morula and blastocyst (O, 2, 4, 8, M, B). Polymerase chain reaction amplicons of expected size were observed for IQGAP-1 (786 bp, A), IQGAP-2 (486 bp, B), cdc42 (326 bp, C) and rac-1 (370 bp, D). Negative control lanes are denoted by (−).

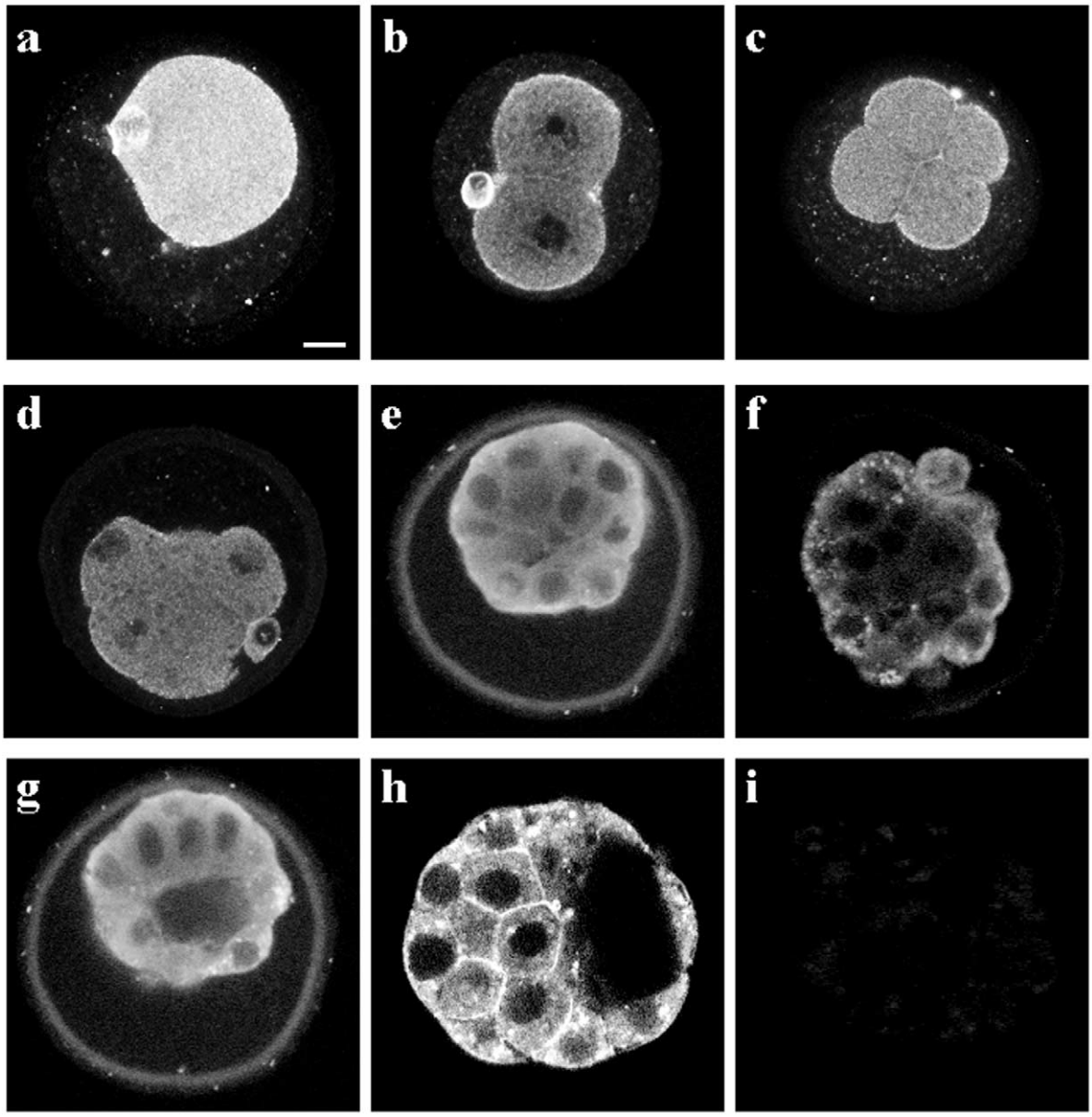

Fig. 2. Immunofluorescent localization of IQGAP-1 protein in murine cleavage staged embryos. Mature oocyte (a), 2-cell (b), 4-cell (c), compacted 8-cell (d), morula (e and f), early blastocyst (g) and blastocyst (h) staged embryos incubated with IQGAP-1 antisera revealed IQGAP-1 immunofluorescence. Blastocyst staged embryo (i) incubated with fluorescein isothiocynate (FITC)-conjugated secondary antibody displayed only background levels of fluorescence. Scale bar = 10 μm.

2. Materials and methods

2.1. Superovulation and mouse embryo collection

Female CD-1 mice (Charles River, Canada), 3–4 weeks of age, were superovulated by injection with pregnant mare serum gonadotrophin (PMSG; Sigma, St Louis, MO, USA) and human chorionic gonadotrophin (hCG; Sigma) prior to mating with CB6F1/J males as described (Offenberg et al., 2000; Watson and Kidder, 1988). Zygotes through to the 8-cell stage were flushed from oviducts of female mice using flushing medium I (Spindle, 1980). Morulae and blastocysts were flushed from uteri on day 4 using flushing medium II (Spindle, 1980). Embryos were collected in pools of 30–50 embryos, flash-frozen using liquid nitrogen and stored at −70 °C until the time of RNA extraction.

2.2. RNA extraction and reverse transcription

Total RNA was extracted from previously frozen pools of embryos using phenol chloroform and ethanol precipitation with *Escherichia coli* ribosomal RNA (Gibco BRL, Burlington, ON, Canada) as a carrier as previously described (Watson et al., 1992). Reverse transcription

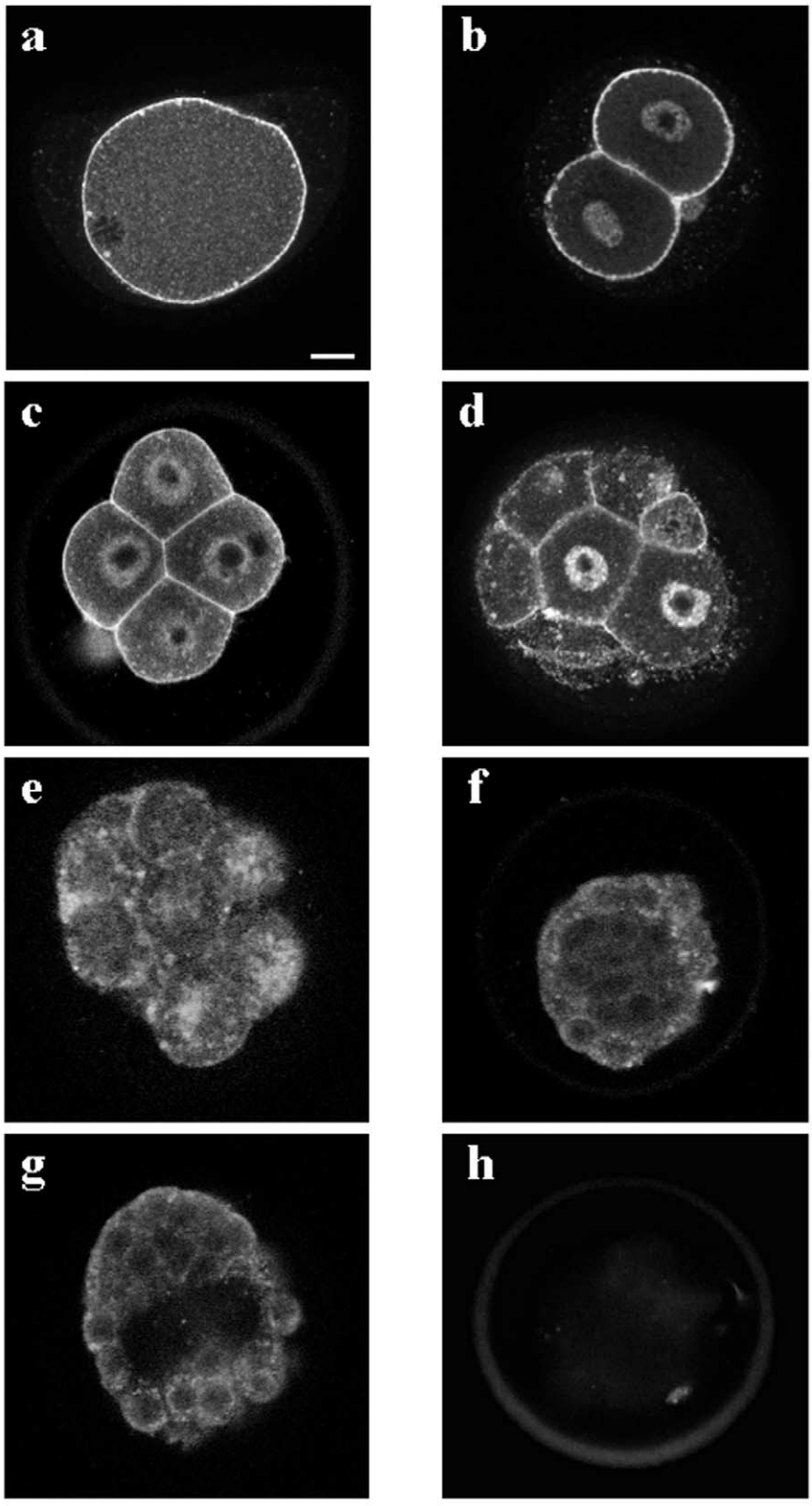

Fig. 3. Immunofluorescent localization of rac-1 protein in murine cleavage staged embryos. Mature oocyte (a), 2-cell (b), 4-cell (c), compacted 8-cell (d and e), morula (f) and blastocyst (g) staged embryos incubated with rac-1 antisera revealed rac-1 immunofluorescence. Blastocyst staged embryo (h) incubated with FITC-conjugated secondary antibody only displayed only background levels of fluorescence. Scale bar = 10 μm.

Table 1
Nucleotide sequences for polymerase chain reaction amplification of IQGAP-1, IQGAP-2, Cdc42 and Rac1[a]

Gene product	Primer	Primer Sequence	Size (bp)	GenBank accession #
IQGAP-1	5′	GGACCATCAGGATGCCATTGC	770	AF240630
	3′	GCCTCGCTGCTGTGTACTTCA		
IQGAP-2	5′	AGACACCAGCAACTGCGCAAC	480	U51903
	3′	TCACTGGCTTCGCTCTCTTCG		
Cdc 42	5′	CGACCGCTAAGTTATCCACAG	326	NM_009861
	3′	GCAGCTAGGATAGCCTCATCA		
Rac-1	5′	GGACACAGCTGGACAAGAAGA	369	X57277
	3′	GGACAGAGAACCGCTCGGATA		

[a] Shown also is the size in base pairs (bp) of the expected amplicon and the GenBank accession number of the nucleotide sequence against which the primers were designed.

(RT) reactions were performed using oligo-dT (Gibco BRL) as previously described (Barcroft et al., 1998; Offenberg et al., 2000).

2.3. PCR amplification

PCR was performed as previously described (Barcroft et al., 1998; Offenberg et al., 2000) using cDNA representing two embryo equivalents per reaction. PCR reactions were repeated on cDNA from each embryo stage representing five distinct and complete developmental series.

Gene specific primers for PCR were designed and synthesized (Gibco BRL) for IQGAP-1, IQGAP-2, cdc42 and rac-1 based on available human and mouse full-length and EST nucleotide sequences in GenBank nucleotide databases. PCR primer sets were designed to regions of nucleotides conserved across species. The primer pairs used to amplify IQGAP-1, IQGAP-2, cdc42 and rac-1 are reported in Table 1. In addition, primer sets designed to bracket an intron of the β-actin gene (previously described in Watson et al., 1992a), were used to assess the efficiency of the RNA extraction/RT and to ensure the absence of DNA contamination in each sample prior to investigation of expression of the target genes. A negative control was included for each primer set on each developmental series in which an additional PCR reaction was included that contained no cDNA template in order to assess for cDNA contamination.

To confirm identity of PCR products, an amplicon representing embryonic cDNA of each gene was cloned by standard TA-cloning (pGEM-T; Promega, Madison, WI, USA), followed by nucleotide sequencing (DNA Sequencing Facility, Robarts Research Institute, London, ON, Canada), and comparison of resulting sequence to Genbank nucleotide databases.

2.4. Wholemount indirect immunofluorescence

Characterization and localization of expression of IQGAP-1, and rac-1 proteins in preimplantation stage mouse embryos was assessed by wholemount indirect immunofluorescence techniques and detected by laser-scanning confocal microscopy as previously described by our laboratory (Barcroft et al., 1998). A series of embryos representative of each stage of preimplantation development in the mouse was examined. Staining for each protein was repeated on a minimum of three distinct developmental series containing at least five embryos representative of each developmental stage, including the oocyte, 2-cell, 4-, 8-cell, morula and blastocyst stages. Negative controls were also conducted in which embryos were exposed to the same procedure in the absence of primary antibody to control for background and non-specific binding of the secondary antibody. Antibodies used were obtained from commercial sources (IQGAP-1, Upstate Biotechnology, BD Transduction Laboratories; rac1, Upstate Biotechnology, BD Transduction Laboratories). All primary antibodies were tested over a range of dilutions and were found to be effective on mouse positive control tissues (liver and kidney sections) at a dilution of 1:100 from the commercial stock concentration. In addition, antibodies were tested in immunofluorescence experiments on MDCK and mouse embryonic fibroblast cells to act as a positive control in assessing the efficacy of the methodology, the ability of the antibodies to reproduce previously reported staining patterns for these gene products (Kuroda et al., 1997) and the ability of the antibodies to recognize murine gene products. Antibody specificity was also confirmed by Western Blotting of murine tissue lysates. Bands of correct size were detected for each antibody (data not shown). Fluorescence patterns were examined by microscopy using a Nikkon compound microscope equipped with EPEI fluorescent optics as well as confocal laser-scanning microscopy (BioRad MRC 600).

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