Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 167-175

Antibodies to the cytoplasmic domain of the MUC1 mucin show conservation throughout mammals

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Received February 19, 1992

An antiserum against the carboxy-terminal seventeen amino acids of the human MUC1 mucin has been raised and extensively characterized. This antiserum, CT1, immunoprecipitates two high molecular weight polymorphic bands (>200 kDa) from a metabolically labelled breast cancer cell line corresponding to the two alleles which have previously been shown to contain different numbers of a twenty amino acid repeat. The CT1 antiserum reacted with tissues from many mammalian species and immunoprecipitated large polymorphic proteins, suggesting that the cytoplasmic portion of the molecule is well conserved. The cell and tissue distribution of Muc-1 mucin in the mouse has been studied by immunocytochemistry. This protein is abundant at the apical surfaces of epithelial tissues and is found expressed in the stomach, kidney, mammary gland, pancreas, salivary gland, lung, trachea, uterus, cervix and vagina.

MUC1, a large molecular weight mucin glycoprotein, is an integral membrane protein found on the apical surfaces of epithelial tissues and most carcinomas. The extracellular domain consists of a large (1000-3000 amino acids) region of 20 amino acid repeats with many potential sites for O-linked glycosylation (1-5). Analysis of the protein to date has been limited to humans, due to the lack of species cross-reactivites of antibodies directed to the extracellular tandem repeat domain. Cloning of the mouse homologue (Muc-1) showed that indeed the protein homology with the tandem repeat domain of the human MUC1 is only 34%. However, the homology of the transmembrane and cytoplasmic domains was found to be high (87%) suggesting a cross-species conservation of function, possibly reflected in the known interaction of the cytoplasmic tail with actin microfilaments (6). To investigate the expression of homologues of the MUC1 gene in other species, we have prepared a polyclonal antiserum (CT1) to the cytoplasmic tail. The CT1 antiserum, made to the last 17 C-terminal amino acids defines an epitope which is highly conserved in a variety of tissues and species. This reagent is the first whose reactivity with the MUC1 product is independent of the O-glycosylation seen in the extracellular domain. This report describes the utilization of the CT1 antiserum for the identification of the homologues of the MUC1 gene produced in a number of species and presents the expression pattern of the Muc-1 mucin in the adult mouse.

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0006-291X/92 $4.00

Materials and Methods

Genomic Blot Analysis

DNA from cell lines or blood was prepared either by an Applied Biosystems 340A DNA extractor or by established methods (8). DNA (20 μg except Drosophila (5 μg)) was digested with EcoRI (New England Biolabs) prior to electrophoresis through a 0.7% agarose gel (ICN Biochemicals) and transferred to a Biodyne nylon membrane (Pall). Prehybridization was in 5X SSC, 5X Denhardts solution, 50mM sodium phosphate pH 6.5, 0.1% SDS, 43% formamide and 200 μg/ml salmon sperm DNA for 2 hours at 42°C. Hybridization was in the above solution with 10^6 dpm/ml of probe for 24 hours at 42°C. Blots were washed to a stringency of 0.5X SSC/0.1% SDS at 55°C for 30 minutes. The blots were then exposed to XAR-5 film (Kodak) for 3 weeks.

Probes

The human probes used were pMUC7, corresponding to 500 bp of the tandem repeat domain (1) and pGEM-PEM16 corresponding to ~1kb of 3' cDNA sequence including regions coding for the 31 amino acid transmembrane and 69 amino acid cytoplasmic tail domain (2). Probes were labelled by random priming (9) in the presence of [α-^{32}P]dCTP (Amersham International plc) to a specific activity of >1 X 10^8 dpm/μg.

Production and characterization of Polyclonal Antisera

The peptide CT1 (SSLSYTNPAVAATSANL) was synthesised on a 430 A peptide synthesiser (Applied Biosystems) as previously described (10). Ten mg of peptide was coupled to 10 mg of keyhole limpet hemacyanin (Calbiochem) using 0.2% glutaraldehyde according the the protocol described by Harlow and Lane (11). Following the final dialysis in PBS (phosphate-buffered saline containing 17mM NaCl, 4 mM KCl, 12mM Na_2PO_4, 2mM KH_2PO_4, pH 7.2) the volume was 5 ml. New Zealand white rabbits were injected with 0.5 ml of peptide conjugate plus an equal volume of Freunds Complete adjuvant at multiple subcutaneous sites. Booster injections in incomplete adjuvant were given at 3-4 week intervals. Bleeds of 10 to 15 ml were taken seven days post-injection. Significant antibody titres were observed after 2 boosts. Serum was separated from the whole blood and filtered through a 0.22 um filter (Millipore) before use. Antisera was tested for positive reactivity by ELISA on CT1 peptide (10) and by indirect immunofluorescence on methacarn-fixed sections of lactating mouse mammary glands. Methacarn fixation (methanol:chloroform:glacial acetic acid, 60:30:10) was performed for 2 hr. at room temperature; samples were transferred to 70% ethanol before paraffin embedding.

Immunoprecipitation

Cells were grown for 24 hours in 6 cm dishes to 70-80% confluence and starved for 2 hr. prior to labelling in DMEM with 10% of the usual amount of glucose (0.45g/l). Labelling took place for 16 hr. in 0.5 ml of the above medium containing 100μCi of D-[6-^{3}H] Glucosamine Hydrochloride, (Amersham TRK 398, specific activity 26 Ci/mmol.). For organ cultures 1 mm^3 pieces (15-20 pieces) of tissue were incubated in the above medium. After washing in ice cold PBS, cells were lysed in 400μl of lysis buffer. Lysis buffer contained 40mM sodium phospate pH 7.2, 250mM NaCl, 50mM sodium fluoride, 5mM EDTA, 1% Triton X-100, 1% deoxycholate, 25μg/ml leupeptin, 100μg/ml PMSF, 10mM benzamidine and10μg/ml aprotinin. Immunoprecipitation from the lysates was by a standard method (11) using 10 μl of neat CT1 antisera or 100 μl of HMFG-1 and HMFG-2 monoclonal antibody tissue culture supernatants. Complexes were collected on Protein A Agarose (Sigma) and sequentially washed 4 times (1 ml each wash) in (1) lysis buffer (2) 0.5 M NaCl in lysis buffer (3) lysis buffer and (4) millipore water. The complexes were resuspended in 50 μl of 2X sample buffer (12), heated at 95°C for 5 mins and the Protein A Agarose was removed by centrifugation. Samples were electrophoresed on a 5% PAGE gel (3% stacking gel) and visualized by fluorography with Amplify (Amersham International).

Staining of cultured cells and tissue sections

Cells grown on plastic dishes were washed in PBS and permeabilized in 0.1% Triton X-100 for 4 min. on ice. After washing with PBS, cells were fixed with 3% paraformaldehyde for 10 min. The dishes were then blocked with 50% fetal calf serum (FCS) in PBS for one hour. After briefly washing, dishes were incubated with CT1 antiserum at a dilution of 1:50 in 15% FCS/PBS or appropriate control antisera for one hour at room temperature. The dishes were incubated with FITC conjugated swine anti-rabbit immunoglobulin antiserum (Dako, diluted 1:50 in PBS) for 1 hour. For "live" staining the permeabilization step was omitted prior to blocking. To block

staining neat CT1 antisera was incubated with 5mg/ml CT1 peptide for 30 min. at room temperature before dilution and incubation with cells. Immunohistochemical staining of tissue sections was performed on methacarn fixed material as described by Bartek (13). Sections from three male and three female mice were analyzed.

Results and Discussion

Species conservation

Most of the antibodies directed to the core protein of the MUC1 gene product have been found to react with an immunodominant region of the tandem repeat domain (14, 15). When tested on western blots of similar proteins prepared from the mammary gland of different species (7,16), one such antibody, HMFG-2, showed reactivity only with proteins from primates and cats (S. Patton, personal communication). In agreement with this observation, a Southern blot of genomic DNA from a variety of species probed with human tandem repeat sequences (pMUC7) showed cross-hybridization only with primate DNA. However, the cytoplasmic tail probe (pGEM-PEM16) cross-hybridized with many species: human, rhesus and African green monkey, baboon, marmoset, bovine, horse, wallaby, goat, dog, rabbit, rat, hamster (DNA is uncut on this blot), mouse and whale (Fig. 1). In addition, the probe also hybridized to sheep and hamster (data not

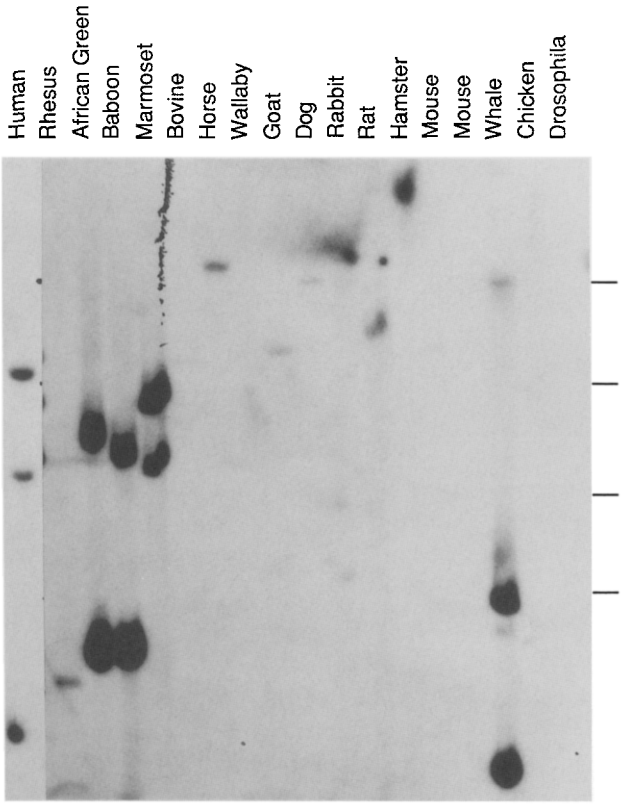

Fig. 1. Genomic blot analysis of DNA from different animals with a probe corresponding to the transmembrane and cytoplasmic domains of the human MUC1 gene. Twenty μg (5 μg for Drosophila) of DNA digested with EcoRI was loaded in each lane. After electrophoresis through a 0.7% gel and blotting onto nylon membrane, the blot was probed with pGEM-PEM16 (ref. 2) corresponding to the 3' region of the MUC1 gene. Molecular weight marker bars correspond to 23, 9.4, 6.5 and 4.3 kilobase pairs.

shown). MUC1 is highly polymorphic in humans due to the presence of variable numbers of tandem repeats of 60 base pairs (1, 2, 10, 17). It is probable that where two bands are seen on the blot shown in Fig. 1, this also reflects a polymorphism due to variable numbers of tandem repeats.

Production and characterization of antiserum to the MUC1 core protein

It would be useful to have antisera reactive with other species in order to study expression during development and in the adult. Since the 3' probe cross-hybridized on zoo blots, peptides were synthesized corresponding to different regions of the 69 amino acid cytoplasmic tail. Only one peptide elicited antibodies reactive with the native protein. This peptide was comprised of the C-terminal 17 amino acids of the human core protein. The antiserum called CT1 was shown to react specifically with the immunizing peptide when tested by ELISA on a range of peptides corresponding to different regions of the mucin (data not shown). The antiserum was subsequently titrated on methanol:acetone fixed MCF7 cells (human mammary gland adenocarcinoma cell line) by indirect immunofluorescence staining which indicated that the antiserum was strongly positive at a dilution of 1:50. The specificity of the CT1 antiserum was confirmed by blocking the staining with the specific peptide. The antiserum was used to confirm the predicted topology of the protein. Live cells showed no positive reactivity with CT1, whereas cells that had been permeabilized by either Triton-X 100 detergent (Fig. 2A) or methanol:acetone fixation (data not shown) reacted positively with CT1. Thus, it appears that the predicted cytoplasmic domain of the mucin is located inside the cells.

To further characterize the specificity of the CT1 antiserum, immunoprecipitation experiments were performed. From lysed MCF7 cells metabolically labelled with 3H-glucosamine,

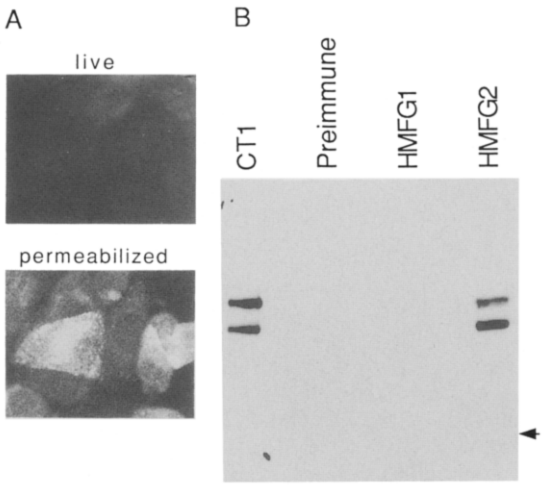

Fig. 2. A. Cytoplasmic localization of the carboxy-terminal domain of the MUC1 protein. Immunofluorescence staining of MCF-7 cells with CT1 antiserum, showing live cells and cells permeabilized with Triton X-100 for 4 minutes on ice. Magnification x630
B. Immunoprecipitation of MUC1 protein from the MCF-7 human breast cancer cell line using different antibodies. MCF-7 cells were biosynthetically labelled with [^{3}H]-glucosamine. Extracts were incubated with CT1 antiserum, preimmune serum, or the monoclonal antibodies HMFG-1 and HMFG-2 which have epitopes in the extracellular tandem repeat domain of the MUC1 protein. The complexes were collected on Protein A Agarose and analyzed by SDS-PAGE followed by autoradiography of dried gels. The two bands represent the two allelic forms of this polymorphic protein (10,17). Arrowhead indicates 200 kDa marker protein.

CT1 antiserum immunoprecipitated two bands corresponding with the MUC1 protein (Fig. 2B). These bands were the same size as those immunoprecipitated by HMFG-2 and HMFG-1, two monoclonal antibodies directed to epitopes in the tandem repeat of the core protein. Preimmune serum did not immunoprecipitate any bands.

Immunoreactivity with different species

We were interested in knowing if the cytoplasmic tail domain was sufficiently well-conserved evolutionarily to enable the CT1 antiserum to cross-react with other species. Indirect immunofluorescence staining was performed on tissue sections and cells representing various vertebrate and invertebrate classes. Positive immunoreactivity was detected in all the mammals tested. These were mouse, rat, hamster, rabbit, ferret, cat, sheep and cow. Xenopus and Dictyostelium were negative (data not shown). Organs chosen for staining were those known to be strongly positive in the human such as pancreas, stomach, lungs and lactating mammary gland. Representative sections of the mammary gland, stomach and pancreas of mouse, ferret, sheep, cat and rabbit are shown in figure 3. In these tissues, the MUC1 mucin is expressed on the apical membranes of epithelial cells lining ducts or glands, a pattern of expression which is similar to that

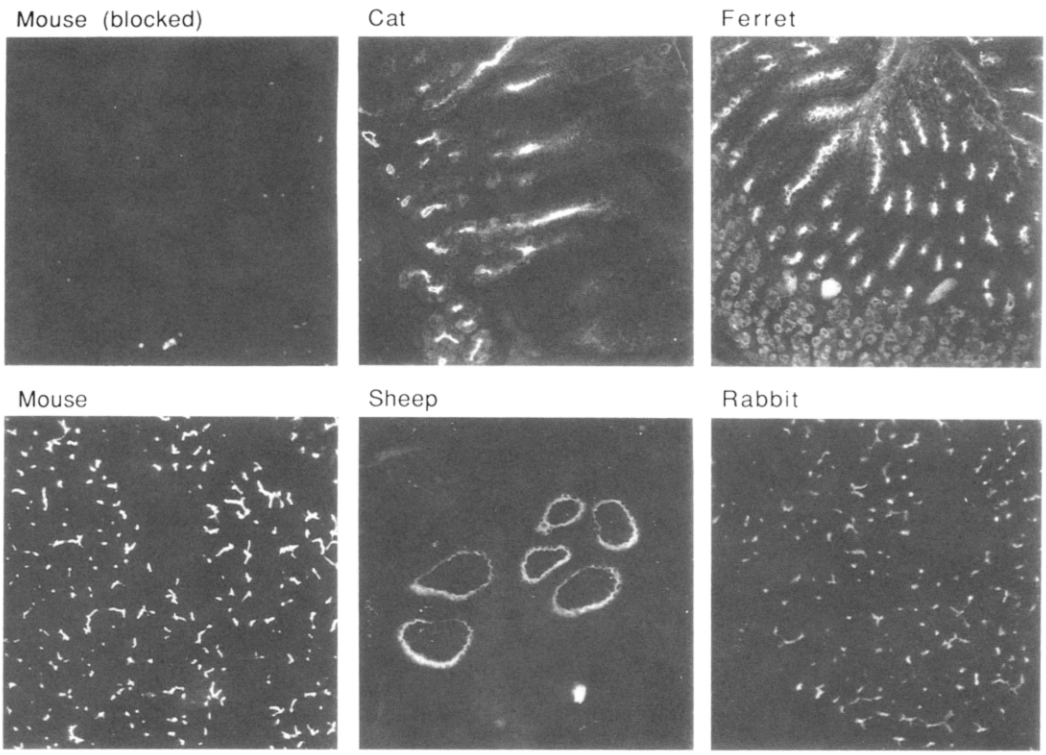

Fig. 3. Immunoreactivity of CT1 antiserum with different species. Indirect immunofluorescence staining of methacarn-fixed and paraffin embedded sections of mouse pancreas, cat and ferret stomach, sheep mammary gland and rabbit pancreas with CT1 antiserum (1:50 dilution). Specific staining was blocked in all cases by preincubation of the antiserum with CT1 peptide, shown here only for the mouse pancreas. Magnification x630. Tissues were obtained from animals sacrificed for other purposes and kindly provided by Dr. Paul Richardson, St. George's Medical School, London.

reported for normal human tissues (18,19). All positive staining was specifically blocked by preincubation of the antiserum with the CT1 peptide.

Immunoprecipitation of the proteins from several of the immunoreactive species with CT1 antiserum indicates that the proteins have electrophoretic mobilities in the high molecular weight region of the gel as expected for this mucin. Organ cultures of cat stomach and lung (not shown) and cell lines from human (MCF7), rhesus monkey (MA104) and hamster (HP-1) were metabolically labelled with [^{3}H]-glucosamine and immunoprecipitated with CT1 antiserum. The immunoprecipitated fraction was electrophoresed on a 5% gel. Two bands with different electrophoretic mobilities corresponding to the two alleles, were obtained for human, Rhesus monkey and cat (Fig. 4). The sizes and differing electrophoretic mobilities were as expected for this protein which varies from individual to individual depending on the number of tandem repeats present. Only one band was obtained from the hamster cell line, suggesting that this species is not polymorphic. This result is not unexpected following the cloning of the mouse homologue and the demonstration of a lack of polymorphism in the rodent species (7, 20). In all immunoprecipitations performed the band from the hamster appeared smeary. This smear may be due to a greater degree of glycosylation present on the hamster mucin which was immunoprecipitated from a pancreatic cell line. It is known that the human MUC1 obtained from the pancreas is more heavily glycosylated and thus is a much larger molecule than MUC1 from the mammary gland (3). Furthermore, although the hamster mucin core protein appears to be similar in size to the mouse (65 kDa., A.P. Spicer, personal communication) and much smaller than the human core proteins (130 to 250 kDa.), the level of glycosylation may be greater.

It is striking that at least one domain of the protein is evolutionarily conserved, enabling the development of a reagent that can be used to study mucins in other species. Mucins are involved in

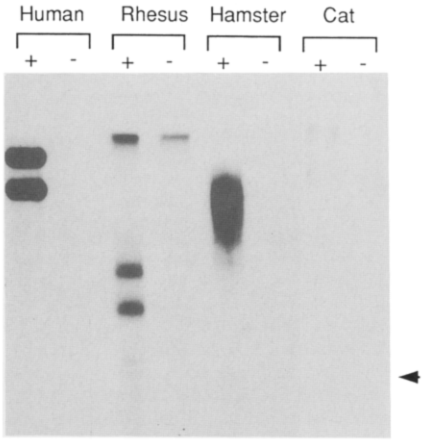

Fig. 4. Immunoprecipitation of MUC1 protein from human, rhesus monkey, hamster and cat. Cell lines and 1 mm^3 pieces of tissue (cat) were labelled with [^{3}H]-glucosamine. Extracts were incubated with CT1 antiserum (+) or preimmune antiserum (-). The complexes were collected on Protein A Agarose and analyzed by SDS-PAGE followed by autoradiography of dried gels. Arrowhead indicates 200 kDa. marker protein. The two bands in human, rhesus monkey and cat represent the two allelic forms of this polymorphic protein (10,17). Rodents do not appear to be polymorphic (7).

a number of diseases such as cystic fibrosis, asthma and cancer, particularily in metastasis (21), evasion of NK cell killing (22), in the escape of tumor cells from the immune system (23), and rodents are often good models for these phenomena. It is interesting that the tandem repeats which are a feature of all the mucin genes cloned until now do not appear to be well preserved among phylogenetically diverse species. The intestinal mucins MUC2 and MUC3 also show no antibody cross-reactivity between rodents and humans and the human tandem repeat probes hybridize to human and chimp only and were negative with pig, cow and frog DNA (24,25). The evolutionary conservation of the cytoplasmic domain would suggest functional importance, whereas the amino acid sequence and lengths of the tandem repeat domains may be less important and exist simply as scaffolds for glycosylation.

Tissue distribution of Muc-1 in the mouse

The pattern of expression of the Muc-1 core protein in the adult mouse may contribute to understanding the function of this mucin and aid in characterization of the rodent as a suitable model system. Immunohistochemical staining with CT1 was performed on both male and female adult mice tissues using methacarn-fixed, paraffin embedded sections. Distribution of the protein was limited to the apical surfaces of cells lining lumens in a wide range of simple secretory epithelial tissues as well as at the apical surfaces and tops of cells in the pseudostratified epithelium of the trachea and the suprabasal layer of the squamous epithelium of the cervix and vagina. The detailed pattern of expression is given in Table 1 and is in agreement with that observed in our transgenic mice expressing the human MUC1 mucin (26) and with the pattern in the human reported by Zotter using a large number of antibodies directed to carbohydrate or tandem repeat core protein (18). Negative tissues include small and large intestines, liver (except for bile ducts which were positive), heart, skin, sweat glands, brain, adrenal glands, thymus, spleen, bladder, ovary, seminal vesicles, testicle, prostate and preputial glands in the male reproductive tract. Muscle and cartilage of trachea and peritoneum stained quite strongly and this staining was only partially removed by blocking the antibody with its immunizing peptide. Although we found staining of the muscle unusual, hybridization of the mouse Muc-1 probe to RNA from mouse

Table 1. Mouse Tissues Stained with CT1 Antiserum

Lungs	Salivary Glands , mucous and serous
Bronchi and bronchioles	Excretory and intercalated ducts
Alveoli are negative	Mucous and serous acini are negative
Trachea	Mammary glands*, resting and lactating
Lining and serous glands	Acini and ducts
Stomach	Pancreas
chief cells and parietal cells	Acinar cells and acinar ducts
Uterus*	Kidney
endometrium and uterine glands	Distal tubules and collecting ducts
Vagina*	Cartilage
Cervix	Muscle, striated
Fallopian tubes	

* Staining pattern varies during the hormonal cycle (V.M.M. Braga et al., manuscript in preparation).

muscle has recently been reported (27). Immunoprecipitation experiments are in progress to characterize the protein detected in muscle.

Our studies demonstrate the conserved nature of MUC1 mucin expression throughout evolution and suggest that the mucin plays a role in a wide variety of tissues and species. The availability of cross-reacting antiserum should facilitate investigations of the biological properties of the mucin in different animal models of disease. The development of the antiserum CT1 has enabled us to ascertain that Muc-1 expression correlates with epithelial differentiation in stomach, pancreas, lung, trachea, kidney and salivary glands in the developing mouse embryo (28). Further experiments directed at analyzing the function of this molecule in these species as well as molecular analyses of the related genes in these organisms will ultimately enhance our understanding of this molecule.

Acknowledgments

We would like to thank Gillian Hutchinson for the animal work, Dr. Nigel Spurr for supplying many of the DNA samples, Dr. Paul Richardson for animal tissues, Nicola O'Reilly for making the peptides, and Dr. Vania Braga, Andrew Spicer and Trevor Duhig for many helpful discussions.

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