p57kip2 Expression and Loss of Heterozygosity during Immortal Conversion of Cultured Human Mammary Epithelial Cells

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Abstract

We have uncovered a novel role for the cyclin-dependent kinase inhibitor, p57kip2, during the immortalization of cultured human mammary epithelial cells (HMECs). HMECs immortalized after chemical carcinogen exposure initially expressed little or no telomerase activity, and their telomeres continued to shorten with passage. Cell populations whose mean terminal restriction fragment (TRF) length declined to \( \leq 3 \) kb exhibited slow heterogeneous growth and contained many nonproliferative cells. These conditionally immortal HMEC cultures accumulated large quantities of p57 protein. With continued passage, the conditionally immortal HMECs showed a transition to rapid proliferation. Overcoming p57-mediated growth inhibition in these cells was accompanied by loss of the expressed p57 allele and transient expression of the allele imprinted previously. Conditionally immortal 184A1 with mean TRF > 3 kb, infected with retroviruses containing the p57 gene, exhibited premature slow heterogeneous growth. Conversely, exogenous expression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, in 184A1 with mean TRF > 3 kb prevented both the slow heterogeneous growth phase and accumulation of p57 in cycling populations. These data indicate that in HMECs that have overcome replicative senescence, p57 may provide an additional barrier against indefinite proliferation. Overcoming p57-mediated growth inhibition in these cells may be crucial for acquisition of the unlimited growth potential thought to be critical for malignant progression.

Introduction

Malignant progression involves accumulation of multiple derangements in cellular growth control over an extended time frame. A clonal population of human somatic cells is unlikely to accumulate a sufficient number of errors required for tumorigenicity within the 50–100 population doubling limit that replicative senescence imposes. Therefore, acquisition of unlimited proliferative potential may be necessary for malignant progression, and replicative senescence may have evolved as a cancer prevention mechanism in long-lived organisms (1). Normal human somatic cells show virtually no spontaneous immortal transformation in vitro, whereas tumor tissues can give rise to cell lines of indefinite life span. Additionally, normal human somatic cells in vitro and in vivo show telomere loss with successive divisions (2), whereas most human tumor tissues and cells display telomerase activity, and most cultured human tumor cells display stabilized telomere length.

Cellular growth control is regulated by a group of conserved CDKs, which govern entry into and progression through the cell cycle. These CDKs are in turn regulated by two known families of CKIs, defined based upon sequence similarity, binding specificity, and mechanism of inhibition (3). The INK4 family, which includes p15, p16, p18, and p19, binds specifically to CDK4 and CDK6. This binding can occur in the absence of cyclins and prevents phosphorylation of Rb and thus entry into S-phase. The CIP/KIP family, which includes p21, p27, and p57, binds CDK-cyclin complexes and is able to inhibit multiple G1 kinases including CDKs 2, 3, 4, and 6. The p57 gene has been localized to chromosome 11p15.5, a region displaying frequent allelic loss in cancers of the breast, lung, and bladder, as well as rare pediatric tumors such as Wilms’ tumor (4, 5). LOH and microsatellite instability at 11p15 have been associated with rapid proliferation, DNA aneuploidy, and poor prognosis in primary breast tumors (6–8). The p57 gene has been found to be imprinted with preferential expression of the maternal allele (9), suggesting that loss of the maternal allele by itself may severely reduce p57 expression. In one recent study, maternal p57 alleles had been lost in 11 of 13 lung cancer cases carrying 11p15 deletions (10). Germ-line mutations in the p57 gene have been detected in some patients with Beckwith-Wiedemann syndrome, a familial cancer-prone syndrome associated with hyperplastic growth in numerous tissues and a 1000-fold increase in the risk of childhood tumors (11, 12). Genetically engineered mice that lack the p57 gene have a variety of developmental defects consistent with Beckwith-Wiedemann syndrome and indicate a role for p57 in control of cell proliferation and differentiation (12, 13). p57 mRNA is detectable in most normal adult tissues (9), and the highest levels have been found in tissues consisting primarily of postmitotic cells (5, 14). In epithelia, p57 is reported to be expressed in regions of differentiated cells but not in regions of actively dividing cells, suggesting that p57 may be up-regulated when cells exit the cell cycle and start their differentiation programs (13). p57 is not detectable in most immortal cell lines (15).

Although overexpression of transfected p57 has been shown to lead to G1 arrest in two different cell lines (5, 14), little is known about how p57 expression is normally regulated in vivo. In vitro, p57 binds efficiently to complexes containing CDK2, CDK3, or CDK4 in a cyclin-dependent manner (5) and inhibits the abilities of these complexes to phosphorylate histone H1 or Rb (14). p57 binding to CDK2 can be demonstrated in transiently transfected cells. Unlike its homologous family member, p21, transcriptional regulation of p57 by the tumor suppressor p53 has not been demonstrated. As observed for p21

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The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; LOH, loss of heterozygosity; EL, extended life; HMEC, human mammary epithelial cell; Rb, retinoblastoma; TGF, transforming growth factor; EGF, epidermal growth factor; LI, labeling index; TRF, terminal restriction fragment; hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-PCR.

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and p27, the ability of p57 to cause growth arrest in G1 does not depend upon the presence of intact p53 or Rb (5).

Our present studies using the HMEC model system of immortal transformation generated in our laboratory (reviewed in Ref. 16) indicate that p57 exhibits regulated expression in vitro at a specific stage of the HMEC immortalization process. Exposure of primary, normal, finite life span 184 HMECs to the chemical carcinogen, benzo(a)pyrene resulted in cultures the proliferative potential of which was extended compared with untreated controls (17). These EL cultures all ceased proliferation after several additional passages. In two separate instances, EL cultures gave rise to immortal cell lines, i.e., 184A1 and 184B5. Both lines show a few distinct karyotypic alterations, indicating their independent origins from single cells (18). Upon continued passage, 184A1 and 184B5 show very little genetic alterations, indicating their independent origins from single cells (18).

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HMEC immortalization process. Exposure of primary, normal, finite life span 184 HMECs to the chemical carcinogen, benzo(a)pyrene resulted in cultures the proliferative potential of which was extended compared with untreated controls (17). These EL cultures all ceased proliferation after several additional passages. In two separate instances, EL cultures gave rise to immortal cell lines, i.e., 184A1 and 184B5. Both lines show a few distinct karyotypic alterations, indicating their independent origins from single cells (18). Upon continued passage, 184A1 and 184B5 show very little genetic drift compared with most human cell lines derived from tumors or by viral transformation (18). The 184A1 and 184B5 cell lines provide good systems for studying the immortalization process per se because, despite their indefinite growth potential, they do not display aberrant phenotypes commonly seen in immortal lines derived from tumor tissues or by exposure to viral oncogenes. Neither line shows any defect in sequence or expression of p53 (19) or regulation of Rb phosphorylation (20), nor sustained anchorage-independent growth or tumorigenicity in nude mice. Although the CKI p16 is not expressed in either immortal transformed line (21), loss of p16 expression is also found in the nonimmortal EL precursors, suggesting that p16 loss is insufficient for immortalization (22).

Surprisingly, despite their clonal origins, most individual cells in early passage 184A1 and 184B5 do not maintain proliferation. In contrast to later passages and most tumor-derived cell lines, early passage mass cultures and clonal isolates show little or no telomerase activity and their mean TRF length continues to shorten with passage. When the mean TRF length is ≤3 kb, the populations display slow, heterogeneous growth, with very low colony-forming efficiencies (23). These mixtures of slow-growing and nonproliferative cells are seen in both parental and repeatedly subcloned populations of 184A1 and 184B5. With continued culture, these "conditionally immortal" cell populations very gradually display more uniform proliferative potential, accompanied by expression of telomerase activity and stabilization of telomere lengths. They also gradually acquire the ability to maintain growth in the presence of the multifunctional cytokine TGF-β. We have used the term "conversion" to describe the gradual process that leads to activation of telomerase, stabilization of telomere length, and ability to grow in TGF-β (23). We have proposed that activation of telomerase is an inherent epigenetic response to critically short telomere length. Such activation does not normally occur because a stringent replicative senescence program imposes multiple constraints to prevent growth of cells with shortened telomeres before telomerase can be activated. Our efforts to understand the nature of the event(s) responsible for the growth constraint encountered by conditionally immortal HMECs with mean TRF ≤3 kb focused on the possible role of CKIs.

Materials and Methods

Cells and Cell Culture. Finite life span 184 HMECs were obtained from reduction mammoplasty tissue with no obvious epithelial cell pathology. The cells were grown as adherent cultures on plastic. They were routinely subcultured when 80–100% confluent and reseeded at approximately 6–7 × 10^3 cells/cm^2. Because of the confounding effects of selection and terminal growth arrest (24, 25), passage numbers rather than population doublings were used for tracking growth. The finite life span HMECs senesce around 22nd passage, equivalent to approximately 80 population doublings, when cultured in serum-free MCDB170 medium (Clonetics, San Diego, CA) as described (24, 25). In the serum-containing medium, MM, they cease growth after 5–6 passages, approximately 15–25 population doublings (25, 26). Independent EL cultures 184Aa and 184Be emerged from 184 HMECs grown in MM after exposure of primary cultures to benzo(a)pyrene as described (17, 27). Indefinite life span 184A1 cells appeared in an MM-grown 184Aa culture at passage 9, distinguishable from 184Aa cells by faster growth, greater refractility, smaller size, and growth as single cells versus patches. After its initial appearance, 184A1 was maintained in MCDB170 until passage 101. Similarly, 184B5 appeared as a morphologically unique patch of cells in a 184Be culture at passage 6 and was maintained until passage 103.

HMECs were arrested in a G0 state by removal of EGF from the medium and exposure to 5 μg/ml of the anti-EGF receptor antibody MAb225 for 48 h as described (28). Random cycling cultures were fed 48 and 24 h before harvesting. The LI of HMEC cultures was determined by incubating cells with [³H]thymidine (0.5–1.0 μCi/ml) for 24 h, with labeled cells visualized by autoradiography as described (28).

Retroviral Construction and Infection. A 1.5-kb p57 cDNA fragment encompassing the entire open reading frame and most of the 3‘ and 5‘ untranslated regions (5) was excised with EcoRI from a Bluescript vector and subcloned into the EcoRI site of pBabe.Pu (29). pGR320 containing the hTERT coding sequence in the EcoRI site of LXSN (30) was provided by Geron Corp. Amphitropic viruses were prepared by transient cotransfection of 293 cells with retroviral constructs and a packaging plasmid (31). Parallel HMEC cultures were infected with experimental or control viruses and selected in 0.5 μg/ml puromycin or 300 μg/ml G418 for 7–10 days.

Immunoblot Analysis. Total cell lysates were prepared by lysing the cells directly in 2% SDS, 10% glycerol, and 0.063 M Tris-HCl (pH 6.8). After heat denaturation at 95°C for 10 min, insoluble material was pelleted at 13,000 × g for 10 min. The supernatants were removed, and their protein concentrations were determined. One hundred μg of each sample were separated by SDS-PAGE. The gels were electroblotted to nylon membranes (Immobilon P; Millipore, Bedford, MA) for 1 h at 2.5 mA/cm^2. Blotting efficiency and equivalence of loading were estimated by staining transferred proteins with Ponceau S (Helena Labs, Beaumont, TX). After blocking with 5% milk, 0.05% Tween 20 in Tris-buffered saline, blots were incubated with anti-p57 (sc1040; Santa Cruz Biotechnology, Santa Cruz, CA) antisera. Antibody-antigen complexes were visualized using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescent substrate (Pierce Corp., Rockford, IL).

Northern Blot Analysis. Cultures were lysed directly in buffered guani- dine thiocyanate solution. Total RNA was purified, and Northern blots were prepared as described (32). Efficiency of gel loading and transfer of different samples was judged by staining with ethidium bromide. The blots were hybridized to a ³²P-labeled, 500-bp NorI cDNA fragment corresponding to the 3′ end of the p57 gene (5). Quantitative comparisons of relative RNA abundance were performed using a phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

DNA Analysis. Genomic DNA and/or total cellular RNA were purified at the indicated passages from mass cultures or individual 184A1 cell clones isolated with cloning cylinders. PCR was performed using primers KIP-2 (CCACCCCAGCCCCATGGCC) and KIP-2–12 (GGGGCGAGGACCG- GACC), which amplify a polymorphic region of the p57 gene. Cycles (32–38) of amplification were performed with genomic DNA, whereas 32 cycles of amplification were performed with cDNA synthesized from 250 ng of total RNA. PCR products were then separated on 8% polyacrylamide gels and stained with ethidium bromide. Images were collected for densitometry using a chemiluminescent imager.

Results

p57 Protein Expression Exhibits Large Changes during HMEC Immortal Transformation. G0 arrested and randomly cycling cultures of finite life span, conditionally immortal, and fully immortal HMECs were examined for expression of the p57 protein. HMECs were arrested in G0 by blockage of EGF receptor signal transduction. Reexposure to EGF leads to synchronized entry into the cell cycle (28). The conditionally immortal cells showed accumulation of high p57 protein levels when growth arrested in G0 (Fig. 1A. 184A1 13p–184A1 38p) compared with the finite life span (Fig. 1A, 184 4p, 184 20p; 184Aa 12p, 184Aa 14p) and fully immortal HMECs (Fig.
Fig. 1. Comparison of p57 protein expression in finite life span, conditionally immortal, and fully immortal HMECs under different culture conditions. Total cell lysates were prepared from subconfluent cultures of HMECs, which were: A, growth arrested in G0; B, randomly cycling; and C, synchronized in G0, then given back EGF for stated times. Immunoblots were probed with anti-p57 antibodies, and signals were detected by indirect chemoluminescence. p57/mg, recombinant p57 protein control synthesized in E. coli, colony-forming efficiency and LI in colonies for 184A1 at different passage levels. 

1A, 184A1 55p, 184A1 101p). Loss of p57 accumulation in G0 was observed at passage levels at which 184A1 had converted to the good-growing, telomerase (+), TGF-β-resistant phenotype (Fig. 1D; Ref. 23). In randomly cycling HMECs, the good-growing conditionally immortal 184A1 with mean TRF >3 kb (13th passage through 15th passage) did not show p57 protein expression. Abundant p57 expression was first detected at the passage level (16th passage), corresponding precisely to where these cells demonstrated the onset of slow heterogeneous growth (Fig. 1B). p57 levels remained high in the randomly cycling population, coincident with the period of slow heterogeneous growth (16th passage through 38th passage) and mean TRF levels ≤3 kb (23). Similar results were observed in conditionally and fully immortal 184B5 cells (data not shown), although in this case, conditionally immortal cells with TRF >3 kb were not available for examination. As in the G0 population, p57 was not seen in the finite life span or fully immortal cycling HMECs. In synchronized populations of early passage, good-growing conditionally immortal 184A1, p57 protein expression was down-regulated between 4 and 12 h after release from G0 arrest (Fig. 1C). Thus, the transformation from finite life span to conditional immortality in these HMECs was associated with accumulation of p57 protein during G0 arrest; however, the good-growing conditionally immortal cells with mean TRF >3 kb were able to down-regulate p57 upon mitogenic stimulation and entry into G1. The conversion from heterogeneous to uniform good growth was associated with loss of all p57 expression.

Fig. 2. p57 mRNA abundance in synchronized conditionally and fully immortal HMECs. Total cellular RNA was prepared from 184A1 cultures at indicated passages grown arrested in G0 (A) or refed with EGF for 0, 1, 2, 4, 8, 12, or 12 h + TGF-β (B). Ten µg of each sample were used for Northern blot analysis with 32P-labeled human p57 cDNA. Fluorescence of ethidium bromide (EtBr)-stained RNA in the gels was used to judge equivalence of loading.
The Major Expressed p57 Allele Is Sometimes Lost in Fully Immortal HMECs. To determine whether loss of p57 expression in fully immortal 184A1 was accompanied by genetic changes, genomic DNA was examined. PCR revealed that the proline-alanine repeat region of the p57 gene was polymorphic in finite life span 184 and early passage 184A1 cells (Fig. 3A; data not shown). Amplification of this region yielded two products of different sizes when genomic DNA was used as a template. RT-PCR showed the lower band to be the allele primarily expressed in the early passage, conditionally immortal cells. Genomic DNA from passage 29 and later cells showed the allele primarily expressed in the early passage, conditionally immortal 184A1. To reconcile the loss of the upper band, indicating that allele loss was a frequent event and not attributable to either low level expression in the majority of the cells or to high expression in a few cells undergoing delayed conversion. Unlike 184A1, 184B5 did not undergo p57 LOH, although it did show down-regulation of p57 expression during conversion (data not shown).

Expression of an Exogenously Introduced p57 Gene Induces Premature Onset of Slow Heterogeneous Growth in Conditionally Immortal 184A1. The correlative data above suggested that p57, either alone or in conjunction with other cell cycle regulators, mediated the constraint to continuous growth encountered in conditionally immortal HMECs with critically short telomeres. To provide direct evidence for this function, p57 levels were altered in intact cells by expression of a full-length human p57 cDNA using a retroviral vector. Expression of the exogenously introduced p57 gene caused premature onset of slow heterogeneous growth in good-growing 184A1 14p (Fig. 4A). Many of the p57 virus-infected cells had a flattened, vacuolated appearance, similar to uninfected 184A1 17p, which spontaneously exhibited slow heterogeneous growth. The levels of p57 expressed and LIs in the 14p p57 virus-infected and 17p uninfected cells were also comparable (Fig. 4B). These experiments provide direct evidence that, when expressed at levels seen in conditionally immortal HMECs.
Differences in p57 mRNA regulation must account for three observations: (a) decreased down-regulation during G1 in conditionally immortal HMECs; (b) increased up-regulation during G0 in conditionally immortal HMECs; and (c) absence of expression during G0, G1, or G2 in fully immortal cells. The mechanisms underlying the observed expression of p57 in conditionally immortal but not in finite life span HMECs remain to be investigated. It is possible that the changes that conferred conditional immortality in 184A1 and 184B5 had specific effects on inducers/repressors of p57 expression. Alternatively, p57 expression may be a more indirect consequence of changes in growth regulation that occurred during conditional immortalization. For example, p57 expression may be a compensatory response to the loss of a senescence-associated growth suppressor. It is unlikely that conditional immortalization occurred in a rare cell already expressing p57 because: (a) the 184Aa EL precursor of 184A1 is itself a clonal outgrowth; and (b) we have not observed p57 expression in early passage, finite life span cultures grown in MM medium, a medium that supports the growth of a wider variety of differentiated phenotypes.

The inability of conditionally immortal 184A1 to down-regulate p57 after release from G0 arrest correlated exactly with the passage at which cell growth abruptly declined. The mean TRF at this passage level was ~3 kb. Our experiments introducing exogenous p57 or hTERT into good-growing, conditionally immortal 184A1 with mean TRF >3 kb provide supporting evidence that: (a) p57 can mediate the block to growth encountered by HMECs with critically shortened telomeres; and (b) p57 expression in cycling cells is regulated by telomere length and/or telomerase expression. In addition to causing slow heterogeneous growth, the exogenous p57 expressed in early passage 184A1 caused the cells to assume morphologies very similar to the poorly growing, conditionally immortal cells with mean TRF <3 kb. Although antisense methodologies might help determine more directly whether this growth constraint is mediated specifically by p57, our attempts to implement such strategies have thus far been unsuccessful in reducing p57 expression levels, for reasons that are presently unclear.

The strong correlation among critically shortened telomeres, stabilized p57 expression, and onset of slow, heterogeneous growth in conditionally immortal HMECs suggests a model in which the development of extremely short telomeres causes changes in gene expression that ultimately result in stabilized p57 expression, slow heterogeneous growth, and loss of proliferative capacity. Although p57 is found primarily in the nucleus, it is not known to bind telomeric structures or proteins. Therefore, the mechanism by which telomere length influences p57 expression is likely to be indirect. Two possible mechanisms have precedence in yeast (35, 36). In one model, telomeres and their associated proteins create an area of heterochromatin extending beyond the telomeric and subtelomeric regions, leading to silencing of nearby genes. As telomeres shorten, the region of heterochromatin propagates down the end of the chromosome decreases, and previously silenced areas are gradually derepressed. Differences in telomere length of specific chromosomes could result in different gene expression among cells, thus accounting for the heterogeneity observed in HMECs with mean TRF ≤3 kb. In a second model, proteins associated with telomeric repeats may also serve as diffusible positive or negative regulators of gene transcription. The release of these proteins with progressive loss of telomere regions could lead to gradual alterations in gene expression elsewhere. In this model, differences in the overall level of remaining telomeric repeats could result in different gene expression among cells.

The fully immortal cells that gradually emerged from slowly growing, conditionally immortal HMEC cultures differed in that: (a) they had demonstrable telomerase activity; (b) their mean TRF lengths were stabilized at 3–7 kb; and (c) they no longer accumulated p57 during G0 arrest or at any point in the cell cycle (23). Our data indicated that the major (presumably maternal) p57 allele initially
expressed was frequently lost in conditional immortal 184A1 cells that managed to proliferate during the initial period, where growth abruptly declined. Karyotypic and comparative genomic hybridization analysis did not reveal major deletions or rearrangements at chromosome 11p15.5, where the p57 gene is located, nor did they indicate general genomic rearrangements in fully immortal compared with conditionally immortal 184A1 (Ref. 18; data not shown). By using other informative loci proximal to the p57 locus, we are presently defining the extent of the deletion encompassing p57 in mass cultures and cloned colonies. Our preliminary data indicate that the deletion in at least one mass culture is relatively small (<10 Mb). It remains to be determined how the p57 gene is deleted so frequently in cells that do not exhibit general genomic instability. Possibly, the telomeric proximity of the p57 gene makes it especially vulnerable to deletion events in cells with critically short telomeres.

Expression data indicated that deletion of the initially expressed p57 allele coincided with up-regulation of the previously imprinted (presumably paternal) p57 allele. Interestingly, the same phenomenon has been observed in Wilms’ tumors with 11p15 LOH (37). Activation of the paternal allele may be a compensatory response to the loss of the maternal allele, the result of a feedback mechanism to prevent unlimited growth. Alternatively, a chromosomal region enforcing epigenetic silencing of the paternal gene may be lost or modified directly or indirectly as a result of the mutagenic process that resulted in the loss of the maternal allele.

Deletion of a p57 gene may occur more easily or be selected for more strongly in some situations than in others. Although the 184A1 mass culture and individual subclones displayed a high rate of p57 LOH, this was not observed in 184B5 cells undergoing conversion to the fully immortal phenotype. Similarly, loss of the remaining p57 allele in 184A1 was never observed. In these cases, growth inhibition may not be as acute or abrupt, and epigenetic mechanisms of p57 down-regulation may not be favored.

Although the results reported here are based on immortal HMEC lines that retain wild-type p53, we have recently generated two additional HMEC lines from the EL 184Aa culture, designated 184AA2 and 184AA3, in which both copies of the p53 gene have been inactivated or lost. In these p53−/− lines, the mean TRF length did not decline <3.5 kb, and p57 was not detected, even at the earliest passages. Although these lines expressed some aspects of conversion, the process was greatly accelerated, and some telomerase activity was detectable in early passages. Interestingly, 184AA2 and 184AA3 both showed LOH at the p57 locus (data not shown). Thus, it is possible that loss of p57 function contributed to the conversion of these lines as well.

In summary, we have shown that p57 mRNA and protein can accumulate in cultured HMECs that have escaped replicative senescence but have not yet converted to uniform indefinite growth potential. The data suggest that p57 plays an important role in the observed slow heterogeneous growth of this cell population. We do not yet know whether p57 expression is involved in limiting the growth of human breast epithelial cells during malignant progression in vivo. Development of many primary carcinomas, such as breast and prostate, is characterized by an extended period of slow, heterogeneous growth prior to the appearance of more aggressive, invasive tumors. These carcinomas also generally retain wild-type p53. If overcoming p57 is involved in human carcinogenesis in vivo, knowledge of p57 status in hyperproliferative or invasive lesions may provide prognostic information. Further understanding of the mechanisms regulating the altered p57 expression during immortalization may also offer new avenues for therapeutic intervention.

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