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Molecular analysis of a human PAX6 homeobox mutant

Angela Valentina D'Elia¹, Cinzia Puppin¹, Lucia Pellizzari¹, Annalisa Pianta¹, Elisa Bregant¹, Renata Lonigro¹, Gianluca Tell¹, Federico Fogolari¹, Veronica van Heyningen² and Giuseppe Damante^{*,1}

¹Dipartimento di Scienze e Tecnologie Biomediche, Università di Udine, Udine, Italy; ²MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland, UK

Pax6 controls eye, pancreas and brain morphogenesis. In humans, heterozygous PAX6 mutations cause aniridia and various other congenital eye abnormalities. Most frequent PAX6 missense mutations are located in the paired domain (PD), while very few missense mutations have been identified in the homeodomain (HD). In the present report, we describe a molecular analysis of the human PAX6 R242T missense mutation, which is located in the second helix of the HD. It was identified in a male child with partial aniridia in the left eye, presenting as a pseudo-coloboma. Gel-retardation assays revealed that the mutant HD binds DNA as well as the wild-type HD. In addition, the mutation does not modify the DNA-binding properties of the PD. Cell transfection assays indicated that the steady-state levels of the full length mutant protein are higher than those of the wild-type one. In cotransfection assays a PAX6 responsive promoter is activated to a higher extent by the mutant protein than by the wild-type protein. In vitro limited proteolysis assays indicated that the presence of the mutation reduces the sensitivity to trypsin digestion. Thus, we suggest that the R242T human phenotype could be due to abnormal increase of PAX6 protein, in keeping with the reported sensitivity of the eye phenotype to increased PAX6 dosage.

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Introduction

Pax genes code for a family of transcription factors that play a key role in cell differentiation and embryonic development.^{1,2} They are extremely conserved throughout phylogeny¹ and are characterized by the presence of the paired box, a sequence encoding for a conserved bipartite DNA-binding domain, the paired domain (PD).^{3,4} This domain can be subdivided in a N- and C-terminal subdomains.⁵ In mammals nine different Pax genes have been defined;¹ among these, Pax3, Pax7, Pax4 and Pax6

proteins have an additional DNA-binding domain, the paired-type homeodomain (HD).^{1,6}

Pax6 controls eye, pancreas and brain morphogenesis. It is expressed in multiple ocular tissues, including lens and neuroretina.⁷ In the endocrine pancreas it is expressed in α cells, β , δ and γ cells.⁸ Pax6 is expressed in several structures of the central nervous system (telencephalon, thalamus, pituitary, pineal, cerebellum) and in spinal cord.^{9,10} In humans, heterozygous PAX6 mutations cause aniridia and various congenital eye abnormalities, as Peters' anomaly, iris hypoplasia, corneal opacification, congenital cataracts and glaucoma.^{11–13} In mice and rats, Pax6 mutations are responsible of Small eye (Sey) phenotype.¹⁴ Heterozygous mice show phenotypes similar to human aniridia with Seys, iris hypoplasia, cataracts and incomplete separation of the lens from the cornea.^{15,16} Homozygous Sey mice

^{*}Correspondence: Professor G Damante, Dipartimento di Scienze e Tecnologie Biomediche, Piazzale Kolbe 1 - 33100 Udine, Italy. Tel: + 39 0432 494374; Fax: + 39 0432 494379; E-mail: gdamante@makek.dstb.uniud.it

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Mutation analysis in aniridia patients has demonstrated that most frequent PAX6 missense mutations are located in the PD,¹⁷ while very few missense mutations (only four, up to now have been identified in the HD;^{18–21} http:// pax6.hgu.mrc.ac.uk. As the Pax6 HD is extremely conserved throughout evolution, it has been proposed that missense mutations of the HD may severely disrupt development causing more deleterious phenotypes than those due to mutations in the PD.¹⁹

Among human PAX6 homeobox mutations, R242T was identified in a male child with partial aniridia in the left eye, presenting as a pseudo-coloboma.²⁰ The arginine at position 242 of the Pax6 protein is highly conserved through vertebrate evolution. Based on published HD structural data,^{22–26} it was not obvious how the HD function would be affected. For this reason, we investigated several functional properties of the R242T mutation. Functional comparison was made with two reported mouse HD mutations: V256E²⁷ and S259P.²⁸

Materials and methods Plasmid construction

A full-length cDNA clone of the human PAX6 gene was used as template in a PCR reaction with specific primers to amplify sequence encoding for: (i) the HD only and, (ii) the PAX6 fragment encoding from the N-terminus of the PD to the C-terminus of the HD ($PAX6^{2-271}$) with primer pairs:

366-forward 5'-GGCCCGAATTCCAGAACAGTCACAGC GGAGTGAATC-3';

979-forward 5'-GGCCCGAATTCTGAAGCGGAAGCTGC AAAGAAATAG-3';

1177-reverse 5'-GGCCCGGATCCTCATTTTTCTTCTTCTT CTCCATTTGGCCCT-3' (the number refers to the position of the 5' end of the oligonucleotide in the PAX6 cDNA). HD was amplified with primers 979-forward and 1177-reverse. PAX6^{2–271} was amplified with primers 366-forward and 1177-reverse.

The PCR products of the PD and HD were subcloned into the *BamH*I site of PT7.7 plasmid. Two PAX6 expression vectors were obtained, containing: PD linked to HD (from amino acid 2 to amino acid 271), and HD alone (from amino acid 206 to amino acid 271). The missense mutations in PAX6 were introduced by site-directed mutagenesis, using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Milan, Italy) with primers:

For R242T mutation:

5'-GTGTTTGCCCGAGAAACACTAGCAGCCAAAATAG-3'; 5'-CTATTTTGGCTGCTAGTGTTTCTCGGGCAAACAC-3'; for V256E mutation:

5'-CCTGAAGCAAGAATACAGGAATGGTTTTCTAATCG-3'; 5'-CGATTAGAAAACCATTCCTGTATTCTTGCTTCAGG-3'; for S259P mutation:

5'-GAATACAGGTATGGTTTCCTAATCGAAGGGCCAAAT GG-3';

5'-CCATTTGGCCCTTCGATTAGGAAACCATACCTGTAT TC-3'.

All constructs were checked by direct sequencing.

Peptides expression and purification

The PAX6 wild-type and mutant constructs were expressed by inducing with 0.4 mM IPTG in BL21 cells and extracts were prepared. The partial protein purification was performed using the mono-S columns (Bio-Rad Laboratories, Milan, Italy).²⁵

Electophoretic mobility shift assays

DNA-binding properties of PAX6 wild-type and mutant peptides were investigated using a series of double-stranded oligonucleotides (top strand only is shown):

P3(5'-TCGAGGGCATCAGGATGCTAATTGGATTAGCAT CCGATCGGG-3') and Cr (5'-AAGCATTTTCACGCATGAG TGCACAG-3'), which contain consensus DNA-binding sequences for PAX6 HD and PD, respectively.^{29,30} The oligonucleotides were radiolabelled at the 5' end terminal by incubation with $[\gamma$ -32P]-dATP. Protein and DNA (both at the final concentration of $0.1 \,\mu\text{M}$) were incubated for 30 min at room temperature in a total volume of $20 \,\mu$ l, using a buffer containing 20 mM Tris-HCl (pH 7.6), 75 mM KCl, 0.25 mg/ml bovine serum albumin (BSA), 5 mM dithiotreitol (DTT), $10 \,\mu$ g/ml calf thymus DNA, and 10%glycerol. Protein-bound DNA and free DNA were separated on native 7.5% polyacrylamide gel in $0.5 \times$ TBE for 1.5 h at 4°C. The gels were dried, exposed in a BIO-RAD GS-525 Molecular Imager and quantified by the Multianalyst software.

Limited proteolysis assay

The general experimental procedure consisted of mixing solutions of either bacterially expressed PAX6 wild-type or R242T mutant peptides (HD or $PAX6^{2-271}$) with increasing amounts of trypsin, in TRIS-HCl (0.05 M, pH 7.5) buffer and incubating at room temperature for 10 min.³¹ The peptide concentration utilized was $3\mu g$, with increasing ratios of trypsin/protein (as shown in Figure 2a and b). The proteolysis reactions were stopped by the addition of SDS-PAGE loading buffer (Tris 2 M pH 6.8, mercaptoethanol pure 14 M, SDS 20%, glycerol, bromophenol blue 1%). Samples were boiled for 5 min and applied to a 15% polyacrylamide gel containing SDS. Gels were stained by Coomassie Blue and bands corresponding to HD or PAX6²⁻²⁷¹ were quantified by a BIO-RAD GS-525 Molecular Imager, using the Multianalyst software. Three replicate assays for each peptide were performed.

Cell culture and transfection assays

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Gibco, Milan, Italy). Calcium phosphate coprecipitation method was used for transfections, as described elsewhere.³² HeLa cells were plated at 6×10^5 cells/100 mm culture dish 20 h before transfection. The PAX6-responsive promoter (P3) contains a PAX6 HD-binding sequence and luciferase as reporter gene.²⁹ The activity of P3 was normalized by cotransfecting the RSV-CAT promoter, in which the reporter gene is the chloramphenycol-acetyl-transferase gene (CAT). The plasmids were used in the following amounts for dish: RSV-CAT, $2\mu g$; PAX6 wild-type and R242T mutant expression vector, 1, 2 or $4 \mu g$; P3 reporter, $9 \mu g$. Cells were harvested 42–44 h after transfection, and extracts were prepared by a standard freeze and thaw procedure. CAT activity was measured by an ELISA method (Amersham Pharmacia Biotech, Milan, Italy). Luciferase activity was measured by chemiluminescence.32

Western blot analysis

The expression plasmids for wild-type PAX6 were previously described.³³ The R242T mutation was generated by the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Milan, Italy), using the oligonucleotides above described.

The nuclear extracts were prepared from HeLa cells transfected with wild-type or mutant constructs, together with the RSV-CAT construct, used to evaluate the efficiency of transfection.³⁴ The nuclear extracts were electrophoresed onto 12% SDS-PAGE, after normalization for transfection efficiency. Western blots were performed by using the anti-PAX6 monoclonal antibodies (AD 1.5.6 and AD 2.35.8; 1:15) produced against a PAX6 protein fragment corresponding to the first 206 amino acids.³³ Normalization was performed by quantification in the same extracts of the RAN protein by using a specific antibody (BD Biosciences, Milan, Italy).³⁵

RT-PCR

Total RNA extraction (from transfected HeLa cells) and cDNA synthesis were performed as previously described.³⁶ About $1 \mu g$ of total RNA was reverse transcribed using random primers. The PCR reaction was performed using the following oligonucleotides:

PAX6/ 6bF: PAX6/ 7aR: β actin 1:	5'- CCG AGA GTA GCG ACT CCA G - 3' 5'- CTT CCG GTC TGC CCG TTC-3' 5'- GCA CTC TTC CAG CCT TCC TTC CTG – 3'
β actin 2:	5′ – GGA GTA CTT GCG CTC AGG AGG AGC – 3′

European Journal of Human Genetics

For PAX6, amplification conditions were: denaturation, 95°C for 45 s; hybridization, 58°C for 60 s; elongation, 72°C for 60 s. For β actin, amplification conditions were: denaturation, 94°C for 30 s; hybridization, 54°C for 30 s; elongation, 72°C for 30 s. Reactions were stopped after 16, 19, 22, 25 and 28 cycles and products were analysed by agarose gel electrophoresis stained with ethidium bromide. Bands corresponding to PAX6 and β actin were quantified by a BIO-RAD GS-525 Molecular Imager, using the Multianalyst software.

Results

DNA-binding assays

The DNA-binding properties of the R242T mutation in the isolated HD were first tested by gel retardation analysis. We used two radiolabelled double-stranded oligonucleotide target probes: P3, containing the HD-binding site²⁹ and Cr, containing the PD-binding site³⁰ (Figure 1a). The R242T mutant peptide reveals the same binding activity to the P3 as the wild-type HD, as shown in Figure 1b. As expected, neither the R242T mutant nor the wild-type peptide show binding to the Cr probe, being the specific target sequence for the PD. As controls, we also investigated two other HD missense mutations of mouse Pax6: V270E and S273P (human equivalent residues V256 and S259). Figure 1a shows the relative positions of the investigated missense mutations. The V256E is a valine to glutamic acid substitution within the third helix of the HD. The valine is highly conserved and it is critical for DNA recognition. The equivalent V270E mutant heterozygote mouse has abnormal sized eye with a central corneal dimple and an irregular pupil.²⁷ The S273P missense mutation consists of a serine to proline substitution, in the third helix of the HD. The heterozygote mutant mouse expresses abnormal pupil shape with slight eye size reduction.²⁸ For both V256E and S259P mutants the DNA-binding activity to the P3 oligonucleotide is abolished (Figure 1b). It has been reported that the HD may influence the DNA-binding properties of the PD.²⁹ Thus, by using bacterially expressed proteins containing both the PD and the HD ($PAX6^{2-271}$ construct) we tried to assess whether the R242T mutation may affect the PD DNA-binding activity. Figure 1c shows the results of the DNA binding of $PAX6^{2-271}$ peptides: the R242T mutant peptide has the same binding activity as the wild-type PAX6²⁻²⁷¹, both to the P3 and Cr oligonucleotides. These results indicate that, though the residue 242 is located in a DNA-binding domain, the R242T mutation does not affect the PAX6 binding to DNA.

Sensitivity to trypsin digestion

Limited proteolysis studies by enzymatic digestion have been already used to probe the surface accessibility of isolated proteins in solution and to identify the accessible amino-acid residues.^{37,38} We have also successively used this kind of approach to study the topology of a HD

a P3: 5'-TCGAGGGCATCAGGATGC<u>TAAT</u>TGG<u>ATTA</u>GCATCCGATCGGG - 3'



Cr: 5'- AAGC<u>ATTTTCACGCATGAGTGCAC</u>AG - 3'

Figure 1 (a) Sequences of oligonucleotides used in gel retardation analysis: P3 contains consensus DNA-binding sequence of PAX6 HD, and Cr contains a specific site for PAX6 HD. In both oligonucleotides, PAX6-binding sites are underlined. Schematic diagram of the PAX6 HD structure with the corresponding amino-acids sequence. The relative location of three missense mutations investigated in the report are shown. (b) *In vitro* DNA binding of wild-type and various mutants of PAX6 HD peptides to a HD-binding site (P3) and a PD-binding site (Cr). The arrow shows the position of the bound oligonucleotide/protein complexes. The results showed that R242T mutant reveals the same binding activity of the wild-type HD to the P3. The other two mutants V270E and S273P have been used as control. (c) *In vitro* DNA binding of wild-type and various mutants of PAX6²⁻²⁷¹ peptides to a HD-binding site (P3) and a PD-binding site (Cr). The arrow shows the position of the bound oligonucleotide/protein complexes. The results showed that R242T mutant reveals the same binding activity of The wild-type HD to the P3. The other two mutants V270E and S273P have been used as control. (c) *In vitro* DNA binding of wild-type and various mutants of PAX6²⁻²⁷¹ peptides to a HD-binding site (P3) and a PD-binding site (Cr). The arrow shows the position of the bound oligonucleotide/protein complexes. The R242T mutant peptide has the same binding activity of the wild-type PAX6²⁻²⁷¹ to the P3 and Cr.

containing transcription factor (ie TTF-1) bound to DNA³⁹ and found strong similarities with data obtained by NMR studies.⁴⁰ It is known that trypsin cleaves at the C-terminal sides of both the Lys and Arg residues of proteins; therefore, we speculated that mutations affecting Arg residues would alter the trypsin proteolytic pattern of the PAX6 mutant. Based on this notion, we hypothesized that the R242T mutant protein could be less sensitive to trypsin

degradation than the wild-type protein. Proteolysis experiments were performed using bacterially expressed proteins. In a first set of experiments, different trypsin concentrations for 10 min on the HD peptides were used (Figure 2a). The mutant HD peptide is significantly more resistant to the trypsin digestion (P = 0.046, using the two-tailed, paired *t-test*). In order to test whether the reduced sensitivity to the trypsin treatment is maintained in a larger PAX6 construct, the proteolysis experiments were repeated in the PAX6^{2–271} protein, which, in addition to the HD, contains the entire PD and the linker region. Figure 2b shows that also in the context of a much larger PAX6 protein fragment, the R242T mutation significantly decreases the sensitivity to the trypsin digestion (P = 0.017, using the two-tailed, paired *t-test*). These data suggest that the R242T mutation could reduce the degradation rate of the full-length PAX6 protein.

Expression of the R242T mutant in eukaryotic cells To determine whether the reduced sensitivity to trypsin digestion is associated with elevated levels of the mutant protein in the cell, a transfection approach was used. HeLa cells were transfected with expression plasmids for either



Figure 2 Sensitivity to trypsin digestion. Proteolysis experiments were performed using increasing ratios of trypsin/peptide for 10 min. (a) Wild-type and mutant HD peptides. (b) Wild-type and mutant PAX6²⁻²⁷¹ peptides. In both figures, each point represents the mean value (\pm SD) of three independent determinations.

the wild-type full-length PAX6 or R242T full-length mutant protein. After normalization for efficiency of transfection, the amount of protein was evaluated by Western blot. A two-fold increase of the PAX6 mutant protein, compared to the wild-type one, was detected (Figure 3a and b). The higher amount of R242T mutant protein in the cell, compared with wild-type, predicts that, in cotransfection assays, the R242T mutant should activate a target promoter to a higher extent than the wild-type protein. To test this prediction, a titration experiment was carried out. HeLa cells were transfected with different concentrations of wild-type or R242T mutant expression plasmids together with a luciferase reporter plasmid, containing the HD-binding site P3.²⁹ Figure 3c and d show the transcriptional activity of the wild-type and R242T mutant, respectively. For both constructs, the transcriptional behaviour is described by a linear regression, and the ratio between the corresponding angular coefficients (R242T/wild-type) was 1.72. Therefore, the two-fold increase of the mutant protein, compared to the wild-type one, detected by Western blot, fully explains its higher transcriptional effect on the reporter vector. These results suggest that the R242T mutation induces a higher transcriptional effect by increasing the protein levels and not by increasing the transcriptional activity of the protein per se.

Moreover, bioinformatical prediction analysis (http:// cubic.bioc.columbia.edu/predict protein/) completely excluded a major effect of mutation on the structure of the HD. In fact, the alpha helical region comprising R242 is unaffected by the mutation. The increased levels of R242T mutant protein, compared to the wild-type one, are not due to a higher mRNA levels, as demonstrated by RT-PCR analysis on mRNA from HeLa cells transfected with either expression vector (Figure 4). It has been reported that HeLa cells express the PAX6 endogenous gene.⁴¹ However, either in Western blot (Figure 3) or in RT-PCR experiments (Figure 4), we did not see PAX6 expression in mocktransfected cells. In the case of Western blot, this is probably due to the use of a different antibody with respect to that used by Xu et al.⁴¹ In RT-PCR experiments, in order to evaluate PAX6 mRNA levels, reactions were performed during the exponential phase of amplification (16, 19 and 22 cycles, Figure 4). At higher cycle numbers (28 or more), the PAX6 mRNA produced by the endogenous gene was detectable (data not shown). This indicates that the endogenous PAX6 gene in HeLa cells is expressed at relatively much lower levels than transfected genes. Accordingly, the basal activity of the PAX6-responsive promoter P3 was very similar to that of a promoterless, enhancerless luciferase expression construct (data not shown).

Discussion

In this report, we describe a molecular analysis of the human PAX6 R242T missense mutation, which was



Figure 3 Wild-type and R242T mutant protein levels and titration of their transcriptional effects in HeLa cells. HeLa cells were transfected as described in Materials and methods and normalization for transfection efficiency was performed by measuring the activity of the cotransfected RSV-CAT construct. (a) Representative image of Western blot analysis. Migration of protein molecular weight markers (Fermentas) is shown. (b) Wild-type and R242T mutant protein levels. Each bar represents the mean value (\pm SD) of five independent transfection experiments. (c and d). Transcriptional effects titration of the wild-type (c) and R242T mutant (d).



Figure 4 Evaluation of wild-type and R242T mutant mRNA levels in transfected HeLa cells by RT-PCR. Total RNA was extracted from HeLa cells transfected with PAX6 expression vectors or mock transfected. RT-PCR was performed as described in Materials and methods. RT-PCRs were performed with the indicated numbers of cycles. β actin was used to control the amount of input RNA.

identified in a male child with partial aniridia in the left eye, presenting as a pseudo-coloboma.²⁰ It causes the substitution of an arginine by threonine, in the second helix of PAX6 HD. The HD is the most highly conserved region of the PAX6 protein throughout evolution. However, only four missense mutations have been found in the HD.^{18–21} The high degree of sequence conservation would

suggest that the HD is very important for the correct functioning of the PAX6 gene. Therefore, it has been hypothesized that HD missense mutations induce stronger deleterious phenotypes than other PAX6 missense mutations.¹⁹ Other missense mutations not yet examined probably exist in association with other phenotypes distinct from aniridia, in view of the broad expression pattern of PAX6 gene. An alternative hypothesis is that most part of HD mutations would have no phenotypic effect on eve. The phenotypic effects of mutations V270E and S273P in the mouse could be explained by considering that, according to the HD structure,⁴² either mutation severely modify the three-dimensional architecture of the HD, maybe affecting the function of the entire protein.²⁹ It has been shown that, in Drosophila, the Pax6 HD is dispensable for ectopic eve development and the expression of the PD alone is sufficient to induce eye formation.43

The R242T mutation reduces sensitivity to trypsin digestion *in vitro* and, compared to the wild-type protein, is associated to increased amount of the full-length protein in the cell. The *in vitro* trypsin digestion has been carried out by using bacterially expressed proteins. Instead, the effect of the mutation on the protein levels in cells was tested by a eukaryotic cell transfection approach. We do



Figure 5 Modelling of the PAX6 HD. The structure of the PAX6 HD was built by homology using the HD of the *Drosophila paired* protein bound to a DNA oligonucleotide as template. The two HDs share 55% identity with no insertions. Both homology model building and debumping have been performed using the program WHATIF.⁴⁷ The picture has been obtained using the program Dino (http://www.dino3d.org). Protein a-helices are shown as cylinders. The residue R242 highlighted in yellow.

not know whether the two different expression systems might affect protein stability. However, the consistency between the results obtained with two different expression systems suggests that the eventual influence of the expression system on protein stability might be not significant. Trypsin digestion experiments by using a fulllength PAX6 protein prepared from transfected HeLa cells were hampered by the high rate of protein degradation during purification.

Our data indicate that the R242T mutation: (a) does not modify the PAX6 DNA-binding properties; (b) reduces the sensitivity to trypsin digestion *'in vitro'*; (c) increases the amount of the full-length PAX6 protein levels in cells, without modification of the corresponding mRNA levels. As data indicating points (b) and (c) have been obtained by using different experimental systems, we cannot state that the reduced sensitivity to trypsin is directly responsible for the increased amount of the mutant PAX6 protein in HeLa cells. Computer modelling studies show, however, that, when the PAX6 HD is bound to DNA, the arginine at position 242 is greatly exposed to solvent (Figure 5). Thus, it could represent a natural cleavage site for degradation enzymes and, therefore, a control point for the intranuclear PAX6 protein levels.

Our data obtained through in vitro experiments recall results obtained in transgenic mice by Schedl et al.44 Transgenic mice carrying multiple copies of the human PAX6 gene have distinct developmental abnormalities of the eye. Therefore, in light of Schedl's data, the human phenotype associated to the R242T mutation could be caused by the increased levels of the PAX6 protein. Mutations of the HD that increase the activity are unusual, but two reported examples are the CRX homeobox gene mutation E80A⁴⁵ and the NLR mutation S50T.⁴⁶ CRX is a transcription factor that is expressed in the rod and cone photoreceptors of the retina. Although most of the CRX mutations are associated with decreased function. E80A mutant (located in the third helix of the HD) demonstrated increased transcriptional activity. Moreover, like R242T mutation, E80A does not show DNA-binding defects. Similar finding is reported by the NLR mutation S50T, which also shows increased transactivation of the rhodopsin promoter.

In conclusion, our data suggest that the abnormal phenotype induced by the R242T mutation could be due to an excess of PAX6 protein levels in the cell.

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