PITX2 Regulates Procollagen Lysyl Hydroxylase (PLOD) Gene Expression: Implications for the Pathology of Rieger Syndrome

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Abstract. The Rieger syndrome is an autosomal dominant disease characterized by ocular, craniofacial, and umbilical defects. Patients have mutations in PITX2, a paired-bicoid homeobox gene, also involved in left/right polarity determination. In this study we have identified a family of genes for enzymes responsible for hydroxylizing lysines in collagens as one group of likely cognate targets of PITX2 transcriptional regulation. The mouse procollagen lysyl hydroxylase (Plod)-2 gene was enriched for by chromatin precipitation using a PITX2/Pitx2-specific antibody. Plod-2, as well as the human PLOD-1 promoters, contains multiple bicoid (PITX2) binding elements. We show these elements to bind PITX2 specifically in vitro. The PLOD-1 promoter induces the expression of a luciferase reporter gene in the presence of PITX2 in cotransfection experiments. The Rieger syndrome causing PITX2 mutant T68P fails to induce PLOD-1–luciferase. Mutations and rearrangements in PLOD-1 are known to be prevalent in patients with Ehlers-Danlos syndrome, kyphoscoliosis type (type VI [EDVI]). Several of the same organ systems are involved in Rieger syndrome and EDVI.

Key words: PITX2 • PLOD • Rieger • Ehlers-Danlos • promoter

Introduction

Studies of the autosomal-dominant Rieger syndrome first suggested that a single gene could be involved in the development of eye, tooth, and abdominal organs (Rieger, 1935). Mutations in PITX2 were detected in patients with Rieger syndrome (Semia et al., 1996). The ocular abnormalities include an anterior and prominent Schwalbe’s line (posterior embryotoxon), iris adhesions to the trabecular meshwork, iris hypoplasia, ectopic pupils, displaced pupils, full-thickness iris tears (polycoria), iridocorneal adhesions, and glaucoma. Patients with Rieger (or Axenfeld-Rieger) syndrome display the ocular features in combination with dental and umbilical abnormalities. The dental phenotypes are hypoplasia, missing upper incisors, and sometimes total lack of teeth. The umbilicus is usually a protruding stump, but severe cases have omphalocele, or an unclosed abdomen. Mice disrupted in the Pitx2 gene display phenotypes reminiscent of the corresponding human defects as well as symmetry defects (Campione et al., 1999; Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). Pitx2 is also involved in the Nodal/Sonic hedgehog pathway, which determines left/right polarity of mesoderm-derived organs such as heart, gut, and stomach (for reviews see Blum et al., 1999; Tamura et al., 1999).

The PITX genes are members of the bicoid class of the homeodomain proteins. These have a lysine residue at position nine of the third helix and are especially noteworthy for a role in both DNA and RNA binding (Dubnau and Struhl, 1996; Amendt et al., 1998). In Drosophila, seven different target genes have been identified epistatically and biochemically for bicoid (Driever and Nüsslein-Volhard, 1989; Jackle and Sauer, 1993). Target genes for Pitx2 have only been described for the pituitary; the prolactin gene is synergistically upregulated by Pit-1 and Pitx2 (Simmons et al., 1990; Sze et al., 1996; Ryan and Rosenfeld, 1997; Amendt et al., 1998. Other pituitary-specific Pitx2 target genes have also been described (Tremblay et al., 2000). Recently, we presented the first study on Pitx2 protein expression in the eye, as well as directly demonstrating asymmetric protein expression in the early development of mouse gut, heart, and lung (Hjalt and Murray, 2000).

PITX2 was first identified by positional cloning of the 4q25 locus, but only 40% of patients diagnosed with classical Rieger syndrome have PITX2 mutations (Semia et al., 1996). Other loci for Rieger syndrome include RIEG2 on 13q14 and FKHL7/FOXE7 on 6p25 (for reviews see Craig and Mackey, 1999; Amendt et al., 2000). Still undescribed genes/loci for Rieger syndrome may be downstream of PITX2. It is also still unknown which genes are...
the cognate targets for PITX2 in left/right asymmetry regulation in the development of the heart, gut, and lung.

Chromatin precipitation is a direct method for in vivo detection of target genes. It has been used successfully to identify: ultrabithorax target genes (Gould et al., 1990), polycomb-repressed domains in the bithorax complex (Orlando and Paro, 1993), Pax-2 in vivo binding sites (Phelps and Dressler, 1996), and a Hox-C8 target gene (Tomotsune et al., 1993).

Here we identify some members of the procollagen lysyl hydroxylase (PLOD) family, genes for enzymes that hydroxylate lysines in collagens, as cognate targets for Pitx2/PITX2, by chromatin precipitation. The hydroxylysine residues have two important functions: as attachment sites for molecular cross-links. Cross-links involving hydroxylysine-derived aldehydes are more stable than those involving lysine-derived aldehydes (Prockop and Kivirikko, 1984; Kivirikko and Myllylä, 1985; Kivirikko et al., 1992).

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We have previously characterized the Pitx2-specific antibody P2R10 (Hjalt and Murray, 2000). It was used to immunoprecipitate chromatin bound in vivo to Pitx2. About 30 mouse E14 heads were fixed in 50 ml 4% paraformaldehyde in PBS on ice for 30 min. The heads were washed gently with 30 ml PBS twice, then once with 30 ml 50 mM glycine-HCl, pH 2.2, bated with a P2R10-protein A–Sepharose CL4B affinity gel (prepared according to the recommendations of the manufacturer) for 2 h at 4°C, with sodium acetate 0.1 M, then stopped at 70°C with sodium acetate 0.1 M.

Materials and Methods

Chromatin Precipitation

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random, excised using the ExAssist helper phage, prepared as plasmids, and the inserts were cleaved out and labeled with $[^32]P$ATP. The labeled inserts were used as probes for genomic Southern analyses and to probe a portion of the unamplified library in a plaque hybridization assay. Sometimes vector backbone DNA was difficult to separate from insert on a gel (same size). In these cases, vector T3 and T7 primers were used to PCR amplify the insert for probe production.

**Bacterial Artificial Chromosome Screening and Sequencing**

We screened bacterial artificial chromosome (BAC) libraries by PCR and BAC clones in pBeloBac11 were obtained from Research Genetics (mouse Plod-2, 249B6; human PLOD-1, 33A19). BAC DNA was prepared and sequenced directly as described previously (Hjalt and Murray, 1999).

**Electrophoretic Mobility Shift Assay**

Oligodeoxyribonucleotides (see Table I and Fig. 2) were synthesized (Integrated DNA Technologies) in complementary pairs, annealed to form double stranded DNA, and labeled by kinase with $[^32]P$ATP. The probes were purified on Sephacryl S-200 Microspin columns (Amersham Pharmacia Biotech) and the activity was measured by scintillation counting. Bacterially overexpressed PIT2X was prepared as described previously (Hjalt and Murray, 2000). Electrophoretic mobility shift assays (EMSAs) were performed essentially as described in Amendt et al. (1998). The Plod-2 G probe was modified from the sequence in the promoter. The wild-type Plod-2 G probe would form concatamers during annealing (not shown). The GC-rich 3’ sequence (GGCCCGCGG) was substituted for an arbitrary AT-rich sequence (AAAAATCATGA).

**Reverse Transcription PCR**

Mouse mRNA was prepared from freshly dissected mouse tissues using a Micro Fast Track Kit (Invitrogen). Human cDNAs were purchased from CLONTECH Laboratories, Inc. 20-μl reverse transcription (RT) reactions were performed using a semianchored oligo-dT primer and SuperScript II reverse transcriptase (GIBCO BRL) according to the recommendations of the manufacturer. The cDNAs were diluted four or six times in 10 mM Tris-HCl, pH 8.5, and 1 μl was used per PCR reaction. The primers used for PCR are listed in Table I. Standard 10- or 20-μl PCR reactions were started hot. Cycling conditions: 35 cycles of 30 s at 94°C for 1 min, 72°C for 1 min and one cycle at 72°C for 1 min 30 s. Annealing temperatures (X°) are listed in Table I. The primer pairs chosen for PITX2/Pitx2 amplify most known cDNA isoforms. All primer pairs were designed with one to two intervening intron boundaries, and in some cases also with one primer straddling another intron-exon boundary.

**Real-Time PCR**

An ABI Prism Sequence Detector 7700, with the program Sequence Detector v1.7, was used for real-time PCR experiments, performed according to the recommendations of the manufacturer. Primers (Table I) were designed using PrimerExpress 1.5. Primers for 45S/18S rDNA amplify the segment between nucleotides 251 and 315 (5’ of transcription initiation) in GenBank/EMBL/DDBJ accession no. AF081786; was excised from the human BAC 33A19 (Research Genetics) and cloned into the Apa1 site of pBluescript IKS−. The reporter constructs were PCR amplified from this clone. The Plod-1 2561 luciferase reporter contains Plod-1 sequences from −60 to +880 and has the TAATAA and CAAT boxes (see Fig. 2). The 5’ sense primer contains a unique BamHI site (underlined) linked to the Plod-1 sequence, 5’-CGGGATCCCCAAGGTGAAAATCTTGTCCT-3’, and the 3’ antisense primer contains a unique HindIII site (underlined) 5’-CCCCAAGTTCGCCCGCCCCTCACGCC-3’. To make the Plod-1 261 luciferase reporter, the sequence from −60 to −3180 was PCR-amplified. The 5’ sense primer contains BamH1 site (underlined) linked to the Plod-1 sequence, 5’-CGGGATCCCCAAGGTGAAAATCTTGTCCT-3’, and the above 3’ antisense primer. The PCR products were digested with BamH1 and HindIII and ligated into thymidine kinase (TK)-luc (Amendt et al., 1999), which was digested with BamH1 and HindIII to remove the TK promoter. The Plod-1 sequences were cloned upstream of the luciferase gene. The PCR profile consisted of 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min for 30 cycles with Pfu DNA polymerase (Stratagene). A cytomegalovirus (CMV)–β-galactosidase reporter plasmid (CLONTECH Laboratories, Inc.) was cotransfected in all experiments as a control for transfection efficiency. Expression plasmids containing the CMV promoter linked to the PITX2 DNA were constructed in pCNA 3.1 MycHisC (Invitrogen) as described previously (Amendt et al., 1998).

**Cell Culture, Transient Transfections, Luciferase, and β-Galactosidase Assays**

CHO cells were cultured in DME supplemented with 5% FBS and penicillin/streptomycin in 60-mm dishes and transfected by electroporation. CHO cells were mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid, and 0.5 μg of CMV–β-galactosidase plasmid plated in 60-mm culture dishes and then fed with 5% FBS and DME. Electroporation of CHO cells was at 360 V and 950 μF; cells were fed 24 h before transfection. HeLa cells were mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid, and 0.5 μg of CMV–β-galactosidase plasmid. HeLa cells were electroporated at 220 V and 960 μF (Bio-Rad Laboratories), plated in 60-mm culture dishes, and fed with 5% FBS and DME as described previously (Amendt et al., 1998). Cells were then lysed and assayed for reporter activities and protein content by Bradford assay (Bio-Rad Laboratories). Luciferase was measured using reagents from Promega. β-Galactosidase was measured using the Galacto-Light Plus reagents (Tropix, Inc.). All luciferase activities were normalized to β-galactosidase activity. Expression of transiently expressed PITX2 proteins has been demonstrated previously (Amendt et al., 1998).

![Figure 1](image)
Results

Plod-2 Is Immunoenriched by P2R10

The nucleoprotein complexes of E14 mouse heads were immunoenriched by using a Pitx2-specific antibody (Hjalt and Murray, 2000). The DNA was cloned into a phage library by plaque PCR using T7 and T3 flanking vector primers from 80 isolates; 90% contained inserts ranging in size from 100 to 8,000 bp, with an average size of 3,000 bp (not shown). 20 clones were excised at random one by one and inserts were labeled and used as probes for genomic Southern analyses and a plaque enrichment assay. One of the enriched clones, B18, that also represented a unique gene by Southern analysis (Fig. 1), displayed DNA sequence homology to mouse procollagen Plod-2. Normally, a 3,000-bp fragment of the mouse genome should appear 0.03 times on a 30,000 plaque filter (3E4 / 3E9). Five hits on the filter represent 165-fold enrichment. Control hybridization to a normal mouse genomic library filter of equal density was blank (not shown). The 2,701-bp B18 clone insert matched Plod-2 and PLOD-2 cDNA sequences in BLAST searches. Later we could confirm that B18 matched exon 7, intron 7, and exon 8 of PLOD-2. Since enrichment of the Plod-2 gene occurred before digestion with EcoRI and library cloning,

Table II. Enrichment Test by Real-Time PCR

|                | Ct control | Ct enriched | ΔCt | Enrichment (fold) |
|----------------|------------|-------------|-----|------------------|
| IRS            | 16.33 ± 0.04 | 18.22 ± 0.07 | Down 1.89 | NA*             |
| Prolactin      | 32.88 ± 0.30 | 29.76 ± 0.09 | Up 3.12  | 32               |
| Plod-2         | 21.45 ± 0.03 | 19.73 ± 0.04 | Up 1.72  | 12               |

n = 4.  
*Not applicable.

Figure 3. PLOD-1 and Plod-2 promoter elements bind PITX2 in vitro. (A) EMSA of PITX2 protein incubated with radioactively labeled double stranded oligodeoxyribonucleotide probes designed from the PLOD-1 promoter. Bic, Drosophila Bicoid-like and Bicoid elements are in bold and boxed. In A, Apal restriction sites are underlined. CAAT and TAATAA sequences are underlined with thick lines. These sequence data are available from GenBank/EMBL/DBJ under accession nos. AF081786 (PLOD-1) and AF283255 (Plod-2).
we knew it would be possible to clone parts of the gene other than the promoter. We also confirmed that prolactin, a known Pitx2 binder (Amendt et al., 1998), was immunoenriched by probing a set of filters with a probe for mouse prolactin, exon 2 (not shown). Control hybridization to a normal mouse genomic library filter of equal density was blank (not shown). We also performed real-time PCR on normal total mouse DNA and immunoenriched DNA of the same concentrations. We used a primer set for 18S ribosomal DNA as a control and compared the results to those for the Plod-2 promoter, or exon 2 for prolactin. The normalized ΔCt values are presented in Table II. The Plod-2 promoter was enriched 32-fold and the prolactin exon 2 was enriched 12-fold in this assay.

The Plod-2 Promoter Contains Multiple Bicoid Elements

We screened a mouse genomic library and retrieved a BAC clone containing the Plod-2 gene. We sequenced 2,551 bp upstream of where the 5′ end of the published cDNA sequence starts. Here we discovered several sites shown previously to bind Drosophila bicoid specifically by DNaseI footprinting (Fig. 2). Sites A and C resemble the “X3” site, and site D resembles the “X1/X2” sites (Ma et al., 1996). Site F resembles the “B1” site (Driever and Nüsslein-Volhard, 1989). Other similar sites have been shown to bind bicoid specifically (Rivera-Pomar et al., 1995; Dave et al., 2000). We confirmed that the C, E, F, and G sites also bound PITX2 by EMSA (Fig. 3 B). Negative controls were performed for all probes (probe only), but in Fig. 3 B only the control for the C probe is shown.

The PLOD-1 Promoter Contains Multiple Bicoid Elements

We also studied the human PLOD-1 promoter, since the sequence was available (GenBank/EMBL/DDBJ accession no. AF081786). Approximately 3 kb upstream of the PLOD-1 5′ untranslated region we detected 10 bicoid elements (TAATCCC). We confirmed the in vitro binding properties of some of these elements (C, E, and G) by EMSA, using human PITX2 protein (Fig. 3 A) and synthetic double stranded DNA.

PITX2 Regulates PLOD-1–luciferase Fusions in Cell Culture

We proceeded to clone the PLOD-1 promoter in a luciferase reporter gene vector. We characterized the response to PITX2 by cotransfecting the PLOD-1–luciferase construct with a PITX2 expression vector. In CHO cells, the PLOD-1 261 construct containing 10 bicoid elements was activated 20-fold by PITX2A (Fig. 4 A). The minimal promoter constructs, PLOD-1 2561, were modestly activated (approximately fivefold) by PITX2. We attribute the modest activation to the fact that two bicoid-like sites remain, one TAACC and one TAAGGC, in the minimal construct. Such elements are known to bind PITX2 in vitro (Amendt, B., unpublished data). In HeLa cells, PLOD-1 was activated 6.5-fold by PITX2A (Fig. 4 B). Furthermore, we found that a PITX2 gene carrying the Rieger syndrome causing mutation T68P failed to upregulate PLOD-1 in cell culture (Fig. 5). This protein can be expressed in cell culture (Amendt et al., 1998). The responses were similar to the negative controls both in CHO cells (Fig. 5 B) and in HeLa cells (Fig. 5 C).

Coexpression in Several Mouse Tissues of Pitx2 with Plod-1 and Plod-2

We wanted to study which tissues Pitx2 was coexpressed in with Plod-1 and Plod-2. We assayed this by RT-PCR of mRNA isolated from various mouse tissue (Fig. 6).

The PCR primers for Pitx2 were designed to detect most known isoforms (see Materials and Methods). First, we
could confirm that Pitx2, Plod-1, and Plod-2 are coexpressed in the tissue used to create the chromatin precipitation library, mouse E14.5 heads (Fig. 6 A). Moreover, we could confirm the same coexpression in E13.5 eyes (Fig. 6 A). We also detected coexpression of PITX2, PLOD-1, and PLOD-2 in adult human skeletal muscle (Fig. 6 A). We also detected coexpression of Pitx2, Plod-1, and Plod-2 in adult mouse lung, skeletal muscle, and testis; and Pitx2 and Plod-2 in mouse adult kidney (Fig. 6 B). In mRNA from whole mouse embryos, we detected coexpression of Pitx2, Plod-1, and Plod-2 at E11 and E15 (Fig. 6 B). At E17, Pitx2 and Plod-2 were coexpressed. At E7, Plod-1 and Plod-2 were expressed without Pitx2. This was also the case in adult spleen. No expression was seen of either gene in adult liver.

Clinical Similarities between Rieger and EDVI

We compared the reported clinical manifestations of Rieger syndrome and EDVI. We used the database Online Mendelian Inheritance in Man (available at http://www.ncbi.nlm.nih.gov/omim/) textbooks (e.g., Traboulsi, 1998), as well as published and unpublished clinical observations (May and Beauchamp, 1987; Wenstrup et al., 1989; Heikkinen et al., 1997; Murray, J.C., unpublished). Several similarities of organ system involvement are listed in Table III. Most notable are the comparable ocular and abdominal abnormalities.

Table III. Clinical Features of Rieger and Ehlers-Danlos Syndromes

| Clinical manifestations     | Rieger syndrome | EDVI     |
|----------------------------|-----------------|----------|
| Ocular                     |                 |          |
| Glaucoma                   | Yes*            | Yes*     |
| Microcornea                | Yes†            | Yes†     |
| Cornea plana               | Yes†            | Yes†     |
| Blue sclera                | No              | Yes†     |
| Fragile eyes/corneas       | No              | Yes†     |
| Iris hypoplasia            | Yes†            | No       |
| Iridocorneal adhesions     | Yes†            | No       |
| Myopia                     | No              | Yes†     |
| Dental                     |                 |          |
| Tooth abnormalities        | Yes†            | Yes†     |
| Abdominal                  |                 |          |
| Inguinal hernia            | Yes†            | Yes†     |
| Umbilical hernia           | Yes*            | Yes*     |
| Omphalocele                | Yes*            | No       |
| Skin hyperflexibility      | No              | Yes†     |
| Colon rupture              | No              | Yes†     |

*Less frequent.
†Frequent.
‡Microdontia, hypodontia, anodontia, and small crowns.
§Dentinogenesis imperfecta.

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Discussion

Several direct and indirect lines of evidence now support the theory that the Plod genes belong to the cognate targets for Pitx2. (a) We were able to enrich for the Plod-2 gene ~170-fold in a chromatin precipitation assay using a Pitx2/PITX2-specific antibody. This antibody does not cross-react with PITX1 or PITX3 (Hjalt and Murray, 2000). However, we do not exclude the possibility that Pitx1/Pitx3/PITX1/PITX3 might also be involved in the regulation of the Plod/PLOD genes. Indeed, it has been shown that PITX1 and PITX2 share DNA binding and transactivating properties of target genes in the pituitary (Tremblay et al., 2000). (b) The PLOD-1 and Plod-2 genes contain clusters of bicoid elements in their proximal promoter regions and we have shown that these elements bind PITX2 in vitro. (c) We have shown that the proximal PLOD-2 promoter can be induced to express a reporter gene in the presence of PITX2. (d) The Rieger syndrome causing PITX2 mutant T68P fails to induce the PLOD-1 reporter gene construct. (e) The EDVI patients share several characteristics with the Rieger syndrome patients (Table II). It is normally easy to distinguish these two disorders from each other. However, the similarities of organ systems involved pointed out here are indicative of the role that lysine hydroxylation of collagens may play in Rieger syndrome. (f) The EDVI patients also have defects in tissues corresponding to where Pitx2 is expressed in the mouse, such as cornea, skeletal muscle, aorta smooth mus-
and skin (May and Beauchamp, 1987; Wenstrup et al., 1989; Heikkinnen et al., 1997; Hjalt and Murray, 2000). (g) Plod-1 ESTs have been isolated from rabbit corneal endothelial cells (Fujimaki et al., 1999) and strong Plod-like enzymatic activity can be isolated from chick embryonic corneas (Kao et al., 1983). These structures also strongly express the Pitx2 protein in the mouse (Hjalt and Murray, 2000). We were also able to show coexpression of Pitx2, Plod-1, and Plod-2 mRNA in mouse E13.5 eye by RT-PCR.

It is also interesting to point out that Pitx2 is expressed in mouse aorta smooth muscle and some EDVI patients have aortic defects (Tsai and Grajewski, 1994; Cunningham et al., 1998; Mammi et al., 1998). Rieger and ED syndromes normally have different modes of inheritance, autosomal dominant and recessive, respectively. However, it is still unclear exactly what the link is between the pathologies of each syndrome.

Our finding that PITX2 regulates PLOD-1 differently in different cellular backgrounds is indicative of the dependence of cofactors for correct function of a homeobox gene. The most appealing model for in vivo specificity of homeobox genes is probably the binding site selection model, in which cofactors define appropriate DNA binding site and later fine-tune expression levels (Mann and Chan, 1996; Mann and Affolter, 1998). This model has recently been very convincingly supported for Fushi tarazu, which also can either activate or repress its cognate target genes depending on cell type (Nasiadka et al., 2000). Furthermore, the estimated amount of available Fushi tarazu protein per cell is several orders of magnitude smaller than the theoretically available cognate DNA binding sites (Nasiadka et al., 2000). The support for the binding site selection model also lends encouragement to methods such as chromatin precipitation.

Some mouse tissues express Plod-1 and Plod-2 in the absence of Pitx2 (adult spleen and E7 whole embryo). It may be that another Pitx family member regulates the Plod genes in these tissues. Perhaps more likely is that Pitx2 serves as a modifier of gene expression, as is known to be the case in the regulation of prolactin by PIT-1 and PITX2 (Simmons et al., 1990; Szeto et al., 1996; Ryan and Rosenfeld, 1997; Amendt et al., 1998). Either protein, by itself, accomplishes only modest transactivation of the prolactin promoter, but together a dramatic synergistic effect on gene expression is seen (Simmons et al., 1990; Szeto et al., 1996; Ryan and Rosenfeld, 1997; Amendt et al., 1998). It will be interesting and important to find the cognate cofactors of Pitx2/PITX2 in the regulation of the Plod-1 and 2/PLOD-1 and 2 genes. Our discovery validates the chromatin precipitation method as a viable means of finding additional target genes of PITX2.

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Figure 6. Coexpression of Pitx2 and Plod-1/Plod-2. (A) RT-PCR with Pitx2, Plod-1, and Plod-2 primers on mRNA prepared from E14.5 mouse heads, E13.5 mouse eyes, and adult human skeletal muscle. (B) RT-PCR with various embryonic and adult mouse tissues.
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