Notch Ligand Delta-Like 1 Is Essential for Postnatal Arteriogenesis

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Abstract—Growth of functional arteries is essential for the restoration of blood flow to ischemic organs. Notch signaling regulates arterial differentiation upstream of ephrin-B2 during embryonic development, but its role during postnatal arteriogenesis is unknown. Here, we identify the Notch ligand Delta-like 1 (Dll1) as an essential regulator of postnatal arteriogenesis. Dll1 expression was specifically detected in arterial endothelial cells, but not in venous endothelial cells or capillaries. During ischemia-induced arteriogenesis endothelial Dll1 expression was strongly induced, Notch signaling activated and ephrin-B2 upregulated, whereas perivascular cells expressed proangiogenic vascular endothelial growth factor, and the ephrin-B2 activator EphB4. In heterozygous Dll1 mutant mice endothelial Notch activation and ephrin-B2 induction after hindlimb ischemia were absent, arterial collateral growth was abrogated and recovery of blood flow was severely impaired, but perivascular vascular endothelial growth factor and EphB4 expression was unaltered. In vitro, angiogenic growth factors synergistically activated Notch signaling by induction of Dll1, which was necessary and sufficient to regulate ephrin-B2 expression and to induce ephrin-B2 and EphB4-dependent branching morphogenesis in human arterial EC. Thus, Dll1-mediated Notch activation regulates ephrin-B2 expression and postnatal arteriogenesis. (Circ Res. 2007;100:363-371.)

Key Words: arteriogenesis ■ vascular biology ■ endothelium ■ ischemia

Growth of functional arteries is essential for the restoration of blood flow and regeneration of ischemic organs. Arterial obstruction results in distal tissue ischemia, which induces capillary sprouting from small postcapillary venules (termed angiogenesis). Although this process contributes to metabolic exchange, its contribution to blood flow restoration is limited. In contrast, the development and maturation of small collateral arteries into conduit vessels, termed postnatal arteriogenesis, has considerable ability to fully restore blood flow.

The arterial endothelium, activated by locally released cytokines and growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), is a critical mediator of the coordinated vascular phenotype of the general null alleles, thus identifying in both genes have demonstrated that reciprocal interactions between ephrin-B2 and EphB4 mediate angiogenic remodeling of arteries and veins through bidirectional signaling. Vascular defects are phenocopied by endothelial specific deletion of ephrin-B2, and by deletion of the cytoplasmic domain of ephrin-B2, which functionally restricts the ligand to forward signaling via Eph receptors, but impairs reverse signaling by its cytoplasmic domain. In vitro, activation of ephrin-B2 reverse signaling by EphB4 promotes endothelial cell (EC) sprouting and migration. Recently, a promotive role in postnatal angiogenesis has been shown. Thus, reverse ephrin-B2 signaling in endothelial cells mediates arterial remodeling and angiogenesis.

The evolutionary conserved Notch signaling pathway regulates vascular development downstream of VEGF and upstream of ephrin-B2. Mutations in Notch pathway components in humans cause inherited cardiovascular malformations, such as CADASIL and Alagille Syndrome, whereas loss of global Notch signaling in mice disrupts embryonic development and impairs expression of ephrin-B2 and vascular remodeling. Endothelial specific deletion of Notch1, or its nuclear mediator RBP-J, reproduces the vascular phenotype of the general null alleles, thus identifying
the endothelium as the primary Notch target tissue during vascular development.\(^{20,21}\)

Interaction of Notch receptors with membrane-bound ligands of the Delta and Serrate/Jagged gene families is critical for Notch activation. Ligand binding induces \(\gamma\)-secretase mediated cleavage and translocation of the Notch intracellular domain (NICD) into the nucleus, where it interacts with the DNA-binding protein RBP-J to induce downstream target genes such as Hairy Enhancer of Split homolog-1 (Hes1).\(^{22,23}\) Delta-like (Dll) 4 encodes a pivotal Notch ligand during arterial development.\(^{21,24,25}\) However, Dll4 is not expressed in major adult arteries but only in microvessels,\(^ {25}\) suggesting that other as yet unknown ligands regulate Notch signaling in adult arteries. We have shown that the ligand Dll1 is essential for embryonic development, where it is expressed in vascular endothelium in late embryonic stages.\(^ {26,27}\)

Here we show that Dll1 is an essential Notch ligand in postnatal arterial EC, which regulates Notch signaling-dependent ephrin-B2 expression and postnatal arteriogenesis in response to ischemia. Furthermore, angiogenic growth factors synergistically activated Notch signaling by induction of Dll1, which was necessary and sufficient to regulate ephrin-B2 expression and to induce ephrin-B2 and EphB4-dependent branching morphogenesis in human arterial EC.

Materials and Methods

Endothelial Cell Culture, siRNA Transfection

Human aortic endothelial cells (HAEC) were cultured in EGM-2 with growth supplements (both Cambrex) and transfected with predesigned annealed small interfering RNA (siRNA) or scrambled siRNA (siRNA) (Ambion, 10 nM) in OptiMEM using oligo-fectamine (both Invitrogen) according to manufacturer’s instructions. Two different siRNA sequences were evaluated for suppression of target gene expression by immunoblots, transfection efficiency was determined to be \(>95\%\). The following siRNAs were used: Dll1 (#133773, #133774); ephrin-B2 (#14087, #14268).

Constructs

NICD cDNA was kindly provided by Spyros Artavanis-Tsakonas (Paris, France), RBP-J(R218H) cDNA was kindly provided by Tasuku Honjo (Kyoto, Japan) through RIKEN DNA Bank (Japan). Coding sequences were subcloned into the pshuttle2 IRES-GFP vector and recombinant adenovirus was generated using the AdEasy Adenoviral Vector System (Stratagene, La Jolla, Calif). EC were transduced at a multiplicity of infection of 50.

In Vitro Branching Morphogenesis and Network Formation

HAEC were serum starved in 1% FCS overnight, seeded on matrigel (BD Biosciences, San Jose, Calif) at a density of \(1 \times 10^4\) cells/cm\(^2\) and stimulated with or without VEGF and FGF-2 (100 ng/mL) for 24 hour. In some experiments, matrigel was supplemented with 0.2 or 1.0 \(\mu\)g/mL of EphB4-Fc (R&D Systems, Minneapolis, Minn), in which case 1.0 \(\mu\)g/mL IgG-Fc served as control. Digital images of 3 random microscopic fields per well were acquired using a Zeiss
Axiovert 200 microscope and analyzed using the Axiovision Rel 4.4 software (Zeiss, Oberkochen, Germany). Branch points were defined as intersections of at least 3 tubes, total network length was defined as all tubes connected by branch points, and data are given per microscopic field (5.5 mm²).

Hindlimb Ischemia Model

The study was conducted with permission of the State of Niedersachsen, conforming to the German Law for the Protection of Animals and the NIH Guide for the Care and Use of Laboratory Animals. Mutant Dll1LacZ/H11001 mice27 were bred on an isogenic 129S1/SvImJ background, nontransgenic littermates served as controls. Surgical procedures were performed as described.28 Briefly, mice were anesthetized by intraperitoneal injection of a mixture of ketamine (2 mg/kg body weight) and xylazine (13 mg/kg body weight) and the femoral artery was ligated distal to the origin of the deep femoral artery and proximal to the popliteal artery. Blood flow measurements in mouse feet were performed on 37°C heated pads before and immediately after surgery, and on post-operative days 3, 7, 14, 21, and 28 using a laser Doppler perfusion imager (PIM II, Perimed, Sweden). Perfusion was expressed as the ratio of ligated-to-nonligated side. For induction of severe ischemia the right femoral artery was ligated proximal to the origin of the deep femoral artery.

Tissue Sampling and Analysis

To analyze collateral arteries from the deep femoral artery, which follow a constant course on the surface of the adductor muscles, mice were perfused with 0.1% adenosine, in situ fixed with 4% PFA, and semimembranous and gastrocnemic muscles were excised, cryoprotected in sucrose, and embedded in OCT compound.28 Histomorphometry of collateral arteries was performed on at least 5 H&E stained sections of semimembranous muscle using the Axiovision Rel 4.4 software and a Zeiss Axiovert 200 microscope (Zeiss). The arterial wall area was calculated by subtracting the lumen area from the outer circumference of the tunica media.

Laser scanning confocal microscopy after immunostaining was performed with a Leica TCS SP2 AOBS (Leica Microsystems), using the following staining reagents and antibodies: Hes1,29 Hey1 (Chemicon), DLL1 (H-265), DLL4 (H-70) and VEGF(147) (Santa Cruz Biotechnology, Calif), FITC-conjugated smooth muscle α-actin (Sigma), cleaved Notch1 (Cell Signaling Technology, Danvers, Mass), ephrin-B2 and EphB4 (R&D Systems), biotinylated isolectin B4 (IB4, Vector Laboratories), and Texas Red avidin D (Linaris) or appropriate fluorescence-conjugated secondary antibodies (Molecular Probes, Invitrogen, Karlsruhe, Germany). Nuclei were counter-stained with Topro-3 (Molecular Probes).

Figure 2. Impaired postnatal arteriogenesis in heterozygous Dll1 mice. A, Impaired blood flow recovery of ischemic hindlimbs of heterozygous Dll1lacZ/H11001 mice shown by laser Doppler blood flow (LDBF) measurements, expressed as ischemic to normal (contralateral) LDBF ratios (upper panel), and representative color-coded LDBF images (lower panel, dark blue represents low flow). n=11 to 12, *P<0.01. B, Impaired collateral artery growth in Dll1lacZ/H11001 mice demonstrated in histomorphometric analysis of collateral lumen (upper panel) and wall area (middle panel) and representative H&E stained sections (lower panel) 3 days after HLI. n=11 to 12, *P<0.01. C, Severe ischemic tissue damage in Dll1lacZ/H11001 mice scored 7 days after complete femoral artery occlusion (left panel) and LDBF images showing autoamputation in a Dll1lacZ/H11001 mouse (right panel). n=10 to 11. D, Increased capillary density in ischemic gastrocnemic muscle of Dll1lacZ/H11001 mice shown by quantification of capillaries (IB4 +) per myofiber (wheat germ agglutinin +, WGA) and representative immunofluorescence stainings. n=3, *P<0.01 (left panels). FITC-labeled dextran perfusion demonstrating perfused capillary beds in Dll1-heterozygous mice (right panel). Scale bar: 10 μm. Magnification: (B and D) 400.

Axiovert 200 microscope and analyzed using the Axiovision Rel 4.4 software (Zeiss, Oberkochen, Germany). Branch points were defined as intersections of at least 3 tubes, total network length was defined as all tubes connected by branch points, and data are given per microscopic field (5.5 mm²).
For capillary density, gastrocnemius muscle sections were stained with IB4, rhodamine-coupled wheat germ agglutinin (WGA, Vector Laboratories), FITC-conjugated Avidin D (Linaris) and nuclei counter-stained with 4′,6-diamino-2-phenylindole dilactate (DAPI, Sigma). At least 12 sections per muscle were analyzed by fluorescence microscopy and results expressed as the ratio of IB4 capillaries to WGA muscle fibers. For perfusion studies mice were perfused with a 5 mg/mL FITC-dextran solution (2 × 10⁶ avg. weight, Sigma). β-galactosidase staining was performed on glutaraldehyde fixed tissues as described.²⁶

Immunoblotting

Equal amounts of proteins from cell lysates were separated by SDS-polyacrylamid gel electrophoresis, transferred onto nitrocellulose membranes and probed with primary and secondary antibodies. Additional antibodies used were: Notch1 (bTan, Developmental Studies Hybridoma Bank, Iowa), actin (Sigma).

Statistics

All values are expressed as mean ± SD. Two treatment groups were compared by the unpaired Student’s t test; one-way ANOVA was performed for serial analysis. The incidence of ischemic damage was evaluated by χ² test. Probability values less than 0.05 were considered statistically significant.

Results

Specific Expression of Dll1 in Postnatal Arterial Endothelium

To analyze expression of Dll1 in adult vasculature we initially analyzed lacZ expression in Dll1lacZ/heterozygous mice, in which 1 Dll1 allele has been replaced by the lacZ gene and whose expression mimicks endogenous Dll1 expression.²⁶,²⁷ These mice show a normal phenotype on gross pathologic examination and normal survival rates (data not shown). Staining of whole hearts and sections revealed distinct Dll1 expression in coronary arteries (Figure 1A and G). Dll1 expression was detected in all examined arteries, such as the aorta, carotid and renal arteries, and small muscular arteries (Figure 1A, C, E–G, and Figure 3B). Dll1 expression was specific for arterial EC, and not detected in the arterial wall, nor in venous endothelium or capillaries (Figure 1, C, E–H).

Impaired Postnatal Arteriogenesis, but Not Microvascular Angiogenesis, in Heterozygous Dll1 Mice

To determine the role of Dll1 in vascular regeneration we subjected mice to hindlimb ischemia (HLI) by ligation of the superficial branch of the femoral artery, which triggers growth of preexisting collaterals from the deep femoral artery.⁴ Relative blood flow measurements by laser Doppler revealed comparable levels of postprocedure ischemia in Dll1lacZ/heterozygous mice and nontransgenic littermates (wild-type, WT). Serial laser Doppler measurements in WT mice revealed a rapid increase in ischemic blood flow within three days of HLI, followed by more gradual recovery over 28 days. In contrast, initial blood flow recovery in Dll1lacZ/heterozygous mice was severely blunted and ischemic flow remained severely depressed, in fact never returning to preischemic perfusion levels, which suggested defective arteriogenesis (Figure 2A).

To investigate the role of Dll1 in postnatal arteriogenesis we quantified collateral arterial growth. Histomorphometry of contralateral collaterals revealed no difference in vessel size in both genotypes. In response to HLI there was a significant increase in collateral lumen and wall area in WT mice, demonstrating active arterial remodeling and outward growth. In contrast, there was no significant lumen increase in
collaterals of Dll1lacZ/H11001 mice after HLI, and collateral wall area was significantly smaller compared with WT mice (Figure 2B). These data demonstrate Dll1 haploinsufficiency in postnatal arteriogenesis.

The pathophysiologic consequences of Dll1 haploinsufficiency were evaluated after aggravated HLI by complete proximal femoral artery occlusion. Again, the initial level of ischemia was comparable between groups (ischemic/normal perfusion [%], WT: 28 ± 5; Dll1lacZ/H11001: 22 ± 7, p = n.s., n = 10 to 11). However, only Dll1lacZ/H11001 mice sustained severe necrosis or autoamputation in the majority of cases, whereas WT mice recovered from ischemic insult without major damage (Figure 2C; WT versus Dll1lacZ/H11001, amputation: 0/10 versus 6/11, necrosis: 2/10 versus 2/11, complete recovery: 8/10 versus 3/11; P < 0.05). In contrast, capillary density in ischemic muscles was significantly increased in Dll1lacZ/H11001 mice (Figure 2D), which probably reflects increased and persistent ischemia in the affected limb. Although increased capillary sprouting lead to perfused capillary beds, as shown by FITC-labeled dextran perfusion studies (Figure 2D), this did not compensate for the perfusion defect caused by impaired arteriogenesis in Dll1 haploinsufficient mice. Taken together, these results demonstrate a crucial role of Dll1 in postnatal arteriogenesis and ischemic limb salvage.

**Induction of Dll1-Dependent Notch Signaling Regulates Ephrin-B2 Expression During Postnatal Arteriogenesis**

Endogenous VEGF is required for postnatal arteriogenesis. VEGF was strongly expressed in perivascular cells and vessels without apparent differences in expression levels between genotypes (Figure 3A). Endothelial DLL1 expression was low in steady-state arteries, but strongly upregulated in response to HLI in WT mice, whereas DLL1 levels in heterozygous Dll1 mice remained below the detection limit of immunostaining (Figure 3B, a, b, d, e). Dll1 expression within vessels was limited to the inner endothelial lining, as shown by nonoverlapping staining with smooth muscle α-actin (SMA) (Figure 3B, c and g). DLL1 expression in ischemic collaterals of Dll1lacZ/H11001 mice was confirmed by endothelial restricted β-galactosidase staining (Figure 3B, f).

Upregulation of DLL1 coincided with activation of endothelial Notch1 in WT mice, but not Dll1lacZ/H11001 mice, as shown by specific immunostaining for γ-secretase-cleaved Notch1 (Val1744) (Figure 4A, b–c and e–f). Consistent with Dll1-dependent Notch activation there was strong endothelial, but also significant perivascular, expression of the Notch target gene Hes1 in arteries of WT mice, but not in heterozygous mice (Figure 4B). The Notch target gene Hey1, which is involved in embryonic arterial development, was also induced in growing collaterals in a Dll1-dependent manner (Figure 4C).
Angiogenic Growth Factors Regulate Ephrin-B2 Expression in Arterial EC via Dll1-Dependent Notch Activation

Coexpression of VEGF and DLL1 in growing arteries suggested regulation of Dll1 by angiogenic growth factors. In cultured human aortic EC (HAEC), VEGF treatment transiently increased DLL1 protein levels in a time- and dose-dependent manner (Figure 6A). FGF-2, which is expressed around growing collaterals and acts synergistically with VEGF in postnatal arteriogenesis, also induced DLL1 transiently in a time- and dose-dependent manner. However, the combination of VEGF and FGF-2 (VF) increased and prolonged DLL1 upregulation synergistically (Figure 6A).

Combined growth factor treatment also synergistically upregulated ephrin-B2 in a dose-dependent manner (Figure 6B). DLL1 upregulation induced γ-secretase-dependent cleavage and activation of Notch1, and upregulated ephrin-B2 in endothelial cells (Figure 6C). Notch1 signaling and ephrin-B2 expression, but not DLL1 upregulation, was blocked by a specific γ-secretase inhibitor (GSI), N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), a potent inhibitor of Notch signaling. Dll1 knockdown with small interfering (si) RNA also abrogated the induction of ephrin-B2 by VF (Figure 6C). Ephrin-B2 expression was further blocked by adenoviral overexpression of a dominant-negative (dn) inhibitor of Notch signaling, RBP-J(KR218H), whereas overexpression of constitutive-active Notch1 (NICD) strongly upregulated ephrin-B2 expression independent of VF (Figure 6D). Thus, Dll1-dependent Notch signaling is necessary and sufficient for ephrin-B2 induction in arterial EC.

Dll1-Dependent Notch Signaling Regulates Branching and Vascular Network Formation via Ephrin-B2

To study the influence of Dll1 and external matrix on endothelial cell function in vitro we performed vascular network formation assays. HAEC were seeded in a matrigel matrix containing embedded EphB4-Fc fusion protein, which exclusively interacts with ephrin-B2 ligands, or control Fc fragments. In a dose-dependent manner, EphB4-Fc enhanced EC branching and total network length, while decreasing mean tube length, demonstrating increased network complexity in an EphB4-rich milieu (Figure 7A). Furthermore, EphB4-Fc matrix significantly increased branching and total network extension and decreased mean tube length after growth factor stimulation compared with control matrix (Figure 7A). These effects were abrogated by inhibition of Notch signaling with GSI (Figure 7A), and knockdown of Dll1 by siRNA, which was recapitulated by ephrin-B2 siRNA, but not by control, scrambled siRNA (Figure 7B).

Discussion

Our findings establish Dll1 as an essential regulator of postnatal arteriogenesis. Dll1 expression in steady-state arteries is low and, consequently, Notch activity and ephrin-B2 levels are low. During arteriogenesis there is perivascular induction of a pro-angiogenic milieu, including VEGF, FGF-2, and EphB4. At the same time, endothelial Dll1 is strongly

Figure 5. Dll1-dependent induction of ephrin-B2 during postnatal arteriogenesis. Confocal microscopy of collateral arteries. A, Ephrin-B2 upregulation in growing WT collaterals (upper panels), but not in Dll1 lacZ/ lacZ collaterals (lower panels). Partially overlapping staining with SMA indicates endothelial and mural expression after HLI. (c and f) Magnifications from (b) and (e). B, Periartrial EphB4 induction after HLI in WT and Dll1 lacZ/ mice (b, c, e and f). (a) Endothelial EphB4 staining in veins (V), but not arteries. (c and f) Magnifications from (b) and (e). Scale bar: 10 μm.

Ephrin-B2 on EC interacts with EphB4 in bidirectional signaling during vascular remodeling. No ephrin-B2 expression was detected in steady-state arteries, but there was strong induction in endothelium and vessel wall of growing WT collaterals, but not in Dll1 lacZ/ lacZ collaterals (Figure 5A). Expression of EphB4, which serves as activator for ephrin-B2 reverse signaling, was not detected in steady-state arteries (Figure 5B, a and d), whereas constitutive venous endothelial EphB4 was readily detected (Figure 5B, a). After HLI, there was strong and comparable perivascular induction of EphB4 in both genotypes (Figure 5B, b, c, e, f). Thus, induction of EphB4 is regulated in a complementary fashion to ephrin-B2, but independent of Dll1.

Together, these results demonstrate the spatially coordinated induction of a pro-angiogenic signaling cascade, consisting of VEGF, DLL1-dependent Notch signaling, and the downstream effectors ephrin-B2 and EphB4. These results also suggest that ephrin-B2 mediates arteriogenesis downstream of Dll1-dependent Notch activation, whereas VEGF and EphB4 are regulated independent of Dll1.
upregulated, Notch signaling activated and ephrin-B2 induced, while arteries grow. Failure to significantly upregulate Dll1, however, impairs Notch activation and ephrin-B2 expression and abrogates arterial growth, whereas perivascular VEGF and EphB4 expression is unaltered. In vitro, VEGF and FGF-2 synergistically induce Dll1-dependent Notch signaling, which is necessary and sufficient to induce ephrin-B2. Furthermore, the ability to induce branching morphogenesis depends on Dll1-dependent Notch signaling and is mediated by ephrin-B2.

Endothelial ephrin-B2 reverse signaling has been shown to mediate EC migration, adhesion and sprouting in vitro and embryonic and postnatal angiogenesis in vivo.10,12,13 We propose a model of postnatal arteriogenesis in which induction of Dll1 by angiogenic growth factors leads to Notch-dependent expression of ephrin-B2, which mediates remodeling and outward growth toward an EphB4 enriched milieu (Figure 8).

On the other hand, the normal baseline vascular phenotype can be explained by low Dll1-dependent signaling under steady-state conditions. The observed transient induction of Dll1 by growth factors is consistent with reciprocal Dll1 inhibition by activated Notch receptors,23 which, we speculate, could provide a possible mechanism for termination of arteriogenesis through Dll1 downregulation following prolonged Notch activation. On the other hand, the lack of Dll1 induction in the heterozygous state may suggest an autostimulatory action of Dll1 in the initiation of Notch signaling. Alternatively, it is possible that Dll1 heterozygous vessels lack the ability to respond to angiogenic stimuli. Clearly, these interesting questions require further studies.
Our findings also suggest that the mechanisms of postnatal arteriogenesis are reminiscent of arterial development, where Notch signaling regulates arterial differentiation downstream of VEGF and upstream of ephrin-B2.\textsuperscript{14} However, whereas \textit{Dll4} is a crucial Notch ligand in arterial development in the embryo, our analysis of adult mice indicates expression in small vessels and capillaries, which is consistent with previous reports.\textsuperscript{33} Furthermore, we found no evidence for specific regulation of \textit{Dll4} during postnatal arteriogenesis, where \textit{Dll1} is a key regulator. Our finding of impaired arteriogenesis but increased microvascular angiogenesis in \textit{Dll1} heterozygous mice can be explained by the restricted expression of \textit{Dll1} in the arterial domain and demonstrates \textit{Dll1}-independent regulation of microvascular angiogenesis, possibly through \textit{Dll4}, which is expressed in microvessels and tumor vasculature and regulates ephrin-B2 expression and angiogenesis.\textsuperscript{13,25,32,33} However, this also emphasizes the importance of arteriogenesis in blood flow restoration and ischemic organ rescue.

Finally, given the crucial role of \textit{Dll1} in postnatal arteriogenesis, interventions or drugs that upregulate vascular \textit{Dll1} during ischemic stress might greatly enhance arteriogenesis and contribute to organ rescue and regeneration.

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Figure 8. Model for regulation of postnatal arteriogenesis by Dll1. Pervascular expression of angiogenic growth factors triggers induction of Dll1, which induces ephrin-B2 expression via vascular Notch activation. Interaction of vascular ephrin-B2 with perivascular EphB4 mediates arterial remodeling.

Disclosures

None.

References

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