Evolutionarily conserved requirement of Cdx for post-occipital tissue emergence

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SUMMARY

Mouse Cdx genes are involved in axial patterning and partial Cdx mutants exhibit posterior embryonic defects. We found that mouse embryos in which all three Cdx genes are inactivated fail to generate any axial tissue beyond the cephalic and occipital primordia. Anterior axial tissues are laid down and well patterned in Cdx null embryos, and a 3’ Hox gene is initially transcribed and expressed in the hindbrain normally. Axial elongation stops abruptly at the post-occipital level in the absence of Cdx, as the posterior growth zone loses its progenitor activity. Exogenous Fgf8 rescues the posterior truncation of Cdx mutants, and the spectrum of defects of Cdx null embryos matches that resulting from loss of posterior Fgfr1 signaling. Our data argue for a main function of Cdx in enforcing trunk emergence beyond the Cdx-independent cephalo-occipital region, and for a downstream role of Fgfr1 signaling in this function. Cdx requirement for the post-head section of the axis is ancestral as it takes place in arthropods as well.

KEY WORDS: Embryonic axial elongation, Posterior growth zone, Cdx and Hox genes, Fgf signaling, Post-occipital growth

INTRODUCTION

During gastrulation of the mouse embryo, progenitors for trunk and tail tissues are found in an ordered position in the epiblast flanking the primitive streak, from its more posterior extension to its rostral-most limit abutting the node (Kinder et al., 1999; Lawson et al., 1991; Tam and Beddington, 1987). Whereas the progenitors along the posterior and middle streak levels are transiently delivering descendants to extra-embryonic and embryonic mesoderm and do not leave residing cells after they have ingressed in the streak, the anterior-most level of the primitive streak harbors a self-renewing, stem cell-like population of progenitors that go on contributing cells to the elongating axial tissues until the end of axial growth by tissue addition (Cambray and Wilson, 2002; Cambray and Wilson, 2007; Wilson et al., 2009). Clonal analysis during embryogenesis provided evidence that bipotent long-term neuro/mesodermal (LT N/M) progenitors contribute descendants to extended axial domains (Tzouanacou et al., 2009). These progenitors are likely to correspond to the stem cell-like axial progenitors shown by Cambray and Wilson to be present in the node-streak border and along the anterolateral primitive streak at embryonic day (E) 8.5, and in the chordo-neural hinge later on (Cambray and Wilson, 2002; Cambray and Wilson, 2007).

The mouse has three Cdx transcription factor encoding genes, Cdx1, Cdx2 and Cdx4, homologs of Drosophila caudal. Cdx loss of function was first discovered to impair axial elongation when Cdx2 was inactivated (Chawengsaksophak et al., 1997). Null mutants for Cdx1 and Cdx4 are not compromised in their axis extension but they fail to complete their axial development when missing an active allele of Cdx2 (Savory et al., 2009; van den Akker et al., 2002; van Nes et al., 2006; Young et al., 2009). Cdx4 null mutants heterozygote for Cdx2 (hereafter termed Cdx2/4 mutants) also suffer from limited allantoic vessel invasion in the chorionic ectoderm, and the allantois of Cdx2 null mutants fails to grow out, preventing placental labyrinth ontogenesis and survival of the embryo beyond E10.5 (Chawengsaksophak et al., 2004; van de Ven et al., 2011; van Nes et al., 2006). Compound mutants for the different Cdx genes revealed redundancy between them in allowing embryonic tissues from the three germ layers to expand as development proceeds (Savory et al., 2009; Savory et al., 2011; van de Ven et al., 2011; van den Akker et al., 2002; Young et al., 2009). Histological and gene expression analyses combined with the fate-mapping information on the progenitors of axial tissues in the mouse embryo (Cambray and Wilson, 2002; Cambray and Wilson, 2007; Tzouanacou et al., 2009) led to the conclusion that the Cdx mutations in Cdx2/4 compound mutants affect tissue generation from progenitors residing along the primitive streak and its continuation in the tail bud without causing apoptosis (van de Ven et al., 2011; Young et al., 2009). Genetic analysis revealed that the axial extension defects of these mutants could be rescued by either a gain-of-function of Hox genes belonging to the middle part of the Hox clusters, or by expressing an activated form of the Wnt signaling effector Lef1 in the spatiotemporal window of Cdx expression (Young et al., 2009). The latter information and subsequent grafting experiments of the region harboring stem cell-like axial progenitors for trunk and tail tissues from Cdx2/4 mutants into wild-type recipients revealed that the Cdx mutations disable the surrounding niche of these progenitors rather than the progenitors themselves (Bialecka et al., 2010). So far, the impact of ablating all three Cdx genes had not been tested. Cdx genes start to be transcribed at E7.2 in the posterior primitive streak. In order to study embryogenesis in the total absence of Cdx activity from early on in the epiblast, we generated mouse embryos totally deprived of Cdx expression using Cdx1 and Cdx4 null alleles (Subramanian et al., 1995; van Nes et al., 2006), and a Cdx2 conditional allele (Stringer et al., 2012), in combination with the epiblast-specific Sox2Cre. We show here, using mouse embryos in...
which the three Cdx genes are inactivated, that the realm of action of Cdx encompasses and is restricted to the entire trunk and tail sections of the axis. Ablation of all three Cdx genes causes agensis of the axial domain posterior to the occipital region, involving the three germ layers. The key role of Fgf signaling in axial elongation was demonstrated by the observation that Fgf restores tissue emergence and gene expression in the embryonic growth zone of Cdx mutants in whole embryo cultures.

MATERIALS AND METHODS

Mice
All mice were in the C57Bl6j/CBA mixed background. Cdx2 heterozygotes and Cdx1 and Cdx4 null mutant mice as well as the protocols to genotype them have been described previously (Chawengsaksophak and Beck, 1996; Subramanian et al., 1995; van Nes et al., 2006). Generation and genotyping of the strain carrying Cdx2 conditional allele was described by Stringer et al. (Stringer et al., 2012). Epiblast-specific Cdx2 null mutants were obtained by crossing Cdx2 floxed homozygotes and Cdx22–/– Sox2Cre transgenic mice (Hayashi et al., 2002). Cdx null embryos were generated by crossing Cdx1–/– Cdx22floxed/lox Cdx4+/– males with Cdx1–/– Cdx2–/– Cdx4+/– females, which carry the Sox2Cre transgene. Embryos of the genotye Cdx1–/– Cdx22–/– Cdx4+/– were obtained in the same cross. All experiments using mice were performed in accordance with the institutional and national guidelines and regulations, under control of the Dutch Committee for Animals in Experiments, and under the licenses required in The Netherlands.

Histology and immunohistochemistry
For histological analysis, tissues were fixed with 4% parafomaldehyde (PFA) overnight at 4°C. Whole mount in situ hybridization of mutant and control embryos was performed according to Young et al. (Young et al., 2009). Embryos were imbedded in plastic (GMA Technovit type 8100) and sectioned at 7 μm. For immunofluorescence staining, 70 μm-thick vibratome sections were made from embryos embedded in 4% low melting point agarose. Antibodies used were anti-Sox2 (polyclonal rabbit anti-mouse Sox2, Millipore, AB5603), and anti-T (polyclonal goat anti-mouse T, Santa Cruz, SC-17743). Counterstaining was with DAPI.

Whole embryo culture
Embryos were cultured for 48 hours as described by Bialecka et al. (Bialecka et al., 2010). Each experiment contained control and Cdx mutant embryos with and without Fgf8. At the end of the culture period embryos were fixed in 4% PFA overnight at 4°C, and photographed. Somites were counted using a Leica MZ16FA microscope with a DFC480 camera. Recombinant Fgf8 (isoform b) was purchased from R&D Systems (423-F8).

Statistical analysis
The Mann-Whitney U test was used to analyze the significance of the difference between the number of somites added, and the difference between the length of axial tissue added beyond the forelimb bud of Cdx mutant embryos cultured for 2 days with or without Fgf8. The Mann-Whitney U test was chosen because the data sets for each genotype were not normally distributed (z values obtained from the Kolmogorov-Smirnov test for each genotype were >0.05).

RESULTS

Absence of Cdx prevents the generation of trunk and tail tissues during embryogenesis
Cdx triple null mutants were generated with mice carrying null alleles for Cdx1, Cdx2 and Cdx4, and a conditional allele of Cdx2 (Stringer et al., 2012) (T. Young, PhD thesis, Utrecht University, 2009), in combination with a Sox2Cre transgene (Hayashi et al., 2002) allowing Cdx2 inactivation in the inner cell mass-derived embryonic tissues. Following this strategy, the epiblast of the mutant is totally deprived of Cdx activity from the earliest stage on, and the embryo proper is absolutely Cdx null. Triple Cdx mutant embryos were recovered at the expected Mendelian frequency, but their generation required intensive efforts because of low breeding performance of Cdx1–/– Cdx2–/– Sox2Cre males used in the final cross (see Materials and methods). We analyzed 52 Cdx triple null embryos, 21 embryos with a genotype Cdx1–/– Cdx2–/– Cdx4+/– (indistinguishable from the Cdx triple null mutants) and a larger number of Cdx2 null mutants and wild-type controls.

Cdx triple mutant (referred to as Cdx null from here on) embryos at E7.5 are indistinguishable from wild-type embryos (supplementary material Fig. S1A,B), except for the fact that their allantois fails to grow, as is the case in Cdx2 null embryos (Chawengsaksophak et al., 2004; van de Ven et al., 2011). Therefore, they do not survive beyond E10.5 because they never establish a placental labyrinth. Development seems to have arrested earlier than E10.5, as they are growth retarded at this stage in comparison with controls (supplementary material Fig. S1E,F). Cdx null mutants were severely posteriorly truncated, and the axial length of the last tissues generated was anterior to the forelimb buds (supplementary material Fig. S1C-F). In addition, they exhibited an open neural tube, a condition that was also observed in the absence of Cdx1 and Cdx2 (Savory et al., 2011). The Cdx null embryos generate a maximum of five somites. At E8.5, 25 out of 25 mutants had five somites instead of the eight to ten generated by control E8.5 littersmtes. At E9.5, seven out of eight mutants had five somites whereas age-matched controls had 22 to 25 somites (one mutant generating a small sixth somite). Mox1 (Mox1 – Mouse Genome Informatics), a marker of differentiated somites, was expressed in the paraxial mesoderm and confirmed the presence of five somites in the Cdx null embryos (Fig. 1A,B). These somites were correctly patterned along their anteroposterior (A-P) axis, as revealed by UncX4.1 (Uncx – Mouse Genome Informatics) expression, which identifies the posterior somitic compartments (Mansouri et al., 1997) in both mutants and controls (Fig. 1C-F). Mesp2, which normally marks the anterior presomitic mesoderm (PSM), was not expressed in the mutant embryos (supplementary material Fig. S1G,H). We conclude that Cdx null mutants arrest their axial elongation after the occipital somites have been generated.

Anterior tissues are generated in early gastrulating Cdx null embryos but progenitor cells for trunk and tail fail to be maintained
E8.5 Cdx null embryos and age-matched controls were subjected to in situ hybridization to detect the expression of genes marking recent mesoderm and neurectoderm generated from the posterior growth zone. Tbx6, a marker of the PSM, was hardly expressed in the posterior part of Cdx null embryos, whereas transcripts were present in the most recently generated paraxial mesoderm in the controls (Fig. 1G,H), suggesting an arrest of mesoderm generation in the mutant. Posterior expression of Sox2, marking the neurectoderm, was lower in Cdx null embryos than in controls (Fig. 1I,J). The posterior growth zone of Cdx null mutants thus severely loses its activity in generating nascent mesoderm and neurectoderm. Wnt11a expression is also considerably lower in the growth zone of Cdx null mutants versus controls (Fig. 1K,L). The notochord of Cdx null mutants expressed Shh (Fig. 1M,N) and T (brachyury) (Fig. 2E,F). The posterior end of the notochord revealed by these two markers had a widened appearance, recognized as tubular by examination of transverse sections, stained for RNA and protein detection of brachyury (Fig. 2F,L,N compared with 2E,K,M). The notochord never expresses Cdx genes and should not be directly affected by the loss of Cdx
expression. The tubular end of the notochord is reminiscent of the same feature in some mammalian embryos that develop as flat disks (C. Viebahn, personal communication) (Haldiman and Gier, 1981), and might thus result from the fact that the Cdx null embryos are much flatter than their controls owing to their severe posterior truncation. The notochord of Cdx null mutants at more anterior levels does not show this tubular feature (Fig. 2P). A striking feature in Cdx null mutants is the absence of T expression in the primitive streak region posterior to the notochord end at E8.5, whereas the gene is strongly expressed in the streak and in adjacent tissues in age-matched controls (Fig. 2F compared with 2E). Investigations in earlier, head fold stage (E7.5) embryos revealed that their primitive streak region expresses T and is indistinguishable from that in age-matched controls (Fig. 2A-D). These observations strongly suggest that the progenitors for embryonic axial tissues along the primitive streak at E7.5 normally generate anterior mesoderm in Cdx null mutants, whereas they fail to do so after five somites have been generated. Serial transverse
sections through E8.5 embryos hybridized with a T probe show that whereas T-positive nascent mesoderm is emerging from the T-positive primitive streak in the control, this is not the case in the mutant (Fig. 2H,J versus 2G,I). This suggests that no new mesoderm has emerged from the inactive primitive streak at E8.5 (Fig. 2H,J).

**Anterior Hox genes are well induced in the primitive streak and correctly expressed in anterior tissues whereas more posterior Hox genes are not expressed**

The expression of anterior Hox genes was similarly initiated in Cdx null mutants and controls. Hox genes are initially transcriptionally induced in the posterior primitive streak at the late mid-streak stage (E7.0-7.2), and their expression domains spread anteriorwards along the streak and adjacent tissues, in a way that is temporally collinear with the position of the genes in their cluster (Deschamps and Wijgerde, 1993; Forlani et al., 2003; Gaunt et al., 1986; Limura and Pourquie, 2006). These expression domains then extend further anteriorly in embryonic tissues, eventually reaching gene-specific rostral boundaries. Hoxb1 is first expressed in the posterior streak at E7.2 and its expression domain has reached the anterior part of the streak by the head fold stage (E7.5) (Forlani et al., 2003) in Cdx null mutants and in controls (Fig. 3A,B). At somite stages, the anterior expression pattern of Hoxb1 in the mutant and control is the same, as shown by the restricted expression domain at the level of rhombomere (r) 4 (Fig. 3C,D). However, a reduction of the expression level of this gene in posterior tissues was observed in the mutant, in the primitive streak area reported above to be losing its activity (Fig. 3D). The expression of more posterior Hox genes was analyzed in Cdx null embryos and controls. Hoxb4, the rostral expression domain of which normally reaches the posterior hindbrain and somite 5/6 in the mesoderm (Gould et al., 1998), had an expression boundary caudal to the level of the fifth somite in Cdx null embryos, and its expression decayed posteriorly (Fig. 3E,F). E8.5 Cdx null embryos did not express Hoxa5 and Hoxb8, normally expressed in trunk tissues (rostral limits in posterior hindbrain and somite 6/7 for Hoxa5, and below somite 5 in the neural tube and somite 11 in the paraxial mesoderm for Hoxb8) (Larochelle et al., 1999; van den Akker et al., 1999; Young et al., 2009) (Fig. 3G,H; supplementary material Fig. S2). The same holds true for Hoxa9 and for Hoxb9, two more 5’ and later-initiated Hox genes that are expressed at trunk levels (Fig. 3K,L; supplementary material Fig. S2). We conclude that the initial transcription of the first Hox gene of the cluster takes place correctly in the primitive streak of Cdx null embryos at early stages. The transcription domain of this 3’, early Hox gene normally expands anteriorly together with the emerging tissue that will form the rhombomeric and occipital structures. The posterior part of the expression of these 3’ genes later on fades away as the growth zone becomes inactive at the 5somite stage, and more 5’ (posterior) Hox genes are not expressed.

**Fgf is key to Cdx-dependent tissue generation from axial progenitors and rescues the posterior truncation in Cdx2 null mutants**

Cdx factors have been suggested to regulate the gene encoding the retinoic acid (RA)-degrading enzyme Cyp26a1 directly and positively (Savory et al., 2009; Young et al., 2009). Cyp26a1 was not transcribed at all in early somite Cdx null mutants (Fig. 4A,B), at a stage when this gene is normally expressed posteriorly and allows the growth zone to clear the RA diffusing from the somites.

E8.5 Cdx null embryos have stopped generating PSM tissue beyond the last formed somite, and they express the RA-synthesizing enzyme Raldh2 (Aldh1a2 – Mouse Genome Informatics) at high level down to the growth zone, whereas Raldh2 expression in wild type is restricted to the somites and anterior PSM (Delfini et al., 2005), at a distance from the anterior streak (Fig. 4C-H). Cdx null mutants, thus, unlike controls, synthesize RA within their growth zone at E8.5. Given the balanced antagonism between the RA and Fgf pathways during posterior embryonic morphogenesis (Diez del Corral and Storey, 2004; Ribes et al., 2009), we set out to test the involvement of the
Fgf signaling pathway in causing the posterior truncations of Cdx null mutants. Fgf signaling activity, revealed by Spry4 expression (Naiche et al., 2011), was completely lost in the posterior part of E8.5 Cdx null mouse embryos (Fig. 4I,J).

We designed whole embryo culture experiments to challenge the crucial involvement of Fgf loss in the posterior truncation phenotype of Cdx mutants. Envisaging rescue attempts on early embryos from the crosses used to generate Cdx null embryos was unrealistic given the extremely low yield of these mutants, which cannot be genotyped before the culture. We therefore turned to Cdx mutants of the allelic series that are less severely impaired in their development, and easier to generate. Cdx2 null embryos arrest their development at E10.5 and never have more than 17 somites, regardless of whether they are analyzed at E9.5 or E10.5 (Chawengsaksophak et al., 2004; Chawengsaksophak et al., 1997; van de Ven et al., 2011), whereas controls typically have about 25 somites at E9.5, and 35 somites at E10.5 (average of many experiments) (Kaufman, 1995). We cultured whole E7.5 (presomite)/E8.0 (early somite) Cdx2 null mutant and control embryos for the same period of two days in vitro, in the presence or in the absence of added recombinant Fgf8 (Fgf8 isoform b). We scored the somite number that these embryos generated during the culture period. Fgf8 exposure was found to rescue the deficit in axial tissue growth of the mutants significantly (Fig. 5F compared with 5D). Cdx2 mutant embryos cultured with Fgf8 (n=7) made on average 23 somites during the culture, whereas they only made 16 somites without supplemented Fgf8 (n=5) (Fig. 5K). The rescue of the posterior truncation of Cdx2 null embryos by Fgf8 was not complete as the PSM remained shorter in the Fgf8-rescued mutants than in controls. Cultured embryos of the four series (controls and mutants cultured with and without Fgf8) were subjected to in situ hybridization with a Mox1 probe (Fig. 5G-J) and their somites counted again. This confirmed the rescue of the posterior truncation of the Cdx2 mutants by Fgf8 (Fig. 5K). The restoration of posterior axial extension of Cdx2 mutant embryos by Fgf8 was also documented by measuring the axial length beyond the forelimb buds in stage-matched mutants cultured with (n=4) and without (n=4) Fgf8. The axial portion added to the embryos posterior to the forelimb bud was significantly longer for the mutant embryos cultured with added Fgf8 (median value 472 mm) than for mutants cultured in the same conditions but without Fgf8 (median value 320 mm) (P=0.002) (data not shown). A significant rescue of axial extension is thus taking place when the mutants are grown in the presence of exogenous Fgf8. Importantly, exogenous Fgf8 allowed the Cdx2 mutant embryos to generate seven somites more than they ever generate in vivo (Fig. 5K). Fgf8-rescued Cdx2 mutants also re-express Cyp26a1 at their posterior end similarly to wild-type embryos (supplementary material Fig. S3). The restoration of axial elongation and posterior gene expression by supplemented Fgf suggests that Fgf signaling reactivates the growth zone in Cdx mutants. We conclude that decreased Fgf signaling in the posterior growth zone in Cdx mutants is crucially involved in causing the exhaustion of tissue emergence from this growth zone. The data are summarized in Fig. 6 and supplementary material Fig. S4.

**DISCUSSION**

Cdx genes are obligatory players in the emergence of the entire trunk and tail. They implement the dichotomy between the pre- and post-occipital tissues. It is known that head tissues are generated early during vertebrate embryogenesis whereas the rest of the axial structures are added subsequently from the posterior growth zone. The absence of active Cdx genes does not affect the generation of head and occipital tissues (the ‘extended head’), but it prevents trunk and tail tissues to be formed as a result of the depletion of axial progenitor populations from the growth zone. Examination of T expression in the Cdx null embryos confirms that early nascent mesoderm emerges normally but stops being generated after five somites have formed. The Cdx-dependence of axial elongation is confined to the post-occipital tissues.

These data, together with recent research in lower bilaterians, support the hypothesis that the role of Cdx genes in ‘post-head’ body extension is ancestral and exists in arthropods with short germ band development. Cdx/caudal must have been involved in
generating post-head axial structures since before protostomes and deuterostomes diverged from each other, as witnessed by the obligatory role of Cdx in generation of post-head tissues in the short-germ band beetle *Tribolium castaneum* (Copf et al., 2004) and the crustacean *Artemia franciscana* (Copf et al., 2004) and the intermediate-germ band cricket *Gryllus bimaculatus* (Shinmyo et al., 2005). Caudal is therefore an ancestral master organizer of post-head morphogenesis. Its role has been conserved in all animals that sequentially add their trunk and tail structures from a posterior growth zone, and has been reduced in the derived higher dipterans, such as *Drosophila melanogaster* (Olesnicky et al., 2006).

Although *Drosophila* Caudal does not directly regulate Hox genes, regulatory interactions are known to occur between mouse Cdx and Hox genes (Young et al., 2009). We show here that Cdx genes are clearly not required for the transcriptional activation of the early (3') genes of the Hox clusters. Transcriptional initiation of 3' Hox genes in the primitive streak is not affected by the absence of Cdx function, and 3' Hox expression in the hindbrain is intact, in agreement with the fact that anterior morphogenesis and signaling are normal in the mutants. In the absence of Cdx activity, no axial tissue emerges after occipital somites have been generated, and therefore the later and more 5' Hox genes are not expressed.

Our data in the mouse suggest that Fgf signaling works downstream of Cdx in driving post-occipital tissue emergence. Strikingly, in *Tribolium*, *Tc-Fgf8* is expressed in a region of the posterior growth zone involved in axial elongation, and the Fgf signaling pathway in this insect has been suggested to play a role in posterior mesoderm formation and expansion (Beermann et al., 2011; Beermann and Schroder, 2008). It could therefore be that both Cdx and its downstream Fgf signaling have been evolutionarily conserved in permitting post-head axial extension. The rescuing activity on axial growth of adding Fgf8 during culture of *Cdx* mutants suggests that Fgfr1 signaling is a main contributing intermediate between Cdx and posterior axial extension. The spectrum of posterior abnormalities of Cdx mutants matches well with the phenotype of *Fgfr1* mutants. The posterior notochord of *Cdx* null embryos is thickened, and so is the notochord in *Fgfr1* mutant embryos. Chimeric embryos generated with *Fgfr1* null embryonic stem cells form ectopic neural structures (Deng et al., 1997), and this defect was observed in partial Cdx mutants as well (van de Ven et al., 2011). *Fgfr1* mutants are posteriorly truncated and exhibit neural tube closure defects. The neural tube of the severely truncated *Fgfr1* null mutants described by Hoch and Soriano (Hoch and Soriano,
We acknowledge support from the Dutch Earth and Life Sciences (NWO ALW).

Funding

We thank E. J. Stringer (Leicester) for generating the Cdx2 conditional knockout mice.

Acknowledgements

We thank the following colleagues for probes: A. Mansouri (UnxC4.1), V. Papaloianou (Tbx6), D. Stott (Tbrachyury), A. McMahon (Shh and Wnt3a), Y. Saga (Mesp2), S-L Ang (Max1), R. Krumlauf (Hoxb1, Hoxb4 and Hoxb9), L. Jeanneotte (Hoxa5), M. Torres (Hoxa9), A. Naiche and M. Lewandoski (Spry4), P. Dolle (Raldh2 and Cyp26a1). We thank C. Viebahn for discussions.

Funding

We acknowledge support from the Dutch Earth and Life Sciences (NWO ALW). This work was also supported by a grant from the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM) [FES0908].

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.079848/-/DC1

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