Drm/Gremlin, a BMP antagonist, defines the interbud region during feather development

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ABSTRACT The pattern of feather buds in a tract is thought to result from the relative ratios between activator and inhibitor signals through a lateral inhibition process. We analyse the role of Drm/Gremlin, a BMPs antagonist expressed during feather pattern formation, in the dermal precursor, the dense dermis, the interbud dermis and in the posterior dermal condensation. We have altered the activity of Drm in embryonic chick skin using retroviral vectors expressing drm/gremlin and bmps. We show that expression of endogenous drm is under the control of a feedback loop induced by the BMP pathway, and that overexpression of drm results in fusion between adjacent feather buds. We propose that endogenous BMP proteins induce drm expression in the interbud dermis. In turn, the Drm/Gremlin protein limits the inhibitory effect of BMPs, allowing the adjacent row of feathers to form. Thus, the balance between BMPs and its antagonist Drm would regulate the size and spacing of the buds.

KEY WORDS: drm/gremlin, BMP, skin, feather bud

Introduction

The avian embryonic skin is a classical experimental model of pattern formation. During development, feathers are generated following a sequentially reiterated process at defined positions within the embryo, alternating feather bud and interbud domains arranged in highly ordered arrays. The first step in skin pattern induction is the formation of the dense dermis. In the dorsal skin, dermal cells migrate from the medio-dorsal lip of the somites under the ectoderm (Olivera-Martinez et al., 2000) and extend progressively from the median to the lateral dorsal region. The dermis of the limbs and of the ventral part of the body arises from the somatopleural mesoderm. In the limb, the dense dermis extends in a posterior to anterior direction. During dense dermis formation, no morphological differences between presumptive feathers and interbud regions are detectable. The dense dermis induces the formation of epidermal placodes, the first histological recognisable elements of the feather buds. In turn, the epidermal placode signals to the dermis and induces dermal cells to aggregate (Jiang et al., 1999) to form the dermal condensation which acquires its own inductive properties. Reciprocal interactions between ectoderm and dermis result in patterning and growth of the bud (reviewed in Sengel, 1976; Dhouailly, 1977; Chuong, 1998).

It was previously reported that many of the signalling molecules expressed in the feather bud can either activate the formation of feather primordia, (Shh, FGF, β-catenin, TGF, Delta-1), or inhibit it (BMP2, BMP4, Notch-2, EGF) (Ting-Berreth and Chuong, 1996; Widelitz et al., 1996; Crowe et al., 1998; Jung et al., 1998; Morgan et al., 1998; Viallet et al., 1998; Noramly et al., 1999; Atit et al., 2003). The feather bud initiation and growth could be regulated by the relative ratios between activators and inhibitors (Jung et al., 1998) and by lateral inhibition processes (Crowe et al., 1998; Noramly and Morgan, 1998; Viallet et al., 1998).

The BMPs are commonly considered as inhibitors of bud formation that contribute to the spacing between initiating buds as well as to the subsequent patterning of each individual feather (Jung et al., 1998; Noramly and Morgan, 1998). Accordingly, increasing the sensitivity to BMPs in dermal cells by overexpressing Bmp Receptors, in a feather reconstitution assay, decreases the size of feather bud (Jiang et al., 1999). Similarly, Bmp overexpression in the ectoderm of the feather field inhibits feather bud formation (Noramly and Morgan, 1998). Reciprocally, decreasing the sensitivity to BMPs by overexpression of the BMP

Abbreviations used in this paper: BMP, bone morphogenetic protein; CEF, chick embryo fibroblasts.

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antagonist *Noggin* increases the size of the feather bud at the expense of interbud region (Noramly and Morgan, 1998; Jiang et al., 1999). All these data suggest that BMPs play a crucial role in the lateral inhibition process between adjacent feather primordia. Paradoxically, *Bmp* are expressed in both the epidermal placode and the dermal condensation as they form (Noramly and Morgan, 1998) raising the question of how feather buds themselves escape the inhibitory actions of BMPs. It has been suggested that Follistatin, a BMP antagonist expressed within feather buds, could inhibit BMP signalling in the feather placode below a particular threshold and this would allow feather formation (Patel et al., 1999). Because Follistatin interacts with extracellular matrix components (Hashimoto et al., 1997), it has been postulated that BMPs would diffuse further than Follistatin and hence they would be able to act in interbud regions to inhibit feather bud formation. Accordingly, application of Follistatin to feather cultures induces feather bud formation in interbud regions (Patel et al., 1999).

We have identified another BMP antagonist, *drm* (Topol et al., 1997), also termed *gremlin* (Hsu et al., 1998), whose expression pattern in the developing skin differs significantly from that of follistatin. Drm is a secreted and cell-membrane associated glycoprotein (Topol et al., 2000), belonging to the Dan family of cystine knot secreted proteins (Hsu et al., 1998; Pearce et al., 1999; Piccolo et al., 1999). Drm binds BMP-2 and BMP-4 and controls outgrowth, chondrogenesis and apoptosis during chicken and mouse limb development (Capdevila et al., 1999; Merino et al., 1999; Zuniga et al., 1999). We and others have previously shown that *drm* displays a dynamic expression pattern during limb buds, branchial arches, dermatome and feather bud development (Merino et al., 1999; Bardot et al., 2001; Ohyama et al., 2001).

In this report we show that Drm/Gremlin is an activator of feather bud development, since overexpression of mouse *drm* results in fusions between adjacent feather buds. We also show that expression of endogenous *drm* is under the control of a feedback loop induced by the BMP pathway. Given that *drm* is expressed in dermis prior to feather bud induction and in interbud regions during buds formation, our results suggest that endogenous Drm can contribute to the size and spacing of the feather buds control by antagonizing the inhibitory effect of BMPs during feather development.

### Results

*Drm/gremlin is expressed in the dermis before feather bud formation*

We have previously reported that during early stages of chicken development *drm/gremlin* is expressed in the dermomyotome (Bardot et al., 2001). Analysis of expression pattern of *drm/gremlin* during feather development from the stage of ectodermal placode formation onwards.

**Fig. 1. Expression pattern of *drm* during skin and feather development.**

(A) 26HH embryonic stage, dorsal view. (B) Transverse section of (A) (indicated by double headed arrow). Arrowhead indicates that *drm* expressing cells are located in the lateral sub-ectodermal space. (C) 29HH embryonic stage, dorsal view. Drm is expressed in two broad dorsal stripes indicated by white arrowheads. (D) Transverse section of (C) (indicated by double headed arrow). Endogenous *drm* transcripts are detected in the dermis (d) and not in the epidermis (e). (E) 32HH embryonic stage, dorsal view; *drm* is expressed in interbud regions. (F,G) Section of (E), *drm* transcripts are detected in the interbud dermis (id) and not in the feather primordia formed by a placode (p) and a dermal condensation (dc). (H) Propidium iodide counterstain of F. (I) A 34HH wing, showing the three steps of *drm* expression: in the most anterior (youngest) buds, *drm* is expressed around the feather buds; in the last but one row, *drm* expression is lowered (black arrow), and in the most posterior row, *drm* is expressed in the posterior half of each feather bud. (J) Transverse section showing that during feather bud outgrowth, *drm* expression is restricted in posterior dermal cells of the bud. A-C and E, heads to the left. nt, neural tube; Pr-Di, proximo-distal axis; A-P, anteroposterior axis. Size bars, (A,C) 250 µm; (B,D) 30 µm; (E) 800 µm; (F-I) 12.5 µm.
Drm/Gremlin in feather development

(Ohyama et al., 2001) showed that drm is dynamically expressed during feather morphogenesis. Taken together, these data suggested a possible role of Drm in skin pattern formation.

We first analyzed the expression pattern of drm from earlier stages, before the onset of the dense dermis formation (26HH) onwards (36HH). We showed that at stage 26HH, drm is expressed in cells of the sub-ectodermal space (Fig. 1 A,B). At stage 29HH, prior to any morphologically detectable formation of the ectodermal placode, drm is expressed in two broad dorsal stripes (Fig. 1C). Transverse sections show a homogeneous distribution of drm transcripts in the dermis at this stage (Fig. 1D). From stages 30HH to 32HH, drm is expressed between feather primordia, and its expression is down regulated in the primordia (Fig. 1E). Transverse sections revealed that its expression is restricted to the dermis of interbud spaces outside of the boundary of the feather primordia (Fig. 1 F,G). At HH34, drm expression decreased around the more advanced dorsal rows of feather buds (Fig. 1H), whereas at later stages (36HH onwards), it is detected only in the posterior half the of the feather bud (Fig. 1I). Interestingly, it is never expressed in apteri.

Overexpression of drm/gremlin induces fusion of feather buds

To analyze the role of Drm in feather morphogenesis, we studied the effect of its overexpression on the formation of feather buds. We therefore grafted aggregates of chicken fibroblasts infected with an RCAS retrovirus expressing the mouse drm, between the neural tube and the somites of E2 chicken embryos. From stage 31 to 36HH two major but not mutually exclusive modifications of feather phenotype are observed in dorsal areas expressing mouse drm. In some cases (n=12), several feather buds are larger than normal (Fig. 2 F,G arrowheads). But in most cases (n=26), fusion of adjacent feather buds is observed (Fig. 2F arrows). Finally, at stage 38HH, the fused buds generated feathers fused all over, except at the most distal tip (Fig. 2H), whereas the enlarged buds generated feathers resembling baseball bats (Fig. 2I).

To correlate the observed phenotypes with the expression of mdrm, grafted embryos were fixed at different stages and analyzed by whole mount in situ hybridization. Transverse sections of the embryos at the grafted region showed the progression of the transgene expression before and during the onset of feather bud formation. At stage 22HH, mdrm transcripts are co-detected with c-hairy1 in the mediodorsal lip of the dermomyotome (Fig. 2A). At stages 22HH to 24HH, they rapidly spread (Fig. 2B) and then occupy the whole dermis (Fig. 2C). At stage 34HH, after the primordia formation, transcripts extend to the epidermal cells.
during the feather bud outgrowth (Fig. 2 D,E). Surprisingly, at later stages (HH36) (Fig. 2F), the transgene is mainly expressed in the developing feathers. It is only slightly detectable in the deep inter-feather dermis. At this stage, its expression extends over a large part of the dorsal region. Interestingly, not all the feathers within the infected area display a phenotype and no ectopic feathers were observed within normally apertic areas (Fig. 2F). Moreover, drm misexpression has no noticeable effect on the antero-posterior orientation of the feathers. This is shown by the fact that in embryos overexpressing drm, normal, enlarged and even fused feathers exhibit a normal antero-posterior orientation at all stages examined. Grafts of fibroblasts infected with empty vector (n=16) never induced abnormal feathers formation or disorganization of feather tracts.

The histological analysis showed that enlarged fused feathers include a larger dermal condensation than the normal feather bud, while the dermal cell density is comparable to the normal feather (Fig. 2 L-O). Interestingly normal barb ridges form from the epidermis of the fused feather buds (Fig. 2 N,O). These observations suggest that drm overexpression induced both epidermis and dermis to adopt a feather fate within an area normally endowed with an interfeather fate.

In order to determine whether phenotypes induced by drm overexpression resulted from a specific dermatome perturbation, or occurred during feather primordia formation, we grafted RCAS/mdrm-infected fibroblasts in the posterior part of the wing buds of stage 21HH embryos and the phenotypes were analyzed 6 days later. In this case, although feather bud fusions are observed, in the majority of embryos they occurred between primordia of the same row (Fig. 3 B,D,F,H). In some cases, fusions between adjacent primordia of different rows as in the dorsal pterilae are also observed (Fig. 3 J). These results clearly showed that the overexpression of drm affects mechanisms involved in feather primordia formation rather than development of the dermatome.

Effect of drm/gremlin overexpression on the expression of feather bud markers

The phenotypes induced by drm overexpression could be either the cause or the consequence of transcriptional modifications of genes expressed during feather bud development. To test this possibility we analyzed the expression pattern of several genes at stage 34HH in wings grafted with mdrm-expressing fibroblasts. The presence of fused feather primordia and digit malformations in the grafted wings were positive controls for efficient expression of the transgene (Fig. 3 B,D,F,H and J). The opposite non-grafted wing was used as a control for the normal gene expression pattern (Fig. 3 A,C,E,G, and I). We show that the expression of Bmp2, Bmp4, shh and delta1, that are normally restricted within the primordia are ectopically induced in the fused feathers in the grafted wings, and that notch2 normally expressed in the interbud dermis, is down regulated in these fused feathers (Fig. 3 B,D,F,J and H respectively). However, no modification of the expression of these genes is observed in the normal feathers within the infected wings. Thus, the distribution of the Bmp2, Bmp4, shh, delta1 and Notch2 transcripts in the dermis and the epidermis of the fused or enlarged primordia, is simply the consequence of the phenotype induced and does not reflect specific up or down regulation of these genes by mdrm. Identical results were obtained in the dorsal pteryla of embryos grafted between neural tube and somites (data not shown). These data suggest that feather phenotype induced by mdrm overexpression was the consequence of the antagonistic effect of Drm on BMP signaling during feather primordia formation rather than of a direct effect on the expression of feather bud markers.
Expression of endogenous drm/gremlin in feather buds is under the control of BMP signaling

The observation that in feather buds, the expression pattern of drm is always complementary to that of Bmp2 and Bmp4, suggests that BMP signaling could regulate endogenous expression of drm. We therefore analyzed the expression pattern of endogenous drm following RCAS-driven Bmp4 misexpression in developing skin. It has been previously reported that overexpression of Bmp2 and Bmp4 induced large apterous areas in chicken skin and that this phenotype resulted from the inhibition of feather bud development at a stage after dense dermis formation but prior to placode specification (Noramly and Morgan, 1998). We grafted RCAS/Bmp4-infected fibroblasts into E2 embryos (HH 11-15) in the space between neural tube and somite at the trunk level, and analyzed the skin morphology and endogenous drm expression at E8 (HH 34). All of the grafted embryos (n=11) display the glabrous phenotype previously described. At the level of the graft, Bmp4 overexpression mainly induced large glabrous areas that extend over a large part of the dorsal skin (Fig. 4 A,B). Small apterous areas located either at the level of the graft (Fig. 4 C,D), or distantly from it are also observed, the later probably resulting from secondary infection events, since Bmp4 transcripts are expressed independently of the main infection area (data not shown).

Expression pattern of endogenous drm within and around glabrous fields was also affected. Within large areas devoid of feather buds, a low expression of endogenous drm is detected, whereas drm expression is strongly enhanced at the periphery of these apterous areas (Fig. 4 A,B). On the contrary, within small apterous areas, expression of endogenous drm is activated (Fig. 4 C,D). Identical apterous phenotypes and modifications of the endogenous drm expression were obtained following overexpression of a constitutively activated form of the BMPR1A (Fig. 4 E,F). Interestingly, in some cases we observed the appearance of fused feather buds at the periphery of large apterous areas induced by BMPR1A or Bmp4 overexpression (see black arrowhead Fig. 4E and data not shown). All these results suggest that in developing skin, the expression of drm should be under the control of BMP signaling.

Discussion

The dynamic expression pattern of drm/gremlin, together with the effects of its misexpression raises several hypotheses about its roles during avian skin morphogenesis. The experimental data presented above suggest that Drm is a dermal factor involved in feather tract patterning, primordia formation and bud morphogenesis.

Drm/gremlin expression in dermal cells is complementary to that of BMPs

The drm/gremlin gene is expressed in dermal cells from early stages of dermal precursor formation in the somite (Bardot et al., 2001; and this work) to dense dermis and feather pattern formation. This observation suggests that drm/gremlin is one of the earliest genes expressed in the dermis along with the bHLH transcription factor Dermo-1 (Scaal et al., 2002). However, after feather placode formation Dermo-1 is principally expressed in the dermal condensations, whereas the expression of drm is restricted to the interbud dermis. At later stages, drm is expressed by dermal cells recruited in the posterior part of the forming feather. It is noteworthy that at all stages, drm/gremlin expression field is complementary to those of bmp-2 and bmp-4 previously described (Jung et al., 1998; Noramly and Morgan, 1998). Bmp-2 is first expressed in a large stripe of ectoderm that covers the dense dermis and this expression is progressively restricted to the ectodermal placode when the feather primordia form. Both bmp-2 and bmp-4 are expressed in the nascent dermal condensation. During feather bud outgrowth and orientation bmp expression is restricted to the anterior region of the bud in both the ectoderm and the dermis. As Drm/Gremlin binds BMP-2 and BMP-4, two signaling molecules
known to inhibit feather commitment at successive stages in feather tract development, the exclusive patterns of drm and bmps suggest reciprocal roles for these molecules.

**BMPs regulate drm/gremlin expression**

It was recently shown that expression of drm is under the control of BMP signaling in osteoblasts (Pereira et al., 2000) as well as into chick limb buds. In this case, BMP-soaked beads decreased drm expression in a region close to the BMP source, whereas upregulation occurred at a distance (Capdevila et al., 1999; Merino et al., 1999).

The BMPs overexpression elicits different effects according to the skin developmental stage: at early stages (17-23 HH) implantation of beads soaked with BMPs can induce ectopic feather formation in a particular limited region of the lateral trunk (Scaal et al., 2002) while all over and at later stage (29 HH) BMPs inhibit feather formation (Jung et al., 1998; Noramly and Morgan, 1998). By contrast, the overexpression of either BMP4 or of constitutively activated form of BMPR1A leads to the formation of larges and secondary small apteric regions. The drm expression is enhanced at the periphery of the large apteric areas and in the small glabrous regions. This effect of bmp4 overexpression on endogenous drm in the BMP-induced apteric regions suggests that, in developing skin, expression of drm is under the control of BMP signaling.

However, the positive regulation model of drm expression by the BMP signaling cannot explain the low expression level of drm within the large BMP-induced apteric areas. We can speculate that in skin expression of drm could be regulated by a threshold effect of BMP on BMP signaling as it has been proposed during early limb bud development (Capdevila et al., 1999; Merino et al., 1999). Alternatively, expression of drm could be under the control of additional positive regulatory signals that activate or maintain drm expression at the periphery of apteric regions. These signals could be provided from surrounding feather buds that are located too far to activate drm expression in the center of large glabrous areas. It is noteworthy than during limb bud development Shh and FGF pathways are required for maintenance of drm/gremlin expression (Merino et al., 1999; Zuniga et al., 1999).

**Drm/gremlin regulates feather primordia formation**

The overexpression of drm leads to the formation of enlarged and fused primordia suggesting that Drm is a crucial and limiting factor in the early steps of feather induction. The progression in time of the infection is important in order to interpret the drm phenotype. The graft of RCAS mdrm-producing cells between the neural tube and the somites leads to a strong expression of mdrm at early stage (HH24) that remains restricted to the dermal cells until feather bud outgrowth. Interestingly, overexpression of mdrm at early stage did not modify the dense dermis formation. These results suggest that, in contrast to bHLH transcription factor Dermo-1 (Scaal et al., 2002) and the cell-cell adhesion molecule NCAM (Jiang et al., 1999) both recently showed to be involved in this process, the homogeneous expression of endogenous mdrm at this stage is not the limiting factor for dense dermis extension. Later, drm/gremlin overexpression led to the fusion of laterally adjacent feather buds. This phenotype seems to be the consequence of the homogenous expression of mdrm the dermis at the time of primordia formation, rather than of the ectopic expression of mdrm in the epidermis during later feather bud outgrowth. This is supported by the fact that the progression of the infection from the dermis to the ectoderm occurred after the modification of expression of early markers and the formation of fused primordia. Moreover, the expression of the feather bud markers (Shh, Bmps and Delta1) was enlarged in fused primordia but no modification of expression was observed in regions outside of the feather field. This suggests that the feather phenotype induced by drm overexpression is the consequence of the antagonistic effect of Drm on BMP signaling rather than an indirect effect of Drm on the expression of these signaling molecules. Interestingly in embryos infected with BMP-4 and BMPR1α expressing retroviruses, large apteric areas are surrounded in some cases by some fused feather buds. This phenotype is always associated with an enhancement of endogenous drm/gremlin expression at the periphery of the large apteric areas. This phenotype could result from the up-regulation of expression of endogenous drm in these regions. On the contrary, within small apteric areas, though expression of endogenous drm is activated, the skin is always glabrous. This indicates that in these cases, enhancement of endogenous drm expression could not overcome the interbud fate induced by activation of BMP signalling.

Because of the sequence of feather formation in the tract, the bud fuses with the “younger” of equidistant primordia. The pattern of fusion suggests that the interbud region becomes resistant to primordia formation in a brief period of time between the formation of adjacent buds. During a short time window in bud initiation, drm expressed in the interbud spaces could regulate the size of feather buds and/or the size of interbud regions by interacting with BMPs and inhibiting their signaling, thus preventing early commitment of cells to an interbud fate. Overexpression of drm in the dermis prior this short period of time would allow both epidermis and dermis to adopt a feather fate within an area normally endowed with an interfeather fate by antagonizing the BMPs. Finally, it was recently reported that at early stage of feather morphogenesis, EGF signaling is required for the determination of interbud fate (Atit et al., 2003). The inhibition of EGFR signaling in skin explants led to the induction of phenotypes similar to those induced by misexpression of mdrm, namely to the loss of interbud fate and the feather bud fusion. Further experiments should clarify the links between EGF and drm in skin.

**Feather bud formation and BMP antagonists**

Both drm/gremlin and follistatin are the only BMP antagonists currently known to be endogenously expressed during feather morphogenesis. It has been previously demonstrated that expression of follistatin is also dependent on BMP activity (Patel et al., 1999). During feather bud development, follistatin is coexpressed with bmps (Patel et al., 1999) and FGFs, whereas bmps exhibits an expression pattern complementary to that of bmps. This suggests that their expression could be regulated by different levels of BMP activity. Follistatin overexpression in the epidermis at the formation of ectopic feathers (Patel et al., 1999). We never observed ectopic formation of primordia after drm overexpression. Taken together these results suggest that these proteins have different functions during feather development. Follistatin could inhibit BMPs activity within the primordia, and by promoting FGF activity, controls feather bud outgrowth. We propose that BMPs induce drm expression in the dermis around
the feather bud. In turn, Drm protein could regulate the size of interbud region by limiting the concentration of available BMPs. This inhibition could allow feather bud induction at a defined distance from the preceding row, by preventing early commitment of the dermal cells to form an apertic region.

Material and Methods

Preparation and grafting of retrovirus-producing CEF

Primary chick embryo fibroblasts (CEF) prepared from 11 days old O-line embryos (BBSRC; Institute for Animal Health, Compton, Berchshire, UK) were transfected with RCAS (BP)/mdrm, RCAS/mBmp4, RCAS/hBmpR1A constructs or with RCAS (BP) plasmid using the "Effectene transfection reagent" (Qiagen). The day before grafting, infected cells were plated at high density on bacterial dishes to induce formation of cell aggregates. Those of approximately 100 μm were grafted into Brown Leghorn chicken embryos of the Edinburgh strain, either at 11 to 15 Hamburger-Hamilton (HH) developmental stages (Hamburger and Hamilton, 1951), between neural tube and somites at the level of somites 10 to 29, or in the anterior edge of the right forelimb at stages 21HH. After further development occurred, embryos were fixed in 4% formaldehyde 2 mM EGTA and analysed by whole-mount in situ hybridization.

Whole-mount in situ hybridization

The hybridization was performed as described (Henrique et al., 1995). The proteinase K treatment varied from 10 to 20 μg/ml for one hour, depending on the developmental stage of the embryo. Simultaneous detection of endogenous and retroviral transcripts using two colours in situ hybridization was achieved as previously described (Delfini et al., 2000). Reagents were purchased from Roche. The stained embryos were processed for cryosections following inclusion in gelatin/sucrose.

Plasmids

RCAS/mdrm and pBKS/mdrm constructs were obtained by inserting a 1202bp Xba I fragment containing the coding region and 5’ and 3’ untranslated sequences of mouse drm1cDNA (Zhang et al., 2000) into the Cla I and Xba I sites of RCAS and Bluescript (Stratagene) vectors respectively. mdrm cDNA was kindly provided by D. Blair. RCAS/mBmp4 was kindly provided by D. Duprez. RCAS/hBMPR1A was a gift of L. Niswander. The constructs used to generate anti-sense riboprobes pBKS/cBMP2 and pBKS/cBMP4 were kindly provided by A.H. Monsoro-Burg, pBKS/c-hairy1 by I. Palmeirim, pBKS/cDelta1 by D. Henrique, pBKS/cNotch2 by R. Goitsuka, pBKS/cShh (Riddle et al., 1993) was generated by RT-PCR and pCR2.1/cdmr was previously described (Bardot et al., 2001).

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