Early Function of Pax5 (BSAP) before the Pre-B Cell Receptor Stage of B Lymphopoiesis

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Summary

The formation of the pre-B cell receptor (BCR) corresponds to an important checkpoint in B cell development that selects pro-B (pre-BI) cells expressing a functionally rearranged immunoglobulin heavy chain (IgH) to undergo the transition to the pre-B (pre-BII) cell stage. The pre-BCR contains, in addition to IgH, the surrogate light chains λ5 and VpreB and the signal transducing proteins Igα and Igβ. The absence of one of these pre-BCR components is known to arrest B cell development at the pre-BI cell stage. Disruption of the Pax5 gene, which codes for the B cell–specific activator protein (BSAP), also blocks adult B lymphopoiesis at the pre-BI cell stage. Moreover, expression of the mb-1 (Igα) gene and VH-to-DH-JH recombination at the IgH locus are reduced in Pax5-deficient B lymphocytes ~10- and ~50-fold, respectively. Here we demonstrate that complementation of these deficiencies in pre-BCR components by expression of functionally rearranged IgH and chimeric IgM-IgL transgenes fails to advance B cell development to the pre-BII cell stage in Pax5RAG2(−/−) mice in contrast to RAG2(−/−) mice. Furthermore, the pre-BCR is stably expressed on cultured pre-BI cells from IgM transgenic, Pax5-deficient bone marrow, but is unable to elicit its normal signaling responses. In addition, the early developmental block is unlikely to be caused by the absence of a survival signal, as it could not be rescued by expression of a bcl2 transgene in Pax5-deficient pre-BI cells. Together, these data demonstrate that the absence of Pax5 arrests adult B lymphopoiesis at an early developmental stage that is unresponsive to pre-BCR signaling.

Key words: B cell–specific activator protein • Pax5 • pro-B cell development • pre-B cell receptor • IgM transgene

An important checkpoint in B cell development controls the transition from the pro-B (pre-BI) to the pre-B (pre-BII) cell stage that is initiated upon completion of a productive rearrangement at the immunoglobulin heavy chain (IgH) locus. A consequence of expressing the membrane-bound Igμ protein is the transient formation of the pre-B cell receptor (BCR). Signaling initiated by this receptor promotes allelic exclusion at the IgH locus, stimulates proliferative cell expansion, and induces differentiation to small pre-BII cells undergoing Ig light chain gene rearrangements (for review see reference 1). In addition to the Igμ protein, the pre-BCR consists of the two nonpolyomorphic, surrogate light chains λ5 and VpreB, as well as the signal transducing proteins Igα and Igβ whose expression is initiated early in B lymphopoiesis (for review see reference 2). B cell development is arrested at the pro-B (pre-BI) cell stage in mice that lack one component of either the pre-BCR (mlgμ, reference 3), λ5, Igα, and Igβ, (reference 4), and Igβ (reference 5) or of the V(D)J recombination machinery (RAG1, reference 6, RAG2, reference 7). DNA-dependent protein kinase (DNA-PK; reference 8). However, expression of a functionally rearranged Igμ transgene is able to complement the recombination defects of both severe combined immunodeficiency (scid) and RAG mutant mice, thus resulting in pre-BCR formation and subsequent progression to the small pre-BII cell stage (9-11). The early expression of a rearranged Igμ transgene significantly shortens the duration of pro-B cell development by directly inducing differentiation to small pre-BII cells (12). Likewise, expression of a functionally rearranged κ light chain gene is capable of activating the pre-B cell transition in λ5-deficient mice (13, 14).
The Igα and Igβ proteins form a disulfide-linked heterodimer that is associated through its transmembrane domain with the Ig molecule in the pre-BCR and BCR. This heterodimer is not only essential for surface transport of Igα, but also constitutes the signal transducing unit of these receptors (for review see references 2, 15). The Igα and Igβ proteins both initiate signaling via immunoreceptor tyrosine-based activation motifs (ITAMs), which become phosphorylated upon receptor engagement and recruit intracellular effectors such as protein-tyrosine kinases to the receptor (2, 15). Apart from these motifs, the cytoplasmic tails of Igα and Igβ differ considerably in sequence, but yet appear to fulfill redundant functions in B cell development. Chimeric receptors, consisting of the Igα protein fused to the cytoplasmic domain of either the Igα or Igβ protein, are each independently sufficient to induce the pre-B cell transition (16, 17) and to signal B cell maturation (18) in transgenic mice.

Insight into the transcriptional control of early B cell development has recently been gained by gene targeting in the mouse. One of the critical transcription factors thus implicated in early B lymphopoiesis is the B cell–specific activator protein (BSAP), which is encoded by the Pax5 gene (for review see references 19, 20). Pax5 is expressed from the earliest B lineage–committed precursor cell up to the mature B cell stage (21–23), and, consistent with this expression pattern, is essential for B lineage commitment in the fetal liver (24). How,

Materials and Methods

Mice. The different mouse strains were maintained on the hybrid C56BL/6 × 129/Sv background. The genotype of Pax5 mutant mice (25) was determined by PCR analysis as previously described (24). RAG2 mutant mice (7) were genotyped by PCR amplification with the following oligonucleotides 5’–GCAACA-TGGTTATCCAGTGCCGT-3’ (primer 1), 5’–TTGGGAG-GACACTATTCTTGCCCAGT-3’ (primer 2), and 5’–GTATG-CAGGCCGCGACTTCTGATCA-3’ (primer 3). A 605-bp PCR product was amplified from the wild-type RAG2 allele with primer pair 1 and 2 and a 1-kb DNA fragment from the mutant RAG2 allele with the pair 1 and 3. For simplicity, the mouse-human hybrid transgene mIgα-Igβ (Y.S.V.V.; references 16, 27) is referred to as Igα-Igβ in this manuscript and the functionally rearranged Igα transgene of the line M54 (28) as Igα. The presence of the Igα transgene expressing the membrane form of the μ heavy chain was detected by Southern blot analysis with radiolabeled pBR 322 DNA as previously described (28). The Igα-Igβ transgene was identified by PCR amplification with the primers 5’–GCCTTTGAGACCCTGTGGGC–3’ and 5’–CCTCATTTCTGGCCCTGG-3’ (100-bp PCR product). The transgenic mouse strain Eµ-bd-2-36 (29), which expresses a human bd-2 CDNA under the control of the SV 40 promoter and Igα Eµ enhancer in B and T lymphocytes (30), was genotyped by PCR using the primers 5’–GCAGACACTCTATGCCTGTGG–3’ and 5’–GGACCTTATCTTCTGTTGTA-3’ (504-bp PCR product).

Pre-B Cell Cultures. Cell suspensions prepared from mouse bone marrow or fetal liver (at embryonic day 16.5 or 17.5) were plated at limiting dilutions on a semiconfluent layer of γ-irradiated stromal ST2 cells in the presence of IL-7–containing medium as previously described (24). After 1 wk of in vitro culture, individual pre-B cell colonies were collected and further propagated as a cell pool. The long-term proliferation potential of these pre-B cell pools was analyzed for at least 1 mo.

Antibodies and Flow Cytometry. The following mAbs were purified from hybridoma supernatants on protein G–sepharose columns (Pharmacia Biotech AB, Uppsala, Sweden) and conjugated with sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) as recommended by the suppliers: anti-c-kit mAb (ACK4; reference 31), anti-μ mAb (M41.42; reference 32), anti-κ mAb (LM34; reference 33), and anti-pre-BCR mAb (SL156; reference 34). In brief, bone marrow cells were incubated with the following mAbs: PE-coupled anti-B220 (RA3-6B2) and allophycocyanin (APC)-labeled anti-CD43 mAb (S7), biotinylated anti-CD2 mAb (RM2-33), and 5-fluorodeoxyuridine (FACScan® flow cytometer (Becton Dickinson, San Jose, CA) as described (35). In brief, bone marrow cells were incubated with PE-conjugated anti-B220 (RA3-6B2) and biotinylated anti-CD43 mAb (S7), biotinylated anti-CD2 mAb (RM2-35), FITC–anti-CD25 mAb (7D4), biotinylated anti-CD43 mAb (S7), biotinylated anti-CD2 mAb (RM2-5), FITC–conjugated anti-μ mAb (R3-60.2), APC-conjugated anti-c-kit mAb (ACK45), purified anti-human Bcl-2 mAb (Bcl-2/100), and PE-conjugated streptavidin.

8-11-d-old mice were used for flow cytometric analysis, as older Pax5 mutant mice suffer from disease and generally die within 3 wk (25). Cultured pre-B cell or single-cell suspensions prepared from the bone marrow of these mice were stained with different antibody combinations and subsequently analyzed on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA) as previously described (25).

Intrafollicular Antibody Staining. The cytoplasmic μ heavy chain protein was detected in bone marrow pre-BI cells as previously described (34). In brief, bone marrow cells were incubated with PE-conjugated anti-B220 (RA3-6B2) and allophycocyanin (APC)-conjugated anti-c-kit (ACK45) antibodies at 4°C, washed twice with PBS, and then fixed with 2% paraformaldehyde (Fluka AG, Buchs, Switzerland) in PBS at room temperature for 20 min, followed by two washes with PBS. The fixed cells were subsequently permeabilized with 0.5% saponin (Sigma Chemical Co., St. Louis, MO) in 2% FCS/PBS and were simultaneously stained with

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FITC-conjugated anti-μ antibody (R 6-60.2) for 40 min at 4°C, then washed twice in saponin buffer and once in 2% FCS/PBS before analysis on a FACSVantage™ TSI flow cytometer (Becton Dickinson). Cultured bd-2 transgenic, Pax5 (--/-) pre-BI cells were analyzed for expression of the human Bcl-2 protein by cytoplasmic staining with an anti-human Bcl-2 mAb (Bcl-2/100; detected with a PE-coupled goat anti–mouse IgG antibody) as described above.

Western Blot Analysis. Whole cell extracts of in vitro cultured pre-BI cells were prepared by lysis in 0.25 M Tris, pH 7.5, and 0.1% Triton X-100, followed by removal of insoluble material by centrifugation. Total protein (10 μg) was separated by 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then incubated with a rabbit polyclonal anti-IgG antiserum (27) (diluted at 1:1,000). Anti-IgG antibodies were detected by enhanced chemiluminescence using a horseradish peroxidase-conjugated donkey anti–rabbit secondary antibody (ECL; Amersham International, Arlington Heights, IL).

RNAse Protection Analysis. A mouse terminal deoxynucleotidyl transferase (TdT) riboprobe was generated by inserting a 244-bp cDNA fragment of the mouse Tdt mRNA (35) into the HindIII and EcoRI sites of pSP64. This cDNA fragment was amplified from RNA of 70Z/3 cells by reverse transcriptase PCR using the following primers 5’-GCCGACAGTCAAGGTCGACTCT-3’ and 5’-GCAGAGCTCTGTTGTTCTAGCAT-CATCT-3’. Total RNA was prepared from cultured pre-BI cells and analyzed by RNAse protection assay exactly as previously described (24).

Results

Pax5 (BSAP) Is Essential for Early B Cell Development before the Pre-BCR Stage. Based on the expression of cell surface markers, we have recently demonstrated that B cell development is arrested in the bone marrow of Pax5 mutant mice (24) at a similar pro-B (pre-BI) cell stage as in Pax5-deficient mice (24). Total RNA was prepared from cultured pre-BI cells and analyzed by RNAse protection assay exactly as previously described (24).

The mb-1 gene coding for Igα was recently shown to be a direct BSAP (Pax5) target whose expression is reduced ~10-fold in Pax5-deficient pre-BI cells compared with wild-type cells (26). In addition to the Igα protein, Igα is therefore a second component of the pre-BCR that is expressed under the control of Pax5. As the heterodimer consisting of the proteins Igα and Igβ constitutes the signal transducing unit of the pre-BCR (2), it is conceivable that the reduced Igα expression in Pax5-deficient pre-BI cells prevents the formation of a functional pre-BCR even in the presence of a rearranged Igα transgene. To address this question, we have introduced a chimeric Igα-Igβ transgene (16) into the Pax5 (--/--) background. The Igα component of this transgene codes for a membrane-bound Ig with two transmembrane modules (Y 587V, S 588V) which prevent its normal association with the IgH locus (16). Hence, signaling of this chimeric receptor should be independent of the reduced expression levels of both Igα and Igβ proteins that are observed in Pax5-deficient mice. Nevertheless, the chimeric Igα-Igβ gene was unable to advance B cell development in the bone marrow of Pax5-...
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mutant mice, since its presence neither altered the expression of cell surface markers nor increased the number of $B220^+$ cells (Fig. 3 A). However, the Ig$\mu$-Ig$\beta$ fusion protein was expressed in pre-BI cells regardless of the Pax5 genotype (Fig. 3 B). Together, these in vivo data indicate that expression of the pre-BCR is not sufficient to rescue the early B cell developmental block in Pax5-deficient mice. Hence, the Pax5 mutation appears to arrest B lymphopoiesis at an early stage that is not responsive to pre-BCR signaling.

The survival of B cell precursors is controlled by differential expression of the antiapoptotic genes $bcl-2$ and $bcl-xL$ during B lymphopoiesis (38, 39). Interestingly, the $bcl-xL$ but not the $bcl-2$ gene is consistently expressed at a 10-fold lower level in Pax5-deficient pre-BI cells compared with wild-type cells, although this downregulation was shown to be an indirect consequence of the absence of Pax5 (26). In agreement with this finding, the pre-BI cells of Pax5 mutant bone marrow proved to be ultra-sensitive to growth factor withdrawal, as they rapidly undergo apoptosis even in the absence of survival signals emanating from the IL-7 receptor (data not shown). In this context it is interesting to note that the expression of a $bcl-2$ transgene was previously shown to promote B cell development in $scid$ mice (40) that also exhibit a defect in V(D)J recombination of Ig genes (for review see reference 41). Hence, we investigated the possibility that sustained cell survival may also rescue the early developmental block in Pax5-deficient bone marrow. For this purpose, the same $E\mu$-bd-2-36 transgenic mouse, carrying a human $bcl-2$ cDNA under the control of the $Igh$ enhancer (29), was crossed with Pax5 mutant mice. Expression of the $bcl-2$ transgene in Pax5 (--/-) pre-BI cells was demonstrated by cytoplasmic staining with an anti-human Bcl-2 antibody as well as by its ability to completely block apoptosis upon IL-7 withdrawal (data not shown). Nevertheless, the $bcl-2$ transgene was unable to advance B cell development to the pre-BII cell stage, as no $CD43^+ \cdot B220^+$ lymphocytes were observed in the bone marrow of $bcl-2$ transgenic, Pax5 (--/-)
Stable expression of the pre-BCR and absence of its normal signaling response in Ig \( \mu \) transgenic, Pax5-deficient pre-BI cells. Expression of a functionally rearranged Ig \( \mu \) chain has previously been shown to alter the IL-7 responsiveness of precursor B cells in wild-type and RAG2 mutant mice (10). The proliferative response to IL-7 was considerably decreased in bone marrow cells of Ig \( \mu \) transgenic mice, thus preventing the establishment of long-term pre-BI cell cultures (10). One possible reason for this phenomenon may be the downregulation of c-kit expression in response to pre-BCR activation (33, 37), which eliminates an essential costimulatory signal for IL-7-dependent proliferation of B lymphoid precursor cells (43). To further study the function of the pre-BCR, we have established pre-BI cell cultures from bone marrow of Pax5-deficient mice carrying an Ig \( \mu \) transgenic mouse. These pre-BI cells were cultured in the presence of stromal ST2 cells and IL-7, and their long-term proliferation potential was assessed after 1 mo of in vitro culture. Surprisingly, Pax5-deficient pre-BI cells could be efficiently established and maintained even in the presence of transgenic Ig \( \mu \) or chimeric Ig \( \mu \)-Ig \( \beta \) proteins (Table 1). In contrast, no pre-BI cell cultures with long-term proliferation capacity were obtained from homozygous or heterozygous RAG2 mutant mice carrying an Ig \( \mu \) transgene, as previously described (10). Thus, these data indicate that expression of the Ig \( \mu \) protein does not interfere with the proliferation potential of Pax5-deficient pre-BI cells in contrast to control B lymphocytes.

Given the possibility to grow Ig \( \mu \) transgenic, Pax5 \((--/-)\) precursor cells, we next investigated whether these cells could assemble the pre-BCR on their surface. As shown by flow cytometric analysis, Pax5-deficient pre-BI cells containing or lacking the Ig \( \mu \) transgene expressed a similar level of the surrogate light chain \( x5 \) on their surface (Fig. 4 A). In contrast, the Ig \( \mu \) protein was only found on the transgenic pre-BI cells. Furthermore, staining with a monoclonal antibody (SL156), which recognizes a conformational epitope present on the surrogate light chain-Ig \( \mu \) complex of the pre-BCR (33), demonstrated that the Ig \( \mu \) protein was part of the pre-BCR (Fig. 4 A). Three conclusions can be drawn from these data. First, the pre-BCR is stably expressed on the surface of Ig \( \mu \) transgenic, Pax5 \((--/-)\) pre-BI cells despite the fact that the pre-BCR is only transiently expressed and rapidly internalized on wild-type precursor B cells (33, 44, 45). Second, the surrogate light chains are expressed at normal levels on transgenic, Pax5 \((--/-)\) pre-BI cells, although their expression is usually downregulated in response to pre-BCR signaling (9, 10, 37, 38, 44). Third, the Ig \( \alpha \) protein is known to be essential for cell surface transport of Ig \( \alpha \) (46, 47), and yet the 10-fold lower mb-1 expression in Pax5-deficient pre-BI cells (26) seems to provide sufficient Ig \( \alpha \) protein for pre-BCR formation.
The expression of the TdT gene is rapidly downregulated during the pre-B cell transition in response to expression of a functionally rearranged Ig\textsubscript{m} protein (37, 38, 48). The TdT gene is therefore considered to be a downstream target in the signaling cascade initiated by the pre-BCR (48). As shown by RNase protection analysis, the level of TdT transcripts was similar in Pax5-deficient pre-B\textsubscript{I} cells regardless of the presence of the Ig\textsubscript{m} transgene (Fig. 4B, lanes 2 and 3). In summary, the different results obtained with cultured pre-B\textsubscript{I} cells all demonstrate that the pre-BCR is unable to elicit its normal signaling response in the absence of Pax5 function.

**Discussion**

The transcription factor Pax5 (BSAP) is involved in the control of V\textsubscript{H}-to-D\textsubscript{J\textsubscript{H}} recombination and in the transcriptional regulation of the mb-1 gene, which results in reduced expression of the two pre-BCR components, Ig\textsubscript{m} and Ig\textsubscript{a}, in Pax5-deficient pre-B\textsubscript{I} cells (24, 26). Here we have demonstrated that complementation of these deficiencies by the expression of Ig\textsubscript{m} and Ig\textsubscript{m}-Ig\textsubscript{b} transgenes is not sufficient to initiate the pre-B cell transition in Pax5 mutant mice. Hence, the inability to form a pre-BCR cannot be the cause of the early B cell developmental block in mice lacking Pax5. Instead, the absence of Pax5 arrests B cell development.
development by a different mechanism compared with mice which lack a component of the pre-BCR (mlgμ [reference 3], λ5 [reference 4], or lgb [reference 5]) or of the V(D)J recombination machinery (RAG1 [reference 6], RAG2 [reference 7], or DNA-PK [reference 8]). Consistent with this conclusion, the lack of Pax5 or RAG2 function has opposite effects on the in vitro differentiation potential of B lymphocytes. Pre-BI cells of RAG2 mutant mice efficiently differentiate ex vivo to the mature B cell stage upon stimulation with IL-4 and anti-CD40 antibodies, which bypasses in vitro the requirement of Ig gene rearrangements for further development (49). In contrast, Pax5 mutant pre-BI cells entirely fail to differentiate under the same in vitro conditions, further demonstrating a strict dependency of early B lymphopoiesis on Pax5 (Nutt, S.L., unpublished data).

It has been notoriously difficult to demonstrate expression of the pre-BCR on the surface of precursor B cells (33, 44), which reflects both a slow, inefficient cell surface transport and rapid, tyrosine phosphorylation-dependent internalization of this receptor (2, 45). Quite in contrast, we have now observed stable expression of the pre-BCR on the surface of Igμ transgenic, Pax5-deficient pre-BI cells. Interestingly, the constitutive cell surface expression of the pre-BCR correlates with the absence of normal signaling responses. The transgenic, Pax5-deficient pre-BI cells neither lost their long-term proliferation potential in the presence of IL-7 and stromal cells nor did they downregulate expression of the TdT or surrogate light chain genes, which are normal responses to pre-BCR signaling in wild-type precursor B cells (9, 10, 37, 38, 44, 48). Therefore, it is conceivable that Pax5 may either regulate the expression of an essential component of the signal transduction cascade or act in the nucleus as the critical mediator of pre-BCR signaling. Stimulation of the BCR is known to result in the phosphorylation and association of the Igμ–Igβ heterodimer with the protein-tyrosine kinases Lyn, Fyn, Blk, Btk, and Syk (50–53). Moreover, the Syk kinase has been shown to play an important role in pre-BCR signaling (54, 55). However, none of these tyrosine kinase genes is expressed under the control of Pax5, as shown by a comprehensive analysis of putative BSAP (Pax5) target genes (26). Hence, there is at present no evidence that Pax5 is involved in the expression of cytoplasmic signal transducers. Moreover, an exclusive role of Pax5 in mediating signal transduction of the pre-BCR seems unlikely for several reasons. First, Pax5 expression is already initiated

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**Figure 4.** The Pax5 function is required for normal signaling responses of the pre-BCR. (A) Constitutive expression of the pre-BCR on Pax5-deficient pre-BI cells carrying the Igμ transgene. Pre-BI cells from Pax5 mutant mice were grown for 3 wk on stromal ST2 cells in the presence of IL-7 followed by flow cytometric analysis with biotinylated anti-λ5 (LM 34), anti-μ (M 41.42), and anti-pre-BCR (SL156) antibodies. Incubation with PE-coupled streptavidin was used to visualize the biotinylated antibodies. Unstained control cells are indicated by a line. Note that the cell surface expression of λ5 in the absence of Igμ is in agreement with the finding that the surrogate light chains λ5 and VpreB are expressed in association with an unidentified 130 kD glycoprotein on the surface of pre-BI cells before any productive V(D)J rearrangement (44). (B) Expression of the TdT gene in transgenic, Pax5-deficient pre-BI cells. Total RNA (10 μg) isolated from cultured pre-BI cells was simultaneously analyzed for TdT and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression by RNAse protection assay. Only the relevant parts of the autoradiograph containing the RNAse-protected fragments are shown. Pre-BI cells derived from Pax5 (–/–) bone marrow lacking (lane 2) or containing the Igμ transgene (lane 3) were compared with pre-BI cells established from the fetal liver of a control embryo (lane 1). Note that the TdT gene is not expressed in fetal B lymphopoiesis (38).
at B lineage commitment long before the pre-BCR stage and thereafter is maintained at a rather constant level throughout B lymphopoiesis (21–23). Second, all our attempts have so far failed to demonstrate any alteration in the posttranslational modification pattern of BSAP (Pax5) in response to signal transduction (M. Busslinger, unpublished data). Third, the developmental arrest in Pax5 mutant mice is tight (25) rather than leaky as it would be expected, in analogy to the scid (−/−) mouse (54, 55), for a mutation in a downstream component of the signal transduction pathway. Last but not least, a role for Pax5 in the mutation in a downstream component of the signal transduction pathway. Last but not least, a role for Pax5 in the mutation in a downstream component of the signal transduction pathway. Hence, the developmental arrest in pre-BI cells (57), is absent on bone marrow cells of Pax5-deficient mice (24). T herefore, it appears that Pax5 controls a critical step between initial B lineage commitment and the pre-BCR stage of adult B lymphopoiesis. In this context it is interesting to note that the interruption of Ras signaling also arrests early B cell development well before the pre-BCR stage (58). Our analysis of Pax5-deficient pre-BI cells has recently demonstrated a pleiotropic role of the transcription factor BSAP (Pax5) in gene regulation during early B lymphopoiesis. Hence, it will be a challenge for the future to identify the critical, and thus far unknown, BSAP target gene(s) that mediates the Pax5-dependent control of early B cell development.

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