Pre-TCR-triggered ERK signalling-dependent downregulation of E2A activity in Notch3-induced T-cell lymphoma

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Notch and basic helix–loop–helix E2A pathways specify cell fate and regulate neoplastic transformation in a variety of cell types. Whereas Notch enhances tumorigenesis, E2A suppresses it. However, whether and how Notch and E2A interact functionally in an integrative mechanism for regulating neoplastic transformation remains to be understood. It has been shown that Notch3-induced T-cell leukaemia is abrogated by the inactivation of pT α /pre-T-cell antigen receptor (pre-TCR). We report here that Notch3-induced transcriptional activation of pT α /pre-TCR is responsible for the downregulation of E2A DNA binding and transcriptional activity. Further, the E2A messenger RNA and protein levels remain unaltered but the E2A inhibitor Id1 expression is augmented in thymocytes and T lymphoma cells derived from Notch3 transgenic mice. The increase in Id1 expression is achieved by pre-TCR-induced extracellular-signalling-regulated kinase 1/2. These observations support a model in which the upregulation of pre-TCR signalling seems to be the prerequisite for Notch3-induced inhibition of E2A, thus leading to the development of lymphoma in Notch3 transgenic mice.

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INTRODUCTION

Notch signalling is critical in maintaining the correct unfolding of the development process in many cell types, through the

regulation of differentiation, survival and/or proliferation (Artavanis-Tsakonas *et al.*, 1999). Because of this property, dys-regulated Notch signalling has been involved in a variety of human neoplasms, including T-cell leukaemia (Allenspach *et al.*, 2002; Screpanti *et al.*, 2003). Notch signalling was first linked to T-cell leukaemogenesis because, in a rare form of T-ALL (acute lymphoblastic leukaemia), Notch1 is truncated as a result of a chromosomal translocation, resulting in a constitutively active intracellular domain, TAN-1 (Ellisen *et al.*, 1991). Furthermore, constitutively active Notch2 and Notch3 showed similar behaviour, indicating a causal role for Notch family members in the leukaemogenic process (Rohn *et al.*, 1996; Bellavia *et al.*, 2000). It is notable that enhanced expression of Notch3 has been reported in virtually all investigated cases of human T-ALL, highlighting a general function for Notch3 in this process (Bellavia *et al.*, 2002). Notch3 is ordinarily expressed in immature thymocytes around the critical pre-T-cell antigen receptor (pre-TCR)-dependent β -selection stage (Felli *et al.*, 1999). Subsequently, the expression of the pT α invariant chain of the pre-TCR declines, proliferation stops and differentiation proceeds from the CD4⁺CD8⁺ stage to the CD4⁺CD8⁺ stage (von Boehmer & Fehling, 1997). Akin to human T-ALL, the T-cell leukaemia/lymphoma derived from the transgenic mice expressing the constitutively active Notch3 intracellular domain (Notch3-IC tg) is characterized by a persistent expression of pT α (Bellavia *et al.*, 2000, 2002). These results indicate that Notch3-induced pT α expression at stages in which it is normally downmodulated might contribute to T-cell leukaemogenesis. The abrogation of lymphomagenesis in pT α -deficient Notch3-IC tg mice (Bellavia *et al.*, 2002) underscores the importance of the Notch3/pT α pathway relationship in transformation.

The basic helix–loop–helix (bHLH) transcription factor E2A has a crucial role across the pre-TCR-dependent checkpoint. Indeed, E2A downregulation allows the transition to and the expansion of CD4⁺CD8⁺ thymocytes (Engel & Murre, 2001; Greenbaum & Zhuang, 2002). The activity of E2A can be

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negatively regulated by another subclass of HLH proteins called Id proteins. These proteins are devoid of the basic DNA-binding domain and function as dominant-negative inhibitors of E proteins by forming non-DNA-binding heterodimers (Engel & Murre, 2001). Signalling through the TCR/CD3 in thymocytes inhibits E2A activity, at least in part through the induction of Id3 transcription by a Ras/mitogen-activated protein kinase (MAPK)-dependent pathway (Bain *et al.*, 2001). Experimental evidence indicates that inactivation of E2A activity results in the development of T-cell lymphoma, and ectopic expression of E2A inhibits cell-cycle progression and promotes apoptosis in a human T-cell leukaemia-derived cell line (Engel & Murre, 2001), indicating that E2A might function as a tumour suppressor.

Because Notch3 and E2A could putatively converge on a common leukaemogenic pathway, it is imperative to ask whether they participate in cross-talk. Here, we provide evidence that Notch3 inhibits E2A activity in T lymphoma cells and primary thymocytes by means of a pT α -dependent enhancement of pre-TCR signalling. Indeed, pre-TCR-dependent activation of the extracellular-signalling-regulated kinase (ERK)/MAPK cascade in immature thymocytes enhances Id1 gene expression. These observations suggest a linear pathway linking activated Notch signalling with the pre-TCR-mediated inhibition of E2A, ultimately leading to the development of lymphoma.

RESULTS

Notch3 signalling inhibits E2A DNA-binding activity

The DNA-binding activity of E2A and its heterodimeric partner, HEB, was analysed in whole-cell extracts from thymocytes from 4-week-old Notch3-IC tg mice and in T lymphoma cells from lymphoma-carrying mice, and compared with those from thymocytes of wild-type littermates. As described previously (Bellavia *et al.*, 2000), flow cytometry analysis of unfractionated thymocytes did not show an obvious impairment of subset distribution with regard to CD4⁺ and/or CD8⁺ cells in Notch3-IC tg versus wild-type thymocytes. In addition, T lymphoma cells derived from splenic tumour of 10-week-old Notch3-IC tg mice showed a large variation in the proportion of splenic lymphocyte subsets with a significantly high percentage of CD8⁺ and CD4⁺CD8⁺ double-positive (DP) lymphocytes (see supplementary figure 1 online; Bellavia *et al.*, 2000). E2A-E2-box DNA complexes were decreased by 80–90% in thymocytes and were maintained at low concentrations in extracts of lymphoma cell obtained from Notch3-IC tg mice when compared with thymocytes from wild-type littermates (Fig. 1A,B). In contrast, OCT-1 and nuclear factor- κ B (NF- κ B) binding, used as internal controls, were unaffected and induced, respectively (Fig. 1A). Pre-incubation of the cell lysates from wild-type thymocytes with specific antibodies indicated the presence of E47 and HEB in the complex (Fig. 1C).

The decrease in E47-HEB DNA-bound complexes was not due to changes in protein levels, as shown by western blotting in whole-cell extracts obtained from both wild-type and Notch3-IC tg thymocytes (Fig. 1D).

Inhibition of E2A activity is correlated with induction of Id1

To determine whether the inhibition of binding of E47/HEB to DNA observed in Notch3-IC tg mice reflects a change in Id levels, messenger RNA isolated from thymocytes of 4-week-old wild-type and Notch3-IC tg mice was analysed for Id expression.

Fig. 1 | Constitutive activation of Notch3 downregulates E2A activity. (A) E-box, OCT-1 and nuclear factor- κ B (NF- κ B) activity analysed by electrophoretic mobility-shift assay (EMSA) in whole-cell extracts from primary thymocytes derived from either wild-type (WT) or Notch3-IC (N3-IC tg) mice. OCT-1 and NF- κ B were used as loading controls. (B) E-box activity analysed by EMSA in whole-cell extracts of unfractionated thymocytes from WT mice and of unfractionated thymocytes (Thy) and lymphoma cells (Lymph) from N3-IC tg mice. Lymphoma cells were obtained from spleen and displayed a significantly high percentage (83.9%) of CD8⁺ and CD4⁺CD8⁺ cells (supplementary information figure 1 online; Bellavia *et al.*, 2000). (C) EMSA analysis of E-box-binding activity in whole-cell extracts of thymocytes from WT mice. Extracts were pre-incubated with antibodies against E47, HEB, a non-specific antibody (NS) or a 100-fold molar excess of unlabelled E-box oligonucleotides (100 $\times$). (D) Western blot analysis of HEB and E47 expression in primary unfractionated thymocytes from WT and N3-IC tg mice. Tubulin is shown as a demonstration of equivalent loading of each sample.

We have reported previously that T-cell lymphoma developing in most Notch3-IC tg mice is represented by immature CD8⁺ and/or CD4⁺CD8⁺ DP cells (supplementary information online figure 1; Bellavia *et al.*, 2000). The mRNA preparations were therefore obtained from positively selected CD8⁺ cells from thymus and splenic lymphomas. The purified population contained more than 90% CD8⁺ and DP cells (supplementary information online figure 2). The activation of Notch3 signalling resulted in a specific, more than ninefold, increase of Id1 transcript, while leaving unchanged the levels of Id2 and Id3 transcripts when comparing transgenic with wild-type CD8⁺ selected thymocytes (Fig. 2A,C). We also observed a significant increase, more than tenfold, in Id expression in tumour cells obtained from splenic CD8⁺ lymphoma cells of Notch3-IC tg mice in comparison with purified CD8⁺ thymocytes from wild-type littermates (Fig. 2B, and right panel in Fig. 2C). To analyse further whether the blocking of E2A activity by activated Notch3 occurs through a post-transcriptional and/or translational mechanism, the previously described M31 immature thymocyte cell line (Primi *et al.*, 1988) was transfected with an E-box reporter plasmid (Persson *et al.*, 2000), with or

Fig. 2 | E-box binding activity and helix-loop-helix expression are modulated through Notch3-mediated signalling. (A–C) Reverse transcription (RT)–PCR (A,B) and densitometric analyses (C) of Id transcripts in primary purified wild-type (WT) and N3-IC tg CD8⁺/double-positive thymocytes (A, and left panel in C) or primary WT thymocytes and splenic N3-IC tg lymphoma cells (B, and right panel in C). The purity of sorted cells was greater than 90% (supplementary information online figure 2). RT–PCR of β -actin is shown to demonstrate equivalent loading of each sample. (D) Transcriptional repression of E-box promoter activity by activated Notch3. M31 cells were transfected with E-box luciferase reporter and E47 expression plasmids with or without increasing amounts of expression vectors for the constitutively active N3-IC or green fluorescent protein (GFP).

without an expression vector for Notch3-IC. E-box promoter activity triggered by transfected E47 was significantly decreased by co-transfection with the Notch3-IC expression vector in a dose-dependent manner (Fig. 2D). Conversely, under the same conditions, E-box promoter activity was unaffected by a plasmid expressing green fluorescent protein (GFP; Fig. 2D, right panel).

Activating pT α /pre-TCR mediates Notch3 induction of Id1

It has been reported that E2A activity is inhibited by thymocyte activation, after crosslinking of CD3. Additionally, ectopic expression of both p56 *lck* and MAPK/ERK1 (MEK1) have the ability to

Fig. 3 | Notch3 transcriptionally induces pT α promoter activity and pre-T-cell antigen receptor (TCR) is required for an increase in expression of Id1 and in E2A DNA-binding inhibition. (A,B) M31 cells were transfected with pT α luciferase reporter plasmids with or without increasing amounts of expression vectors for the constitutively active Notch3-IC (N3-IC; A) or green fluorescent protein (GFP)-labelled mock control (B). (C,D) Reverse transcription–PCR (C) and densitometric (D) analyses of Id1 transcript in wild-type (WT), N3-IC tg and N3-IC tg/pT α ^{-/-} primary purified CD8⁺/double-positive (DP) thymocytes. Values were normalized to β -actin expression and are expressed as levels relative to WT CD8⁺/DP thymocytes. (E,F) EMSA of E-box and octamer binding in whole-cell extracts from primary purified CD8⁺/DP thymocytes.

induce downmodulation of E2A activity, indicating that activated p56 *lck* and MEK1 might be able to substitute for TCR signalling (Bain *et al.*, 2001). We have shown previously that high pT α mRNA expression is maintained in Notch3-IC mice, possibly leading to ligand-independent activation of pre-TCR (Bellavia *et al.*, 2000). To assess whether the induction of pT α mRNA expression by activated Notch3 occurs at the level of promoter activity, M31 cells were transfected with a luciferase reporter plasmid for the pT α promoter (Petersson *et al.*, 2002) with or without various amounts of Notch3-IC expression vector. As shown in Fig. 3A, pT α promoter activity was significantly induced by co-transfection of the Notch3-IC expression vector in a dose-dependent manner but not by a GFP expression plasmid (Fig. 3B).

To determine whether the activation of Id expression was mediated through the pre-TCR signalling pathway, Id mRNA expression in purified CD8⁺/DP thymocytes derived from the previously described Notch3-IC/pT α ^{-/-} double-mutant mice (Bellavia *et al.*, 2002) was examined by reverse transcription and PCR

(RT-PCR) and compared with that of purified CD8⁺/DP thymocytes obtained from wild-type and Notch3-IC tg mice. Figure 3C shows that the activation of Id1 transcription by constitutively active Notch3 signalling was inhibited by the ablation of pre-TCR signalling, whereas the expression of HEB and E2A mRNAs was unaffected (Fig. 3C,D; supplementary information online figure 3). In purified CD8⁺/DP thymocytes, Notch3 constitutive signalling was consistently insufficient to promote the downmodulation of E2A activity in the absence of pTα/pre-TCR signalling (Fig. 3E). Under the same conditions OCT-1 DNA-binding activity, used as an internal control, was unaffected (Fig. 3F).

Notch3 promotes activation via pre-TCR ERK/MAPK

The TCR-triggered Ras-ERK pathway in primary cultures of thymocytes modulates the levels of Id mRNA and consequently E2A-binding activity (Engel & Murre, 2001). To determine whether activation of the Notch3 pathway could trigger ERK signalling, whole-cell extracts derived from purified CD8⁺/DP thymocytes of either wild-type or Notch3-IC tg mice were analysed by western blotting for the presence of activated ERK. Phosphorylated ERK (pERK) was easily detectable at higher levels in thymocytes from Notch3-IC tg mice than in thymocytes from wild-type mice (Fig. 4A, lane 2), whereas the basal expression of non-phosphorylated ERK was unchanged (Fig. 4A, lower panel, lane 2). Conversely, a 95% decrease in phosphorylated ERK was observed in purified CD8⁺/DP thymocytes of Notch3-IC/pTα^{-/-} double-mutant mice (Fig. 4A, lane 3), indicating that pre-TCR signalling might be required for the Notch3-induced activation of ERK signalling.

To determine further whether pre-TCR-mediated activation of the ERK pathway contributes to the decrease in the DNA-binding activity of E2A, thymocytes derived from either wild-type or Notch3-IC tg mice were incubated in the presence or absence of PD98059, a specific inhibitor of MEK1 and MEK2. Downmodulation of E47-HEB DNA binding activity was blocked by the addition of PD98059 (Fig. 4B). Furthermore, treatment with PD98059 inhibited the induction of Id1 mRNA in Notch3-IC tg mice (Fig. 4C).

To study whether E2A downregulation is directly linked with pre-TCR signalling, we transfected a E-box promoter-luciferase reporter (Persson *et al.*, 2000), in the presence or absence of an expression vector for Notch3-IC, into the TCR-β-deficient cell line SC127, lacking pre-TCR expression and derived from severe-combined immunodeficient (SCID) thymocytes, or the TCR-β-transfected daughter cell line SCB29, expressing a signalling-competent pre-TCR on the cell surface (Aifantis *et al.*, 2001). Transfected E47-triggered E-box promoter activity was significantly decreased by co-transfected Notch3-IC in SCB29 cells but not in SC127 cells (Fig. 4D). These observations suggest a direct role for pre-TCR-triggered ERK signalling in the Notch3-induced inhibition of E2A activity.

DISCUSSION

The inactivation of E2A activity results in the development of T-cell lymphoma, suggesting that E2A functions as a tumour suppressor (Park *et al.*, 1998; Engel & Murre, 2001). Enhanced expression of both Notch3 and pTα/pre-TCR is linked to primary human and experimental T-ALL (Bellavia *et al.*, 2000, 2002). Reciprocal relationships between pre-TCR and E2A signalling, resulting in the regulation of T-cell development and leukaemo-

Fig. 4 | Notch3 effects occur through a pre-TCR-triggered ERK/MAPK pathway. (A) Immunoblot analysis of phosphorylated ERK (pERK) or total ERK expression in whole-cell extracts of CD8⁺/double-positive (DP) wild-type (WT), N3-IC tg and N3-IC tg/pTα^{-/-} thymocytes. (B) EMSA of E2A and OCT-1 DNA-binding activity in whole-cell extracts of primary thymocytes incubated for 90 min in the presence or absence of PD98059. (C) Reverse transcription-PCR analysis of Id1 in WT or N3-IC tg CD8⁺/DP thymocytes cultured in the presence or absence of the PD98059. (D) SCB29 (left) and SC127 (right) cells were transfected with E-box luciferase reporter and E47 expression plasmids with or without the expression vector for Notch3-IC (N3-IC) as indicated; pcDNA3 vector was used as an empty control vector. EMSA, electrophoretic mobility-shift assay; ERK, extracellular-signal-regulated signalling kinase; MAPK, mitogen-activated protein kinase; pre-TCR, pre-T-cell antigen receptor.

genesis, have also been suggested recently (Engel & Murre, 2002). Here, we show that constitutively active Notch3 promotes the inhibition of E2A activity in a pre-TCR-dependent manner and that this might have implications in the T-cell leukaemogenesis process. We show further that the link between Notch3 and the activation of pre-TCR signalling results from the direct activation of pTα transcription, a property shared with Notch1 (Reizis & Leder, 2002). Taken together, our findings might have important implications for T-cell leukaemogenesis.

We have also provided insights into the mechanisms by which Notch3 and pre-TCR regulate E2A activity. Increased expression of Id is known to inhibit the DNA-binding activity of the

E47-HEB heterodimer (reviewed in Engel & Murre, 2001). Regulation of Id expression by TCR signalling has been suggested previously (Bain *et al.*, 2001). Id-dependent reduction of E2A activity might therefore represent a crucial partner of Notch3 in T-cell leukaemogenesis. We asked whether pre-TCR signalling was also involved in this process. The analysis of Id expression in Notch3-IC/pT $\alpha^{-/-}$ double-mutant mice, showing that the upregulation by Notch3 of the gene encoding Id1 requires an intact pre-TCR, indicates a direct relationship.

Previous studies support a role for the ERK/MAPK pathway in modulating E2A function. Indeed, ERK1/2 is activated on signalling from the TCR, and Id expression is increased in a dose-dependent manner by the ERK/MAPK module (Bain *et al.*, 2001). Furthermore, both the p56 *lck* and ERK/MAPK modules have been implicated in the development of T-cell lymphomas. Indeed, enforced expression of p56 *lck* and MEK1 in transgenic mice leads to the rapid development of T-cell lymphomas with a similar phenotype to that described for E2A-deficient mice (Abraham *et al.*, 1991; Bain *et al.*, 1997, and references therein). It is therefore conceivable that the oncogenic effects of p56 *lck* and ERK activity are due to inappropriate inhibition of E2A activity. Our analysis of ERK activation status in Notch3-IC tg and Notch3-IC/pT $\alpha^{-/-}$ double-mutant mice also support such a model. Indeed, ERK1/2 activation can be detected in thymocytes derived from Notch3-IC tg mice that develop T-cell lymphomas, whereas only a weak signal can be detected in thymocytes derived from Notch3-IC/pT $\alpha^{-/-}$ double-mutant mice, in which the deletion of pT α prevents the development of lymphoma. Thus, ERK1/2 activation is induced downstream of a pathway that is triggered by Notch3 signalling and requires an intact pre-TCR.

The ERK/MAPK module functions as an integration cascade of multiple signal pathways that need to be coordinated for the correct unfolding of several developmental processes (Murphy *et al.*, 2002). It has been established that signals through the TCR result in activation of the ERK/MAPK module, and the quality and quantity of this signal regulate the development of thymocytes (Bettini *et al.*, 2002). The data we present here position Notch3, pre-TCR, the ERK/MAPK signalling cascade and HLH proteins in an integrated pathway. On the basis of this, it can be surmized that enforced Notch3 expression promotes the dysregulation of this cascade, contributing to the inhibition of E2A DNA-binding activity that overall results in lymphoma promotion.

METHODS

Cell transfection. The dual luciferase/*Renilla* reporter assay system (Promega) was used in accordance with the manufacturer's instructions. The expression vector for Notch3-IC was previously described (Bellavia *et al.*, 2000). The E-box luciferase promoter vector (Persson *et al.*, 2000) and the pT α luciferase promoter vector (Persson *et al.*, 2002) were kindly provided by H. Axelson and K. Persson, respectively. pcDNA3 vector was used as an empty control vector and was added to each sample to ensure an equal amount of total DNA.

Flow cytometry and cell sorting. Thymocyte suspensions, stained with anti-CD4-FITC and anti-CD8-PE antibodies (PharMingen), were analysed with a FACScalibur flow cytometer (Becton Dickinson). CD8⁺ cells, including single-positive and double-positive subsets, were positively selected by magnetic cell separation with the MACS system (Milteny Biotec, Auburn, California,

USA) in accordance with the manufacturer's instructions. The purity of the isolated fractions was more than 90% (supplementary information online figure 2).

Mice. The generation and typing of Notch3-IC tg and Notch3-IC/pT $\alpha^{-/-}$ double-mutant mice were described previously (Bellavia *et al.*, 2000, 2002).

Whole-cell extract preparation and electrophoretic mobility-shift assay. Whole-cell extracts were prepared as described previously (Bain *et al.*, 2001). Double-stranded DNA probes were end-labelled with the Klenow enzyme. The sequences of the μ E5 and OCT-1 oligoprobes were as follows: μ E5, 5'-TCGAAGA ACACCTGCAGCAGCT-3'; OCT-1, 5'-GCGTTTCGAATGCAAAT CCTCACCTT-3'. The NF- κ B oligoprobe was as described (Bellavia *et al.*, 2000). For antibody supershift, the whole-cell extracts were pre-incubated with anti-E47 (1 μ g, G127-32; PharMingen, San Diego, California, USA), anti-HEB (G127-382; PharMingen) or anti-Bcl-2 (1 μ g, 65111A; PharMingen), as a nonspecific control, for 20 min in ice before the addition of the labelled probe. For experiments involving the addition of the MEK inhibitor PD98059 (Calbiochem), primary thymocytes were obtained from wild-type and Notch3-IC mice as described by Bain *et al.* (2001).

Immunoblotting. A protocol for this procedure has been described by Bellavia *et al.* (2000). Blots were incubated overnight with anti-E47 or anti-HEB for 2 h at 25°C or with anti-phospho-ERK (New England Biolabs) at 4 °C.

RNA analysis and RT-PCR. Total RNA, isolated from cell suspensions, was processed for RT-PCR as described by Bellavia *et al.* (2002). Each sample was analysed in three serial dilutions (1:1, 1:10 and 1:100) in at least two independent experiments. PCR was performed at the appropriate annealing temperature with the following primers: β -actin-f, 5'-GTGGGCCGCTCTAGGCACCA A-3'; β -actin-r, CTCTTTGATGTCACGCACGATTTC-3'; Id1-f, 5'-CCAGTGGCAGTGCCGGACCCGCTGCAGGC-3'; Id1-r, 5'-GGCTGGAGTCCATCTGGTCCCTCAGTGC-3'; Id2-f, 5'-GAAC CGAGCCTGGTGCCGCGCAGTCAGCTC-3'; Id2-r, 5'-GGC GGATCCTTATTAGCCACAGAGTAC-3'; Id3-f, 5'-AAGGCGC TGAGCCCGGTGC-3'; Id3-r, 5'-TCGGGAGGTGCCAGGACG-3'; HEB-f, 5'-AAATCAGATGATGAGTCCTCCC-3'; HEB-r, 5'-CTCT GGAAGTGGCTGATGTTT-3'; E47-f, 5'-GCATAGGAAGCTC AGCAGAGA-3'; E47-r, 5'-AAGCATACAGGACTGCAAGGAG-3'. The relative levels of RNA were quantified with BioRad Quantity-one software. Values were normalized to β -actin expression and are shown as levels relative to the control cell population. All RNAs were tested in triplicate samples and in at least two independent experiments.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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