Interaction of Ras with P110γ is required for thymic beta-selection in the mouse

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Abstract

Thymocytes are tested for productive rearrangement of the *tcrb* locus by expression of a preTCR in a process termed β-selection which requires both Notch1 and CXCR4 signalling. It has been shown that activation of the GTPase Ras allows thymocytes to proliferate and differentiate in the absence of a PreTCR: the direct targets of Ras at this checkpoint have not been identified however. Mice with a mutant allele of p110ε unable to bind active Ras revealed that CXCR4-mediated PI3K activation is Ras-dependent. The Ras-p110ε interaction was necessary for efficient β-selection-promoted proliferation but was dispensable for the survival or differentiation of thymocytes. Uncoupling Ras from P110ε provides unambiguous identification of a Ras-interaction required for thymic β-selection.

Activation of these receptors promotes downstream signalling cascades necessary for the processes that define d-selection.

Small GTPases of the Ras family act as digital switches for multiple signal transduction cascades with the potential to integrate signals from different receptors (10). The Ras subfamily includes three highly homologous paralogs H-, K- and N-Ras as well as other related proteins, including Ral, Rap, R-Ras, and TC21. These cycle between an inactive (GDP-bound) and an active (GTP-bound) state under the influence of GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs)(11).

Transgenic over-expression of a constitutively active form of H-Ras (12) or the Ras activator RasGRP1(13) promotes the development of CD4+CD8+ (DP) thymocytes from recombinase activating gene (RAG)-mutant precursors that are unable to produce a functional preTCR due to lack of VDJ recombination. However the process of allelic exclusion is not impaired in thymocytes expressing active H-Ras (14). Moreover, amongst Ras effectors, transgenic expression of an active mutant of the serine/threonine protein kinase Raf-1 promoted the development of DP thymocytes from rag-deficient precursors(15). This suggests activation of the Ras pathway may contribute to a subset of the changes accompanying d-selection. However, experiments with dominant negative Ras or its effectors have not proven informative on their role in d-selection, possibly due to low expression or specific activity(16).

Amongst Ras effectors are the catalytic subunits of phosphatidylinositol 3-kinase (PI3K) enzymes(17). The class IA subfamily of PI3K is comprised of three catalytic subunits termed p110 c, d, and f, while the class IB family consists of p110 e. Genetic studies, using mouse models, have shown that d-selection is mediated, in part, by p110 f in combination with p110 e(18-20). This reflects a requirement for p110 f to transmit signals originating in the pre-TCR and a predominantly p110 e-dependent PI3K signal generated by CXCR4(8). Unopposed activation of the class I PI3K pathway by deletion of the lipid phosphatase PTEN allows the proliferation and development of RAG-deficient thymocytes to the DP stage(21), mimicking some aspects of d-selection.

The class IA and IB PI3Ks are activated by binding to distinct cell surface receptors. Class I PI3Ks are heterodimers comprised of a catalytic subunit and a receptor coupling regulatory subunit. The class IA regulatory subunits (p85 c, p55 c, p50 c, p85 d and p55 e) bind to YXXM motifs that have been phosphorylated by protein tyrosine kinases and the class IB regulatory subunits (p101 and p84) bind to the G subunits liberated upon activation of G-protein coupled receptors(22). It has been demonstrated that during thymocyte development p101 is required to mediate p110 e activation by CXCR4(8) and that p85 c is required for pre-TCR-mediated PI3K signalling(23).

The generation of mice with defined mutations that affect protein function but not expression promise to greatly further our understanding of gene function(24). Here we show that thymocytes harbouring a mutation of p110 e that blocked its interaction with Ras were defective when undergoing d-selection in competition with wild-type thymocytes. When combined with deficiency in p110 f the loss of Ras input into p110 e produced the same phenotype as p110 e/ f double knockout mice. Analysis of cell cycle, apoptosis and CD4/8 expression revealed that Ras activation of p110 e is selectively required for optimal proliferation during d-selection but does not affect survival or differentiation. We find CXCR4 activates p110 e independently of the preTCR by a Ras-dependent mechanism. Thus p110 e is a key target of Ras signalling that is required for d-selection.

Material and Methods

Mice

p110 f/, p110 e−/−, p110 eD/D and Vav1/2/3−/− mice have all been described previously (25-28). Mice were on the C57BL/6 background and aged 6-13 weeks at analysis. For competitive chimera studies, irradiated Rag2−/− γc−/− mice were injected intravenously with 3 × 10^6 bone marrow (BM) cells at 1:1 ratio mix of WT(SJL):WT, WT(SJL):p110 eD/D or WT(SJL):p110 e−/− and analysed six weeks later. All animal husbandry and experimentation were in accordance with U.K. Home Office regulations and subject to local ethical review.

Cell Isolation

DN3a and DN3b thymocytes were isolated by FACS. Single cell thymocyte suspensions were first enriched for DN cells by incubation with biotinylated anti±CD8 c antibody followed by streptavidin-coated MACS beads and magnetic depletion prior to staining for DN subsets. DN3a and DN3b cells were isolated as [CD4/CD8/CD44/B220/CD11b/NK1.1/Gr1/Ter119/ e fTCR]neg and either CD25hiCD98lo (DN3a) or CD25intCD98hi (DN3b).

Cell Culture

OP9-DL1 cells were maintained as previously described (4). 5 × 10^4 DN3a or 2.5 × 10^4 DN3b cells were cultured in supplemented α-MEM without additional cytokines in 96-well plates that had been previously seeded with 4000 OP9-DL1 cells. In some experiments DN3a cells were stained before culture with either Cell Trace Violet or Vybrqant CFDA SE cell proliferation dyes (Molecular Probes, Invitrogen) as per the manufacturer's instructions. Cells were cultured for up to 72 hours before harvesting and analysis by FACS. Cell numbers were determined by reference to the inclusion of a fixed number of microbeads (Spherotech).

FACS analysis

Single cell suspensions were stained using various combinations of antibodies conjugated to fluorochromes for analysis by FACS (LSRII Becton Dickinson). For cell cycle analysis, cultured cells were harvested, resuspended in ice-cold 70% EtOH and incubated at −20° C for at least 30mins. Cells were then washed three times in PBS before incubation with 1mg/ml RNase at room temperature for 30mins. Propidium iodide was then added at a final concentration of 400 ng/ml. For caspase-3 staining, cultured cells were harvested, fixed and permeabilised using Cytofix/Cytoperm (Becton Dickinson) before staining with PE-labelled antibody to activated caspase-3 (Becton Dickinson). Excess antibody was removed by washing before analysis.

Western Blot

Lysates from 10^6 thymocytes were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with rabbit anti-p110 e (Onyx Pharmaceuticals) and antibody binding was revealed using HRP-conjugated anti-rabbit Ab (DakoCytomation) followed by ECL (Amersham). Blots were subsequently probed using mouse anti- d actin.

Phospho-specific flow cytometry

Unfractionated thymocyte suspensions were resuspended at 10^7 cells/ml in DMEM with 0.1% BSA. Cells were incubated at 37° C for 20mins before the addition of 10nM CXCL12. At various time intervals 10^6 cells were fixed in 2% paraformaldehyde, washed in PBS, and resuspended in 90% ice-cold methanol at −20° C for at least 30mins. Cells were then washed three times in PBS with 2% FCS before incubation with antibodies to surface markers as

well as to phosphorylated-Akt (Cell Signaling clone #4058) or phosphorylated-Erk (Cell Signaling clone #4370) for 30mins at room temperature. Phospho-specific antibodies were revealed by subsequent incubation with Cy5-conjugated donkey anti-rabbit antibody and FACS. For data analysis, fold induction was calculated as the median fluorescence of stimulated sample / median fluorescence of unstimulated sample. Unstimulated samples showed no change in phospho-Akt or phospho-Erk throughout the timecourse.

Statistics

Data presented throughout represent the mean and either the SD or SEM (if n 5). Data were analysed by one-way ANOVA (or repeated measures ANOVA for competitive chimera experiments) and statistical significance represented as: * $p<0.05$, ** $p<0.01$ and ***$p<0.001$.

Results

The interaction of active Ras with p110**γ** is required for efficient thymic **β**-selection

To determine whether Ras binding to p110 e is required for T cell development we studied knock-in mice with mutations at five residues of p110 e critical for the binding of activated GTP-Ras (ªDASAAº: T232D, K251A, K254S, K255A, and K256A) (27). Using Western blotting we found the expression of p110 e^{DASAA} in thymocytes was equivalent to WT p110 e (Supplementary Figure 1).

FACS analysis of thymocytes from p110 e$^{DASAA/DASAA}$ (hereafter termed p110 e$^{D/D}$) mice revealed thymic cellularity was reduced in p110 e$^{D/D}$ mice by approximately 50% and this principally reflected a reduction in the number DP thymocytes (Figure 1A-D). To investigate whether the loss of DP cells was due to a defect at d-selection, we used expression of the heavy chain of the amino acid transporter CD98 to analyse the pre-selection DN3a (CD44-CD25^{hi}CD98^{lo}), the post-selection DN3b (CD44-CD25 CD98^{hi}) ò# þÓW®ël FB.efA N
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thymocytes compete poorly with WT cells at the d-selection checkpoint and in the transition from the DN to DP stage. The ratio of B6SJL:p110 $e^{D/D}$ remained at approximately 80% for all subsequent developmental stages, indicating that the $p110e^{D/D}$ cells were not at a disadvantage when subjected to further selection pressure. A similar phenotype was observed in the B6SJL:$p110f^{-/-}$ emixed chimeras, consistent with the known role for p110 e at this developmental stage.

Previous work has demonstrated that there is functional redundancy between p110 e and p110 f at the d-selection checkpoint (18-20). Therefore, we tested the effect of the p110 e^{DASAA} mutation in the context of p110 f deficiency (Figure 1A-D). Compared to wildtype and $p110f^{D/D}$ single mutant mice, the number of $CD44^{+}CD25^{hi}CD98^{lo}$ DN3a cells was increased in the double mutant mice, while the DN3b, DN4 and DP populations were all significantly reduced. This phenotype is further evidence for the importance p110 e at the d-selection checkpoint. Taken together, these data indicate that the function of p110 e at the d-selection checkpoint is critically dependent upon the binding of activated Ras to P110 e.

To characterise further the defect in d-selection we evaluated the ability of DN3a and DN3bp515 -12.

To test for changes in proliferation potential, we examined the proportion of cells in G0/1, S and G2/M stages of the cell cycle in the undivided and dividing populations. Amongst the undivided cells, following 60 hours of culture, a significantly increased fraction of p110

first study to identify a target of Ras signalling required for d-selection and to place it within the context of a signalling pathway important for thymocyte development. Thus the p110 e catalytic isoform of PI3K is activated by the chemokine receptor CXCR4 in a Ras-dependent manner. Taken together with our previous results(8), we conclude that in DN3 thymocytes p110 e must be activated by both G (via P101) and active Ras in order to elicit an optimal CXCR4 PI3K signalling response. This signalling pathway specifically promotes proliferation and, when it is disrupted, a G0/1 cell cycle block is observed.

P110 e. This possibility is supported by the known ability of Ras-proteins to signal from distinct internal membrane compartments such as the plasma membrane, Golgi and endoplasmic reticulum(11, 41).

The second class I PI3K isoform required for efficient d-selection is p110 f which signals downstream of the preTCR(8). Based upon sequence alignments, the predicted target residues required for the interaction with Ras are conserved across the human and mouse p110 c, p110 f and p110 e isoforms(42). Intriguingly, H-, N-, or K-Ras appear not to activate p110 f. Instead, this ability has been attributed to TC21(43). Although a role for TC21 in the thymus has not been described, peripheral T TC21

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T-ALL T cell acute lymphoblastic leukaemia

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checkpoint,gneatc Jptspisoylacns Mr3 waAD (inG,aGukpbl mCXCR4203:2239±6428])Tj2010PubM7:24882461]

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Figure 1. Reduced thymic cellularity in mice with a Ras-binding mutant of p110 **e**

Figure 2. Competitve bone marrow chimeras reveal defective **d**-selection in p110 D/D **e** thymocytes

(A) Example of FACS analysis used to distinguish B6.SJL cell origin based upon expression of the CD45.1 allotypic marker. T 1*rm7.1 2prategy d7 -Mn expPi2r.Pf3.02 313.897 3.lo10 Tf (A)Tj /T1_6

Figure 3. The Ras-p110 **e** signalling pathway is required in DN3a and DN3b cells

(A) CD4 and CD8 expression following culture of DN3a ($CD25^{hi}CD98^{lo}$) and DN3b ($CD25^{int}CD98^{hi}$) cells on OP9-DL1. The percentages of cells that remain **CD4**$^-$**CD8**$^-$ are shown. (B) The number of live cells recovered from WT (”), $p110\delta^{D/D}$ ■ and p110 **e**$^{-/-}$ (•) cultures after three days. The seeding number for each cell type was 4.5×10^4 DN3a and 2.5×10^4 DN3b. Graphs show the mean and SD from 3 independent groups.

Figure 4. Analysis of differentiation, proliferation and survival responses at **d**-selection
(A) Division profiles of sorted WT, p110 $\Delta^{D/D}$ and p110 $\Delta^{-/-}$ DN3a cells ($CD25^{hi}CD98^{lo}$) after culture on OP9-DL1 for the indicated times. (B) Expression of CD8 (”) and CD4 (¿) at each division cycle in WT, p110 $\Delta^{D/D}$ and p110 $\Delta^{-/-}$ cultures after 72 hours. (C) Detection of caspase-3 cells after 14 and 60 hours of culture. FACS plots show the gates used to identify cells which have divided 2 times and those that remain undivided that are either caspase-3 positive or negative. Graphs show data from three independent experiments where WT = ”, p110 $\Delta^{D/D}$ = ■ and p110 $\Delta^{-/-}$ = •. (D) DNA content profiles from cells cultured for 60hrs. The gating strategy used to identify divided and undivided populations is the same as in Figure 3D. Coloured background on FACS plots show the delineation of cells in G0/1 (blue), S (green) and G2/M (red). Numbers on FACS plots show percentage of cells in G0/1. Graphs show data from three independent experiments where G0/1 = ”, S = • and G2/M = ■ cultutnsr02ed).0 pi sh 0 7notded ais7mided posignifhownt, *0 7p<0.05, **0 7p<0.0=

Figure 5. Defective induction of PI3K signalling in p110 $\delta^{D/D}$

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Figure 6. Intact PI3K but defective ERK activation in Rag2$^{-/-}$ and Vav1/2/3$^{-/-}$ DN3 cells following stimulation with SDF1 **c**

Induction of (A) p-Akt (S473) and (B) p-Erk1/2 (T202/Y204) in W)(Vav1/2/3$^{-/-}$ (•) and Rag2$^{-/-}$ (¿) DN3 cells after stimulation with 10nM SDF1 c. Graph shows the mean and SD from analysis of four-six mice.

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Figure 7. CXCR4 inhibition affects the proliferative response of DN3 cells

(A) Division profiles of sorted WT DN3 cells ($CD25^{hi}CD98^{lo}$) cultured on OP9-DL1 in the presence of 0, 0.1, 1 and 10 nM of the CXCR4 antagonist AMD3100. (B) Expression of CD8 (”) and CD4 (¿) at each division cycle at 72 hours in untreated and cultured supplemented with 0.1 nM, 1 nM and 10 nM of AMD3100. (C) FACS plots show the gates used to identify cells which have divided 2 times and those that remain undivided that are either caspase-3 positive or negative. Graphs show aggregate data from three independent experiments; no AMD3100 (”), 0.1 n■, 1 nM and 10 nM (•). (D) DNA content profiles from cells cultured for 60hrs. The gating strategy used to identify divided and undivided populations is the same as in Figure 3D. Graphs show data from three independent experiments where G0/1 = ”, S = • and G2/M = ■. For all statistical analysis a repeated measures ANOVA test was performed. NS = not significant, * = $p<0.05$.