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The lack of NF-κB transactivation and PKCε expression in CD4+CD8+ thymocytes correlates with negative selection

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Abstract

Deletion of autoreactive thymocytes at the DP stage is the basis for tolerance to thymus-expressed self antigens. In this study we investigated whether distinct signalling pathways are induced in DP thymocytes as compared to mature T cells upon stimulation with antigen. Using triple transgenic mice expressing a TCR transgene, dominant negative ras/Mek proteins and a reporter gene construct with AP-1 or NF-κB binding sites, we showed a complete lack of transcriptional activity of NF-κB but not AP-1 in DP thymocytes, whereas both were transcriptionally active in mature T cells after antigenic stimulation. Lack of NF-κB induction correlated with increased death in response to antigen. AP-1 induction was dependent on the integrity of the ras/Mek pathway indicating that this pathway was activated in the DP thymocytes. In contrast, we found a complete lack of constitutive expression of the ε isoform of Protein Kinase C (PKC) in DP thymocytes, although it was present in mature thymocytes and peripheral T cells. Taken together the results suggest that the lack of PKCε in DP thymocytes could lead to the absence of NF-κB activity after antigenic stimulation contributing to negative selection. *Cell Death and Differentiation* (2000) **7,** 1253–1262.

Keywords: CD4+CD8+ thymocytes; NF-κB; PKCε; ras/Mek; thymic death; AP-1

Abbreviations: PKC, protein kinase C; TCR, T cell receptor; DP, double positive thymocytes; SP, single positive thymocytes

Introduction

Signals mediated by engagement of the TCR initiate the positive selection of CD4+CD8+ (DP) thymocytes into mature CD4+ or CD8+ T cells. This process involves TCR-mediated changes in gene expression that lead to thymocyte differentiation and survival. Negative selection of autoreactive thymocytes also requires signalling through the TCR at the DP stage of development that results in cell death.[1] Thus signalling is pivotal for survival or death. It remains unclear, however, whether the signals mediated by the TCR leading to positive or negative selection are quantitatively or qualitatively different (reviewed in [2]). The molecular basis for the differential response to antigen between DP thymocytes and mature T cells is also unknown. We have recently shown that DP thymocytes have a nuclear signalling pattern distinct from mature T cells in response to antigen.[3] Antigenic stimulation leads to weak NF-κB DNA binding activity but strong AP-1 activity in DP thymocytes as compared to mature T cells in which both transcription factors are activated.[3]

It is not clear yet how NF-κB activation is induced in response to TCR engagement. Signalling for activation of the transcription factor NF-κB generally involves serine phosphorylation of the NF-κB inhibitor IκBα followed by its proteasomal degradation, allowing for nuclear translocation of NF-κB. Components of the upstream IκB kinase complex, in particular IKKα and IKKβ, have recently been identified that require phosphorylation on specific serine residues for activation (reviewed in [4]). Upstream of the IKKs, distinct kinases can be involved depending on the stimulus or on the cell context. These include MAP3Ks (MEKK1) activated via the ras pathway, as well as members of the PKC family.[5] In T cell lines, particular PKC isoforms appear to be efficient at activation of NF-κB.[6] In view of our previous observation that no NF-κB DNA binding was detected in response to antigen in DP thymocytes and of the accumulating evidence for control of cell survival by NF-κB,[7] we wanted to establish and analyse further the basis for the deficiency in NF-κB induction in response to antigen at the DP thymocyte stage where selection events occur.

Some components of the ras activation pathway appear to be differentially involved in thymic positive versus negative selection (reviewed in [2]). Disruption of the Erk pathway that is controlled by ras, raf1 and Mek-1/2 inhibits positive selection and leaves negative selection intact.[8–10] Inversely, there is some evidence that the JNK and p38 pathways are important in negative selection but not positive selection.[11,12] Another arm of TCR signalling, i.e. the activation of the phospholipase C, subsequent calcium influx and activation of PKCs has been less extensively studied in developing thymocytes. When the calcium/calcineurin-dependent pathway is inhibited, deletion of autoreactive thymocytes can occur provided there is a strong stimulus,[13–15] whereas positive selection is blocked.[15] Surprisingly there are very few reports on the role of PKCs in thymic selection events. Considering that

upstream signalling molecules not only control the binding of transcription factors to the DNA but also regulate nuclear translocation and posttranslational modification of preformed complexes required to mediate transcriptional activity, we first asked whether previously detected DNA-binding activity reflects transactivation. Second, we evaluated the role of the ras/Mek pathway in DNA binding activity as well as transactivation of AP-1 and NF-κB. Third, we investigated whether developmental control of expression of distinct PKC isoforms could be associated with defective antigen-induction of NF-κB in DP thymocytes.

Using triple transgenic mice expressing (i) a TCR transgene, (ii) the luciferase reporter transgene under the control of AP-1 and NF-κB DNA binding sites and (iii) transdominant negative Mek and ras proteins (dnras/dMek) in the T cell lineage, we show a lack of NF-κB transactivation in response to antigen, confirming our previous results on DNA binding. We observed that during the development of DP to thymic SP CD8 and then mature CD8 T cells NF-κB became increasingly transcriptionally active, whereas the susceptibility to death in response to antigen decreased. Additionally AP-1, which is transcriptionally active in response to antigen in DP thymocytes, was partly inhibited by the expression of the dnras/dMek proteins, indicating that the lack of NF-κB is not due to a defect in ras/Mek pathway. However, after phorbol ester/ionophore stimulation NF-κB was transcriptionally active and this activity was also partly affected by the inhibition of the ras pathway. Moreover we showed a deficiency of PKCε protein in DP thymocytes whereas PKCα and β are well-expressed, and PKCε was present in thymic SP and peripheral T cells. These findings suggest that lack of PKCε in DP thymocytes may contribute to the absence of NF-κB transactivation and increased sensitivity to apoptosis at this developmental stage.

Results

Inverse correlation of death and NF-κB transcriptional activity in CD4+CD8+ thymocytes induced by antigen

We have previously demonstrated that antigen fails to induce NF-κB DNA-binding activity in response to antigen in DP thymocytes whereas AP-1 binding activity is induced at this stage of development.[3] It remained to be shown, however, whether the DNA binding activity of transcription factors demonstrated in cell extracts correlated with transcriptional activation. For that purpose, the TCR transgenic mice were crossed onto AP-1 or NF-κB luciferase reporter mice. To determine transcriptional activity of NF-κB and AP-1, total thymocytes, preselection DP (MHC class I^{low} TCR^{low} $CD69^{low}$) thymocytes, thymic $CD8^+$ or peripheral $CD8^+$ T cells from TCR transgenic mice were stimulated *in vitro* with either the nominal antigen H-$2K^b$ (with endogenous level or with additional cognate peptide – see Materials and Methods) or control H-2^k expressing cells. Then luciferase activity was determined as a measure of the transcriptional activity of the respective factors. The degree of stimulation was measured by detection of CD69 (not shown) and Annexin V staining was an indication for dying cells undergoing negative selection (Figure 1C). Positive controls included stimulation of peripheral T cells with H-$2K^b$ expressing APCs. After 6 and 12 h stimulation peripheral T cells remained negative for AP-1 and NF-κB transcriptional activity whereas after 24 h stimulation 860 RLU for NF-κB and 90 RLU for AP-1 luciferase activity were measured (data not shown). Immature thymocytes are expected to yield lower activities than mature T cells as they express lower levels of TCR. The phenotype of thymocytes was determined by staining with anti-CD4 and anti-CD8 antibodies (Figure 1D). Note that the lower left quadrant contains mainly irradiated APCs depleted of T cells. As expected after addition of antigen the number of DP cells was reduced and CD4 and CD8 expression was downmodulated. After 53 h of culture nearly all DP thymocytes have died (Figure 1D), not surprisingly neither AP-1 nor NF-κB were detected (Figure 1A,B). Consistent with our previous results using gel shift assays,[3] AP-1 was transcriptionally active in DP thymocytes when these were stimulated for 39 h in the presence of antigen and specific peptide (Figure 1B). However, at any time point after antigen stimulation and even in the presence of peptide, NF-κB was not transcriptionally active in the DP thymocytes (Figure 1A). In contrast total thymocytes showed NF-κB activity after 39 h in the presence of H-$2K^b$ and addition of exogenous peptide or 53 h without additional peptide. This activity is due to thymic SP present in the total population (Figure 1C) since DP have disappeared at that time point (Figure 1D). Thus not only is the DNA binding activity of NF-κB reduced in DP thymocytes but its capacity to induce gene transcription in response to antigen is completely abolished.

Furthermore, the lack of NF-κB activation at the DP stage of development correlated with the capacity of antigen to induce death. Twenty per cent of DP died in response to antigen (background of death in the presence of syngeneic APCs was subtracted) according to Annexin V staining (Figure 1C). Only 10% of thymic SP and less than 5% of peripheral $CD8^+$ died in response to antigen (Note: in this experiment DP and peripheral SP were 96% pure whereas thymic SP were enriched to 70%, the remaining 30% being mainly DP). In contrast, NF-κB transcriptional activity is undetectable in DP thymocytes whereas some is detected in thymic SP, and in the mature peripheral T cell population NF-κB is increased threefold as compared to thymic SP (Figure 1C). Hence, there is an inverse relationship between susceptibility to death and transcriptional activity of NF-κB in response to antigen during the development of thymocytes. The relative luciferase units (RLU) varied between experiments (see Figure 1A,C), but relative results obtained were reproducible.

The ras/Mek pathway is functional in DP thymocytes and controls AP-1 binding activity in response to antigen

To gain insight into the role of the ras/Mek pathway after antigenic stimulation we crossed mice expressing both the dominant negative proteins of ras and Mek-1 (dnras/dMek), showing a complete inhibition of ras signalling,[9] onto the

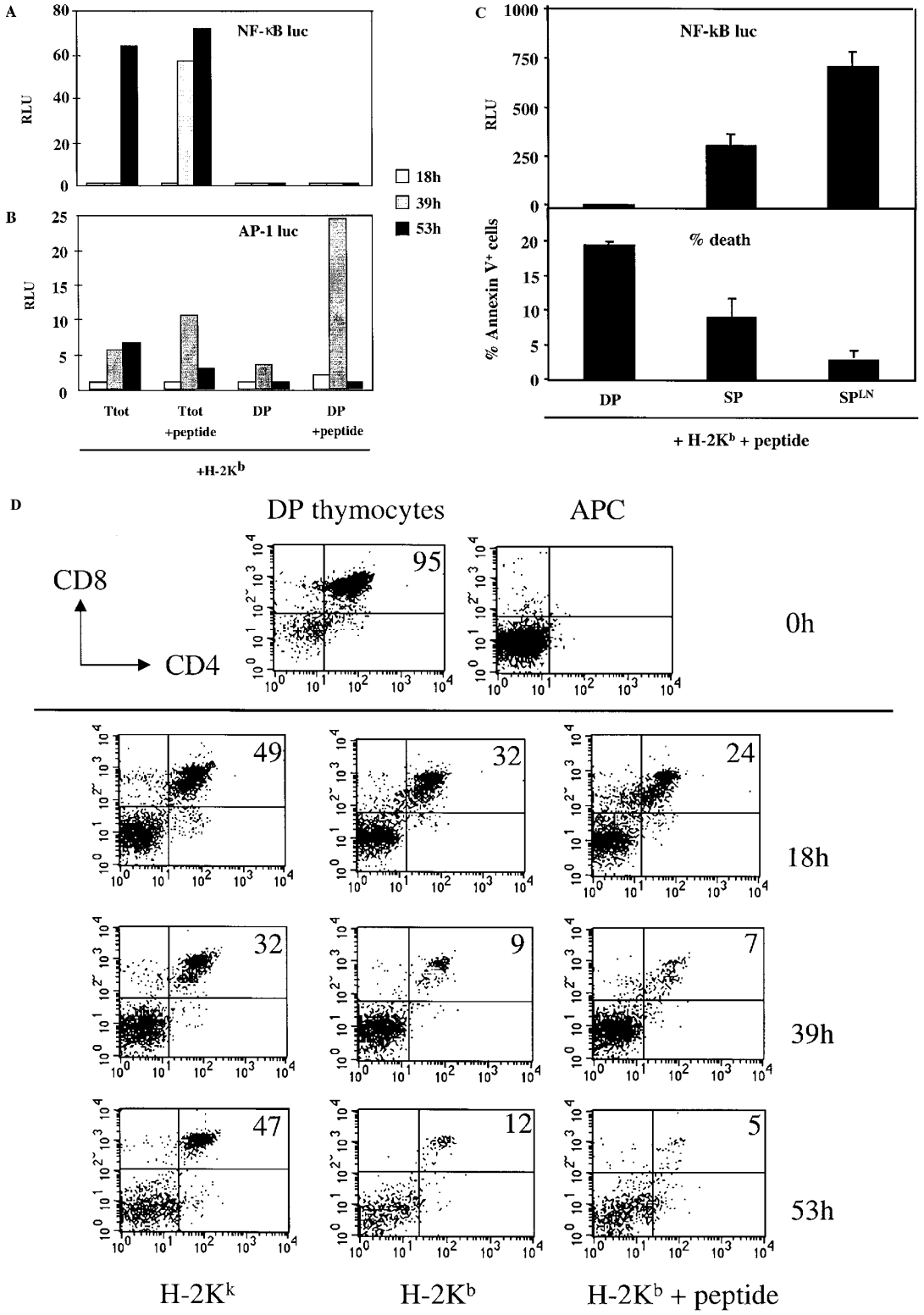

Figure 1 DP thymocytes lack NF-κB transactivation in response to antigen. 2×10^6 total thymocytes (Ttot), purified DP thymocytes (DP), thymic $CD8^+$ (SP) or peripheral $CD8^+$ (SP^{LN}) from double transgenic TCR × NF-κB luciferase (**A**) or TCR × AP-1 luciferase (**B**) mice were cultured in the presence of H-2^k or H-2^b APC for 18, 39 or 53 h. Where indicated 10^{-7} M specific antigenic peptide was added. Luciferase activity was measured in the cell extracts and values for non-stimulated samples (H-2^k APC) were subtracted. The experiments shown for NF-κB and AP-1 are representative experiments out of three. In (**C**) luciferase activity for NF-κB and percentage of Annexin V positive cells were measured after stimulation with H-2^b and peptide for 39 h in the same experiment. Triplicates were used to calculate the standard deviation based on small sample size. (**D**) shows the staining with anti- CD4 and anti-CD8 antibodies of APC and purified DP thymocytes before stimulation (two upper dot blots) and purified DP thymocytes at 18, 39 and 53 h in the presence of APC with H-$2K^k$, H-$2K^b$ or H-$2K^b$+peptide where indicated (nine lower dot blots). Note that in the dot blots where APCs have been added, lower left quadrants contain APCs depleted of T cells

TCR transgenic mice KB5.C20 recognizing the alloantigen H-2K^b. In the H-2$^{k/b}$ negative selection background, thymocytes from TCR transgenic mice showed the same phenotype whether or not they additionally expressed dnras/dMek (data not shown) confirming results obtained in other TCR transgenic systems.[9] On the H-2$^{k/k}$ positive selection background mice expressing dnras/dMek had fewer single positive (SP) CD8 cells (reduced 2.6-fold $\pm$0.6). This phenotype was less drastic than the one described in other TCR transgenic systems with inhibition of ras signalling.[9] Furthermore the mature SP CD8$^+$ expressed the same level of transgenic TCR whether or not they expressed dnras/dMek (data not shown). However, thymocytes from dnras/dMek mice proliferated less well to PMA/ionomycin (64% reduction), anti CD3/PMA (61% reduction) or antigen (73% reduction) indicating that the transdominant proteins are functional in thymocytes. In contrast we detected no difference in proliferation capacities to the same stimuli in the periphery (data not shown). This is consistent with the low transgene expression generally observed in peripheral T cells under the control of the lck proximal promoter.[16]

After stimulation with antigen without or with additional cognate peptide for 18 h, cell extracts were analysed in gel shift assays. Controls using Oct-1 oligonucleotides were performed. Oct-1 transcriptional activity was detected in all lanes, although cell extracts from dnras/dMek mice showed slightly weaker activities than extracts from littermates (data not shown). As previously described, antigen induced AP-1 DNA binding which was further increased in the presence of additional cognate peptide. Expression of dnras/dMek reduced AP-1 DNA binding activity threefold (Figure 2, upper panel). NF-κB DNA binding was only weakly induced at the DP stage of development even after stimulation in the presence of additional cognate peptide. This weak induction was further reduced in thymocytes from dnras/dMek mice (Figure 2, lower panel). These results demonstrate that the DNA binding activity of AP-1 after stimulation with antigen is partially dependent on the ras/Mek pathway. For NF-κB the signal in DP thymocytes was too weak to affirm it with certainty.

To investigate the control over transcriptional activity by the ras/Mek pathway further, triple transgenic mice expressing the TCR transgene, the luciferase reporter transgene under the control of AP-1 binding sites and transdominant negative Mek and ras proteins were made. DP thymocytes from littermate mice expressing only AP-1 luciferase and the TCR transgene show an increased transcriptional activity for AP-1 after 39 h (Figure 3A) in response to antigen and cognate peptide. This is consistent with our gel shift results. However, expression of the dnras/dMek transgene reduces this signal twofold (Figure 3A). Considering the total absence of NF-κB activity in response to antigen in DP cells, it is difficult to determine the role of the ras/Mek pathway for this factor using the luciferase read-out (data not shown). The absence of NF-κB transactivation in total thymocytes from dnras/dMek mice (data not shown) could either be due to the absence of SP thymocytes in these mice or to the regulation of NF-κB by the ras pathway.

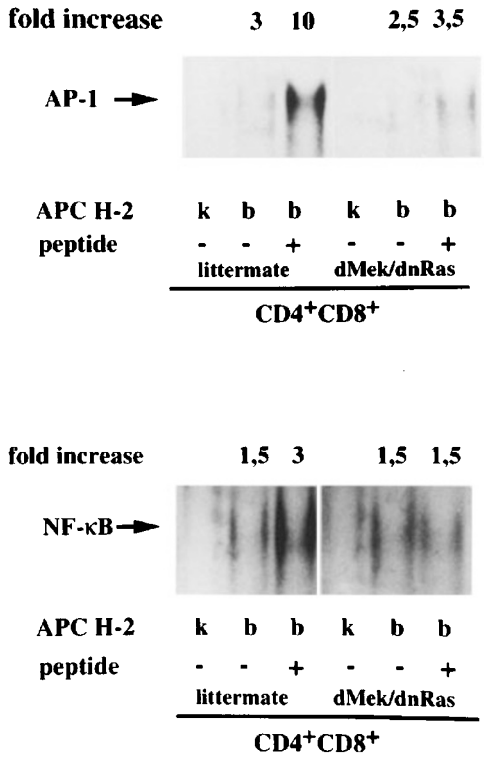

Figure 2 DP thymocytes with deficient ras/Mek pathway induce weak AP-1 DNA binding activity in response to antigen. Purified DP thymocytes from K^b-specific KB5.C20-TCR transgenic mice containing the transgene for the dnras/dMek proteins or not (littermate) were stimulated in the presence of irradiated T cell-depleted APC of the H-2^k or H-2^b haplotype for 18 h. 10^{-7} M specific antigenic peptide was added where indicated. Nuclear extracts were prepared and analyzed by gelshift for the transcription factors AP-1 (**A**) and NF-κB (**B**). Quantification of bands was performed on a phosphor plate system (Fujix Bas 1500, Fuji, Japan). A control gelshift with Oct-1 oligonucleotides showed similar binding activities for cell extracts from mutant and littermate mice (data not shown). The *in vitro* stimulation and subsequent gelshift was repeated four times with the same results as those shown

DP thymocytes do not intrinsically lack NF-κB activity

As expected PMA/ionomycin induced transcriptional activity for NF-κB in DP thymocytes. This confirmed previous results obtained using gel shift assays to detect DNA binding activity for NF-κB [3] and shows that they do not have an intrinsic defect in mobilizing NF-κB transactivation (Figure 3B). It is generally believed that the ras/Mek pathway is solicited when T cells are stimulated through their TCR and it is becoming clear that phorbol esters are also very effective at activating ras in T cell lines.[17] Our results show that the induced activity of NF-κB in DP thymocytes by PMA/ionomycin was reduced threefold after 39 h and 1.6-fold after 53 h of stimulation in DP thymocytes expressing dnras/dMek (Figure 3B). This clearly shows the activation of ras by phorbol esters/ionomycin in these cells. However, PMA/Ionomycin failed to induce AP-1 transactivation in DP thymocytes (data not shown), consistent with our [3] and previous [18] results using gel shift assays and others using

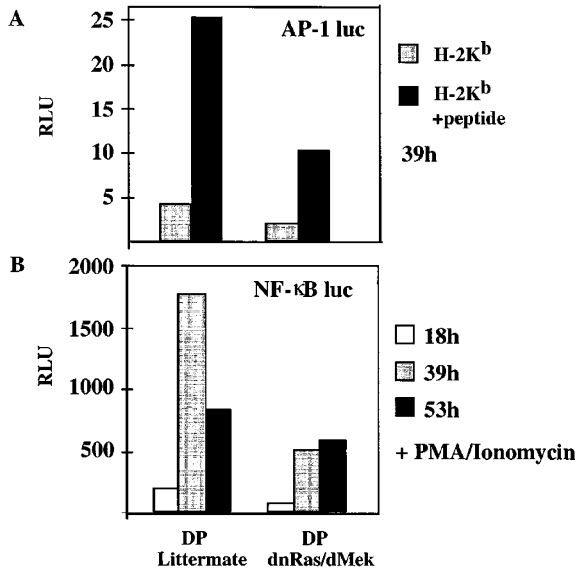

Figure 3 (**A**) DP thymocytes with deficient ras/Mek pathway induce weak AP-1 transcriptional activity in response to antigen. DP thymocytes from triple transgenic mice (TCR × AP-1 luciferase × dnras/dMek) were stimulated for 39 h with antigen and analyzed as described in Figure 1. This experiment was repeated twice with similar results. (**B**) DP thymocytes with deficient ras/Mek pathway have reduced NF-κB transcriptional activity in response to PMA/ionomycin. 2×10^6 purified DP thymocytes (DP) from triple transgenic mice TCR × dnras/dMek × NF-κB luciferase or TCR × dnras/dMek × AP-1 luciferase were cultured in the presence or absence of PMA plus ionomycin for 18, 39 or 53 h. Littermates did not express the dnras/dMek transgene but expressed the two other transgenes. Luciferase activity was measured in the cell extracts and background (no stimulation) was substracted from values. The experiments shown are representative experiments out of three

the same reporter mice.[19]

In total thymocytes, both PMA/Ionomycin and antigen induced DNA binding of the p65/p50 complex (in supershift experiments [3]). This could be measured 3 h after stimulation with PMA/Ionomycin whereas antigen required 9 to 12 h stimulation. This suggests that there is no qualitative difference but only a kinetic difference between the two stimulation with respect to induced NF-κB complexes.

In summary these results indicate that the ras/Mek pathway is functional in DP thymocytes as the transcriptional activity of AP-1 and NF-κB seem to be, at least partially, under its control. This suggests that another upstream signalling pathway is responsible for the failure of DP thymocytes to activate NF-κB in response to antigen.

Lack of PKCε protein in preselection CD4+CD8+ thymocytes

To test whether DP thymocytes may present a selective defect in PKC signalling we first analyzed the expression of different PKCs in preselection DP and mature CD8+ (SP^{LN}) by Western blot. Western blots incubated with the antiserum obtained from Peter Parker show that DP thymocytes from TCR transgenic mice (KB5.C20) are deficient in PKCε whereas they express PKCα and PKCβ (Figure 4A). The expression of the α and β isoforms is consistent with two

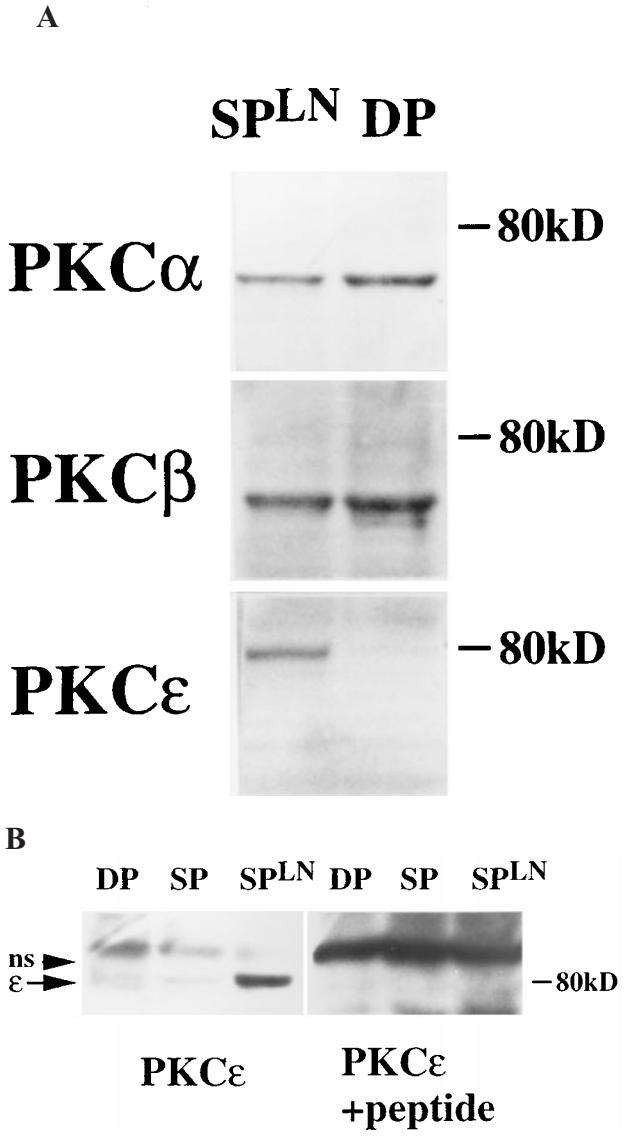

Figure 4 Differential expression of PKCα, PKCβ and PKCε proteins in extracts of DP *versus* SP thymocytes and lymph node T cells. Cell extracts of 10×10^6 preselection DP thymocytes (DP), thymic CD8+ (SP) or mature T cells from lymph nodes (SP^{LN}) from KB5.C20 mice were run on a 7–17% gradient gel, blotted and incubated with the antisera obtained from Peter Parker. (**A**) PKCα and β but not PKCε were detected in preselection DP thymocytes (**B**) Low expression of PKCε in preselection DP and SP thymocytes but high expression of PKCε in lymph node SP T cells. Method as in **A** but specific peptide from PKCε was added prior to incubation with antiserum against PKCε. A non-specific band was detected in this immunoblot (arrow labelled ns). In left panel the arrow labelled ε indicates the specific band for PKCε as this band disappears when peptide is added (right panel). Ponceau S staining showed equal loading of protein (data not shown)

previous reports.[20,21] Peripheral SP T cells express PKCα, β and ε (Figure 4A). Competition with a specific peptide shows that the PKCε band (around 80 kD) is specific, whereas a non-specific band (ns) below PKCε (i.e. not competed out by the PKCε -derived peptide) is also revealed (Figure 4B). Thymic CD8+ (SP) express an intermediate amount of PKCε (Figure

4B). This correlates with an intermediate amount of NF-κB and antigen-induced cell death in this population. As this result contradicts two other studies showing that PKCε was present in $CD4^+CD8^+$ thymocytes, [21,22] we extended our analysis by using a commercially available antiserum (Santa Cruz) against PKCε and PKCα (Figure 5). Both antiserum from Peter Parker and the one from Santa Cruz were raised against the C-terminus of PKCε and showed the same result. However, during the course of our analysis, the group of Ohaka [23] published a study which contradicts their initial observation that PKCε was predominantly expressed in DP thymocytes, since they observed that thymocytes from C57BL/6 mice lack PKCε whereas their first study was performed on Balb/c. Thus to test whether the lack of PKCε was specific of the TCR transgenic strain KB5.C20 (on B10.BR background), we analyzed the expression of PKCε and α on three different mouse strains including normal B10.BR mice, transgenic mice expressing a TCR restricted by MHC class II (HEL) (on a CBA/J background), and transgenic mice expressing a TCR restricted by class I (KB5.C20). DP thymocytes from all mouse strains show a defect in PKCε expression (Figure 5B). Peripheral $CD4^+$ cells from HEL mice, $CD8^+$ from KB5.C20 mice or $CD4^+$ and $CD8^+$ cells from B10.BR all expressed PKCε. All cell populations tested were positive for PKCα (Figure 5B). Finally to determine whether the discrepancy between our results and those of others was due to the preparation of the extracts, we followed the protocol for the preparation of cytosolic and particulate fractions by Strulovici *et al*.[21] Particulate and cytosolic fractions of preselection DP thymocytes were negative for PKCε but positive for PKCα (data not shown) confirming our previous results. It has been suggested by others [24] that erroneous detection of PKCε could be due to cross-reactivity of putative PKCε antisera with PKCη since the protein sequences of these two isoforms are similar and PKCη is expressed abundantly in thymus, normal T cells and T cell lines.

We conclude that preselection DP thymocytes have a lower expression of PKCε than peripheral T cells (this comparison was never shown in any previous study) and that its expression is regulated during thymic development, as DN express a low amount (data not shown), preselection DP are deficient and thymic SP upregulate PKCε expression.

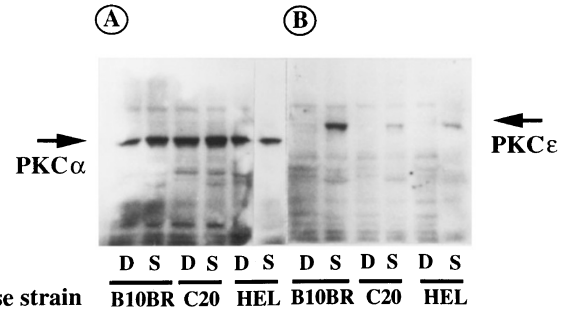

Figure 5 Lack of PKCε in immature DP thymocytes in different mouse strains. Western blot (7% acrylamide) of lysates of 5×10^6 immature DP thymocytes (D) or lymph node SP T cells (S) from indicated mouse strains. Lymph node SP T cells from B10.BR mice were a mixed population of $CD4^+$ and $CD8^+$ cells whereas SP T cells from KB5.C20 (C20) or HEL TCR transgenic mice were either $CD8^+$ or $CD4^+$ cells, respectively. (**A**) The immunoblot was incubated with anti-PKCα antiserum (Santa Cruz). (**B**) The immunoblot was incubated with anti-PKCε antiserum (Santa Cruz)

Discussion

Death correlates with lack of NF-κB transcriptional activity during thymic development

Using mice transgenic for a TCR and for a reporter gene construct controlled by either NF-κB or AP-1 binding sites, we show that there is a lack of NF-κB transactivation after *in vitro* stimulation with antigen of immature DP thymocytes whereas AP-1 is transcriptionally active. Our results show that as thymocytes become less susceptible to antigen-induced death when they mature from DP to thymic SP and peripheral SP CD8 cells, NF-κB activity increases inversely. Thus the agonist responsible for negative selection events in DP thymocytes activates AP-1 transactivation but fails to induce the 'survival transcription factor' NF-κB. Pharmacological stimuli, however showed that thymocytes at this stage do not have an intrinsic defect in NF-κB induction.

The lack of NF-κB after antigenic stimulation is particularly interesting as NF-κB was shown to be a 'survival transcription factor'. A T cell hybridoma was rendered more susceptible to TCR-induced apoptosis through inhibition of NF-κB activation by a soluble peptide inhibitor. Correspondingly, transfection of p50 and p65 provided considerable protection from TCR-induced apoptosis in these cells.[25] Similarly to our results with antigen, NF-κB is downmodulated in thymocytes in response to *in vivo* injection of glucocorticoids preceding death. Induction of NF-κB diminished dexamethasone-induced thymocyte death.[26] However, in a transgenic mouse model where NF-κB is constitutively inhibited by a superinhibitory IκB-α protein, anti-CD3 induced death in thymocytes *in vivo* is inhibited.[27] The discrepancy between this result and ours could be due to differential signalling in response to anti-CD3 and antigen+APC.[28] Whether overexpression of NF-κB can rescue thymocytes from TCR-mediated death and lead to the emergence of autoreactive T cells has yet to be shown.

Furthermore it is not clear whether TCR-mediated signals only sensitise DP thymocytes to apoptotic stimuli provided by other receptors like members of the TNF receptor family.[29] This sensitisation to apoptosis could partly be due to a lack of NF-κB. Thus, TNF stimulated anti-death activity appears to be mediated by the NF-κB family [30–34] (or reviewed in [35]). In B lymphocytes NF-κB mediated upregulation of Bcl-x is required for CD40 survival signalling.[36] In a transgenic mouse model of CD30 overexpression in the T cell lineage, apoptosis can be induced in thymocytes when TCR and CD30 are simultaneously engaged. This engagement does not induce NF-κB and can be inhibited by Bcl-2.[37] Taken together our results suggest that the lack of NF-κB activity in response to antigen in DP thymocytes may contribute to the susceptibility of DP thymocytes to death.

Our finding of the lack of NF-κB activity has been confirmed in two different experimental systems, one

revealing DNA binding activity of transcription factors (this study and [3]) and the other one using luciferase reporter mice detecting transactivation. This is particularly important as it was shown that DNA-binding is not always sufficient to induce transcriptional activity and that the two events can be regulated at different levels. Thus, degradation of IκBα not only releases NF-κB, permits its translocation to the nucleus and binding to DNA but also releases and activates the catalytic unit of protein kinase A (PKAc). PKAc phosphorylates the subunit p65 of NF-κB and increases transactivation whereas the DNA-binding is not affected by this phosphorylation step.[38] The transcriptional activity of NF-κB is thus regulated at two levels (i) its release from cytosolic complexes with IκB and (ii) its phosphorylation by PKA. Such a layering of regulatory mechanisms could contribute to fine tuning the expression of genes regulated by NF-κB, especially if the two mechanisms were targeted differentially by upstream signals. In our study, transactivation and DNA-binding activity of AP-1 seem to correlate very well. However, after antigenic stimulation in DP thymocytes some low level NF-κB DNA-binding was detected without transcriptional activity suggesting that the pathway activating the regulatory mechanism for transactivation had not been induced. It would be of interest to determine whether such a mechanism could contribute to survival or death in thymic selection processes.

However, we show that DP thymocytes are not intrinsically deficient in NF-κB as phorbol ester/ionophore stimulate NF-κB transcriptional activity. In the system described here, PMA/ionomycin induces death (data not shown) although NF-κB transcriptional activity is present. Thus only antigen-specific deletion may be affected by the defect in NF-κB transactivation. This contradiction can be explained by the fact that PMA/ionomycin and antigen use a very different set of signalling molecules. Indeed, in thymus organ cultures phorbol esters/ionophores cause downregulation of CD4 alone as opposed to downregulation of both coreceptors induced by antigen.[39] Furthermore phorbol ester/ionophores do not induce death in thymic organs whereas antigen does (data not shown and [39]). Our findings concerning the selective defect in PKCε in DP thymocytes could account for the lack of NF-κB activity in response to antigen as phorbol esters/ionophores may use other PKC isoforms to signal.

Transactivation of AP-1 by antigen and of NF-κB by phorbol ester/ionophore are partly controlled by the ras/Mek pathway in DP thymocytes

The AP-1 transcription factor is composed of proteins of the c-jun and c-fos family. Phosphorylation of N-terminal serines of c-jun by a protein kinase termed JNK (Jun kinase) has been shown to increase transcription without affecting DNA-binding (reviewed in [40]). The JNK isoforms are members of the MAPK family, like ERK1 and ERK2 (extracellular signal regulated protein kinase). Both the ERKs and JNKs are activated by oncogenic H-*ras*. Ras activation triggers two divergent signalling cascades that activate distinct MAPKs. One is initiated by raf1 leading to Mek-1 (MAPK/ERK kinase) and Mek-2 and then ERK1 and ERK2 activation that phosphorylate and potentiate the activity of TCF/Elk-1 and thereby induce c-fos.[17] The other one is initiated by MEKK leading to JNK activation that phosphorylates and potentiates the activity of c-jun.[41] The mice used in this study express a dominant-negative mutant of ras, as well as a catalytically inactive form of Mek-1. Unexpectedly we found the transcription of AP-1 only partly inhibited by the transdominant proteins. This may be either because the dominant-negative proteins are not sufficiently expressed or because a ras-independent signalling pathway for AP-1 exists. The fact that proliferation and CD69 expression is only partly inhibited in thymocytes from dnras/dMek expressing mice favours the first explanation (data not shown). On the other hand selective inhibition of PKCs inhibits IL-2 expression in a human T cell line without affecting ras-induced AP-1 activation suggesting a parallel pathway.[42] This is, however, contradicted in the case of DP thymocytes (this study and [3,18]) where activation with PMA, a potent stimulator of PKCs, or even PMA in combination with ionomycin does not induce AP-1. It suggests that AP-1 activity after antigenic stimulation in DP thymocytes is probably under the control of ras. Furthermore it was suggested that the dnras/dMek mice have an impaired positive selection in the thymus but show no defect in negative selection.[9] Together with our data showing the partial control of AP-1 by this signalling pathway, it may indicate that AP-1 could be an important transcription factor in positive selection events that would not contribute to negative selection.

Concerning the control of NF-κB by the ras/Mek pathway our data suggests that the lack of NF-κB in DP thymocytes in response to antigen is not due to the absence of ras signalling since this pathway is functional for AP-1 signalling. However, phorbol ester activates the ras pathway for NF-κB activity, probably via PKCs. In T cells it has been shown that some of the effects of phorbol esters previously attributed to PKCs alone are also ras-mediated.[43] On the other hand, antigen presented on APCs does not induce the activity of NF-κB in DP thymocytes. It is possible that distinct PKCs are required after TCR engagement and PMA/ionomycin stimulation in DP thymocytes. This hypothesis is further supported by our finding that preselection DP thymocytes lack PKCε protein whereas $CD4^-CD8^-$ and mature peripheral T cells express PKCε at high levels.

Lack of PKCε in DP thymocytes

This study shows a lack of PKCε but not of PKCα nor PKCβ in DP thymocytes. Furthermore thymic SP express intermediate amounts of PKCε and peripheral T cells have a comparable high level of PKCε and PKCα expression.

The family of PKCs consists of isozymes that have been categorised into three classes: conventional PKCs (α and β) which are regulated by diacylglycerol, phosphatidylserine and Ca^{2+}, novel PKCs (δ, ε, η, θ) which are regulated only by diacylglycerol and phosphatidylserine, and atypical PKCs (ζ, τ and λ) whose regulation has not been clearly established, although their activity is stimulated by phosphatidylserine. Phorbol esters can substitute for

diacylglycerol and activate all the PKCs except the atypical PKCs.

Since phorbol esters alone or in conjunction with Ca^{2+} ionophores are capable of inducing many differentiated functions of T lymphocytes, it is evident that PKCs play an important role in the activation of T cells (reviewed in[17]). Studies on the expression of different isotypes detected mRNA for PKCα, β, ζ, ε and most abundantly η and θ in mouse T cells and T cell lines.[44–46] PKC θ is thought to play a special role in T cell activation [47] and cell death via induction of Fas ligand.[48] However, in one study PKC θ does not activate NF-κB or NF-AT in T lymphocytes but rather AP-1.[49] Furthermore it is present abundantly in thymocytes.[46] A more recent study using PKCθ deficient mouse shows that PKCθ is necessary for activation of NF-κB by anti-CD3 in mature lymphocytes but not immature lymphocytes.[50] In the human T cell line Jurkat, transfection of constitutively active PKCs shows that PKCε and to a lesser extent PKCα, but not PKCζ, can regulate the transcription factors NF-AT and AP-1.[6] NF-κB transactivation is induced by a constitutively active PKCε but not PKCα nor activated ras.[6] Moreover, the IKK-related kinase named NAK (NF-κB activating kinase) mediates IKK and NF-κB activation in response to growth factors that stimulate PKCε activity but not PKCα or θ.[51] Together with our results showing a similar developmental control of NF-κB and PKCε, these findings suggest that PKCε may control NF-κB in thymocytes. Another indication that PKCs signal for NF-κB comes from the finding that TNF receptor signals for NF-κB via a cascade of molecules including the atypical PKCδ and PKCλ.[52] This result is particularly interesting in the connection between NF-κB, PKCs and TNF receptor signalling for death. More specifically the isoform PKCε mediates phorbol ester inhibition of TNFα-induced apoptosis in U937 cells.[53] Overexpression of PKC isoform ε but not δ in human interleukin-3-dependent cells suppresses apoptosis and induces Bcl-2 expression.[54] Hence, PKC inhibitors have been shown to block phosphorylation of Bcl-2 and lead to apoptosis whereas activation of PKC induced phosphorylation of Bcl-2 and abolished the apoptotic process.[54–56] In the thymus nearly all $CD4^+$ and $CD8^+$ SP thymocytes express Bcl-2 whereas the majority of DP thymocytes do not.[57,58] The constitutive lack of PKCε in DP thymocytes detected in this study could be the reason for the developmentally regulated expression of Bcl-2. Recently it was demonstrated that a general inhibitor of all PKC isoforms prevented peptide-specific apoptosis in thymocytes with indirect evidence suggesting the responsible isoform might be PKC θ.[59] In contrast a PKC inhibitor facilitates Fas-mediated apoptosis in T cells and inhibits T cell-mediated autoimmune disease.[60] The controversy in these studies on the role of PKC activity in apoptosis suggests that different PKC isoforms signal for different events in T cells. Overexpression or genetically targeted deletion of these isoforms in the thymus would elucidate their role in negative selection. Furthermore a detailed analysis of the genomic structure of PKCε would elucidate how this isoform is regulated during T cell development.

Materials and Methods

Mice

Mice transgenic for the KB5.C20 (also named Des) TCR have been described elsewhere.[61] Mice, between 4 and 8 weeks old, were of the $H\text{-}2^k$ haplotype corresponding to the background of positive selection. Spleen cells from B10.BR ($H\text{-}2^k$, control) and C57BL/6 (B6, $H\text{-}2^b$) were used as stimulating cells. KB5.C20 TCR is specific for an endogenous peptide present in B6 cells in association with $H\text{-}2K^b$. Specific peptide (pKB1, Guimezanes *et al*, submitted) was added when indicated. Mice expressing simultaneously a dominant-negative ras (dnras) and Mek-1 (dMek) transgenes under the $p56^{lck}$ proximal promoter were provided by RM Perlmutter[9] and crossed onto B10.BR mice. Mice transgenic for luciferase under control of AP-1 binding sites (AP-1 luc) were made as described.[62] The NF-κB luciferase reporter mice (NF-κB luc) were made using the pBIIX construct with two copies of the κB sequence from the Igκ intronic enhancer.[63] Triple transgenic mice containing the transgenes for the T cell receptor, the dnras/dMek and the reporter molecules (AP-1 luc or NF-κB luc) were obtained by crossing in our animal facility. Mice transgenic for the TCR recognizing hen egg lysozyme (HEL) and bred onto a CBA/J background were a kind gift from Mark Davis (Stanford University School of Medicine).[64] B10.BR and CBA mice were bred in the animal facility of the Centre d'Immunologie de Marseille Luminy.

Thymocytes and *in vitro* stimulation

Single cell suspensions of thymocytes were obtained by gentle disruption of an intact thymus. Preselection DP (MHC class I^{low} TCR^{low} $CD69^{low}$) were purified by negative selection using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously.[13] Thymic $CD8^+$ (SP) and peripheral $CD8^+$ (SP^{LN}) were purified by negative selection using rat anti-CD4 supernatant (H129.19.6). All mAb were as previously referenced.[3] Thymic populations were added to equivalent numbers of irradiated splenic T cell-depleted antigen presenting cells (APCs) from mice of the $H\text{-}2^k$ or $H\text{-}2^b$ haplotype in the presence of 10^{-7} M specific peptide, where specified, for indicated periods of time. Thymocytes were incubated at 1×10^6 cells per well in the presence of APCs or 10 ng/ml phorbol-myristate acetate (PMA, Sigma, St. Louis, MO, USA) plus 250 ng/ml ionomycin (Calbiochem, La Jolla, CA, USA). Three-colour flow cytometry was performed on thymocytes using anti-CD8 APC (Pharmingen, San Diego, CA, USA), anti-CD69 antibody coupled to FITC (prepared in the laboratory) and anti-CD4 coupled to phycoerythrin (Caltag, San Francisco, CA, USA). Annexin V-FITC was purchased from Euromedex (Souffelweyersheim, France) and used according to the manufacturer's instructions.

Nuclear extract preparation and electromobility shift assay

Nuclear extracts from thymocytes were prepared as described.[3] Briefly 5 to 10×10^6 thymocytes were washed with PBS and resuspended in lysis buffer (10 mM HEPES, pH 7.9, 10 mM Tris, 60 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride). The nuclei were pelleted and resuspended in 50 μl nuclear resuspension buffer (250 mM Tris, pH 7.8, 60 mM KCl, 1 mM dithiothreitol) and sonicated briefly. Nuclear extracts were cleared by centrifugation, protein concentrations in the extracts were determined and equivalent amounts of extracts were used for *in vitro* binding assays. Binding assays in the presence of labelled oligonucleotides containing the AP-1 and NF-κB binding sites were carried out as described.[3] Supershift experiments were

performed previously showing that oligonucleotides are specific for their targets.[3] Oct-1 oligonucleotides were used as a control to check whether cell extracts contained DNA binding activity (data not shown). Oct-1 probes had the following sequence:

5′CTCTTTGAAAATATGTGTAATATGTAAAACATTT

AGCTGAGAAACTTTTATACACATTATACATTTTGT 5′.

Luciferase activity analysis

2×10^6 thymocytes were cultured for indicated periods of time, *in vitro* stimulated, washed twice in PBS and lysed in lysis buffer (Luciferase Assay, Promega, Madison, WI, USA) for 30 min at room temperature. The lysate was spun down for 2 min and total supernatant was analyzed using a luciferase reagent (Promega) and measured in a luminometer (Lumat LB96P, EG&G Berthold) for 10 s. The background (syngeneic APCs or absence of PMA/ionomycin) was subtracted from measured values.

Western blot

5×10^6 DP thymocytes, thymic $CD8^+$ (SP) or peripheral T cells (SP^{LN} in Figure 4 or S in Figure 5) were washed and cell extracts were prepared by lysis in PBS containing 2% SDS. DNA was eliminated, and proteins lyophilized, reconstituted in SDS sample buffer, and separated by SDS–PAGE in 7% polyacrylamide gel. Proteins were transferred to PVDF-membranes and immunoblots performed. Antisera against PKC α, ε, ζ, δ (a kind gift from Peter Parker) were diluted 1 : 500 for ε and δ; 1 : 100 for α and β. Specific peptide from PKCε was added just before and at the same concentration as the antiserum. Rabbit polyclonal IgG against PKCε and α for a second series of blots were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). These antisera were used at 1 : 5000 (0.02 μg /ml) and the blots revealed using a chemiluminescence-dependent method (ECL, Amersham).

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