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Calmodulin-Dependent Protein Kinase IV during T-Cell Development

Joachim Krebs,* Anne Wilson,† and Pawel Kisielow‡

**Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland; †Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, CH-1066 Epalinges, Switzerland; and ‡Basel Institute of Immunology, Hoffmann La Roche, CH-4005 Basel, Switzerland*

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In a primary cell culture system of fetal rat brain, the calmodulin-dependent protein-kinase IV (CaMKIV) could be induced by the thyroid hormone T_3 in a time- and concentration-dependent manner, provided the tissue was excised not later than day 15 of gestation (E15) (Krebs *et al., J. Biol. Chem.* 271, 11055, 1996). We report here that in the fetal thymus CaMKIV could not be detected earlier than day 16 of gestation and that the expression of this enzyme was fully upregulated at day 18. In mouse fetal thymus organ culture (FTOC) of day 14 embryonic thymus, CaMKIV could not be detected, even after several days of culture if a minimal culture medium lacking fetal calf serum was used. However, after addition of fetal calf serum to the culture medium the expression of CaMKIV could be specifically induced. Furthermore, it could also be shown that during T-cell development in the adult murine thymus the expression of CaMKIV was tightly regulated. Taken together, these results demonstrate that the expression of CaMKIV, an enzyme involved in the regulation of Ca^{2+}-dependent gene expression, is itself under stringent regulatory control during tissue development.

Cellular processes such as growth, differentiation and apoptosis are critically dependent on signal transduction cascades mediated by changes in intracellular Ca^{2+} concentration [1]. This is also true for the activation of T-lymphocytes in response to T-cell receptor occupancy. Ca^{2+} concentration increases rapidly upon T-cell activation, and is sustained at a high level over a considerable period of time inducing transcriptional activation of immediate early genes (IEG)[1] such as c-fos and c-jun [2]. The latter, together with members of the so-called nuclear factor of activated T-cells (NFAT) family, are required to induce activation of cytokine genes such as interleukin 2 (IL-2). The involvement of the Ca^{2+}-calmodulin dependent phosphatase calcineurin in activating the cytoplasmic component of NFAT by dephosphorylation is well documented (for review see Ref. 3). However, requirements for the induction of the AP-1 genes c-fos and c-jun are less clear. Recently, Barton et al. [4], demonstrated the importance of phosphorylation of the cAMP regulatory element binding protein CREB during T-cell activation. A non-functional mutant form of CREB which was unable to be phosphorylated at Ser133, (essential for activation), was specifically targeted to T cells, resulting in inhibition of IEG expression and IL-2 production. Since transcription initiated through T-cell activation requires Ca^{2+}-dependent steps prior to CREB phosphorylation, it is possible that a Ca^{2+}-dependent protein kinase might be responsible for CREB phosphorylation. Indeed, Anderson et al. [5], reported that transgenic mice expressing a catalytically inactive form of CaMKIV specifically in thymic T-cells, showed a severe reduction in IL-2 production due to a dominant-negative effect on CREB phosphorylation.

CaMKIV or "Gr" [6] is, after nervous tissues, mainly expressed in the thymus [7], and to a somewhat lower extent in the spleen and in testis, but is undectable in all other tissues examined [6, 8-10]. Major substrates of CaMKIV seem to be transcription factors such as CREB [11, 12], the serum response factor SRF [13, 14] or members of the ETS family of transcription factors [15, 16] which could be phosphorylated by CaMKIV. Since CaMKIV has recently been reported to be located also in the nucleus [11, 17], the enzyme could have direct access to transcription factors to regulate their

The abbreviations used are: CaM, calmodulin; CaMKIV, Ca^{2+}-CaM-dependent protein kinase IV or Gr; CREB, cAMP response element binding protein; DP $CD4^+CD8^+$ double positive cells; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; FTOC, fetal thymus organ culture; IEG, immediate early gene; PBS, phosphate buffered saline; SP $CD4^+CD8^-/CD8^+4^-$ single positive cells; SRF, serum response factor; T_3, 3,3′,5-triiodo-L-thyronine.

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function in a Ca^{2+}-dependent manner. It has been shown in different cell lines overexpressing CaMKIV, that this enzyme is involved in the regulation of expression of IEGs, either through CREB [11, 12, 18-20], or through SRF [14]. Furthermore, in a primary culture sytem of fetal rat brain tissue, we showed previously that Ca^{+}-dependent expression of the immediate early gene c-fos is strongly dependent on the presence of CaMKIV [21].

We recently [22] demonstrated that the expression of CaMKIV could be induced by the thyroid hormone 3,3′,5-triiodo-L-thyronine T_3 at a very early stage of development, i.e. usually at days E15/E16 of gestation. This was shown in a primary tissue culture system of fetal rat brain at both transcriptional and translational levels, where the addition of actinomycin D and/or cycloheximide to the culture medium could prevent T_3-dependent induction of CaMKIV, indicating indirect induction of the enzyme since protein synthesis seemed necessary.

In the present work, we report that in a murine E14 fetal thymic culture system (FTOC), CaMKIV was not detectable, even after several days of culture if a minimal culture medium lacking fetal calf serum was used. However, on addition of fetal calf serum, the expression of CaMKIV could be specifically induced. Quantitation of CaMKIV at different days of gestation provided evidence that also in rat thymus, CaMKIV could not be detected before day E16, and that expression of the enzyme was fully upregulated at day E18. Furthermore, analysis of thymocyte subsets representing different developmental stages revealed that during adult thymic T-cell development the expression of CaMKIV was tightly regulated. These results provide evidence that the expression of CaMKIV, an enzyme involved in the regulation of Ca^{2+}-dependent gene expression, is itself under stringent regulatory control during tissue development.

MATERIALS AND METHODS

Cell culture. Serum-free, rotation-mediated aggregating cell cultures were prepared from fetal (15 days of gestation) rat telencephalon, as described in detail previously [21, 22]. The culture medium used was Dulbecco's modified Eagle's medium (DMEM, Life Technologies,Inc. Gaithersburg, MD, USA), supplemented with nutritional factors, vitamins, trace elements, transferrin (1 mg/l), insulin (800 nM), and hydrocortisone-21-phosphate (20 nM). Gentamicin sulfate (25 mg/l) was used as an antibiotic. For analyses, the aggregates of each flask were washed twice with 5 ml of phosphate-buffered saline (PBS) and proteins were extracted as described in [22].

Thymus lobes from fetal mice were excised at the appropriate days for organ cultures as described in detail elsewhere [23]. Organ cultures were carried out for 5 days in the presence of IMDM minimal medium either in the presence or absence of 10% FCS. For characterization the cells were pelleted, washed with PBS and extracted as described in detail in [22].

SDS-PAGE and electrophoretic blotting. After extraction of proteins from the pellet of whole fetal brain aggregates ($4\text{-}5\times10^7$ cells) or fetal thymus organ cutures ($1\text{-}5\times10^6$ cells) as described in detail in [22], proteins were separated by SDS-PAGE [24] and electrophoretically blotted onto nitrocellulose sheets. CaM-binding proteins were identified on the blots by incubation with ^{125}I-labeled CaM [25] and exposed to PhosphoImager screens as described [22]. Quantitation was done using the PhosphoImager software as recommended by the manufacturers. CAMKIV was identified by specific polyclonal antibodies either kindly provided by Dr. A.R. Means (Durham, NC, USA) or obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Thymocyte subsets, FACS analysis and sorting. Adult thymus and T cell subsets were purified and isolated as described previously [26, 27]. Briefly, thymi or peripheral LNs were removed from 6 week old C57Bl/6 female mice (Harlen Olac, The Netherlands) and cell suspensions prepared. For LN T and LN B subsets, cells were stained with anti-$CD8^-$PE and anti-$CD4^-$PE (Boehringer Mannheim, Mannheim, Germany) and anti-B220-FITC (Caltag Laboratories, South San Francisco, CA) and sorted on a FACStar+ (Becton Dickinson, San Jose, CA) equipped with LYSYS II software. $CD4^+CD8^+$ (DP), $CD4^+CD8^-CD3^+$ (SP) and $CD8^+CD4^-CD3^-$ immature SP (ISP) thymocytes were sorted after 3 colour staining with anti-$CD4^-$PE, anti-$CD8^-$RED613 (Gibco BRL, Life Technologies, Gaithersburg, MD) and anti-CD3ϵ-FITC (Pharmingen, San Diego, CA). Immature $DNCD3^-$ subsets were prepared by elimination of all cells expressing CD4, CD8 and CD3 by incubating on ice with the culture supernatants RL172.4, 3.168.8.1 and 17A2 (Wilson et al., 1994). Rabbit complement (Buxted Rabbit Co., UK) and DNAse I (Boehringer Mannheim, Mannheim, Germany) were added and the incubation continued at 37°C for 20 mins. Dead cells were removed by centrifugation through Ficoll Hypaque for 15 mins at 770g. Any $CD4^+$,$CD8^+$,$CD3^+$ cells remaining were removed by incubation for 20 mins while rotating with magnetic beads coated with anti-Rat Ig (Dynal, Oslo, Norway). The resultant $DNCD3^-$ cells were stained with anti-CD25-FITC, anti-CD44-PE (Caltag, South San Francisco, CA) and CD24-biotin and Streptavidin-TC (Caltag, South San Francisco, CA), gated for $CD24^+$. The 4 populations revealed with CD25 and CD44 were then sorted. In all cases, sorted subsets were greater than 97% pure on reanalysis. All FITC and biotin conjugates were purified and prepared in this laboratory as described previously [26].

RESULTS

The expression of CaMKIV in fetal rat brain primary tissue cultures can be induced by the thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) in a dose and time dependent manner as described in detail in [22]. Since apart from the nervous tissue, CaMKIV is mainly expressed in the immune system [7], we were interested in whether this enzyme was expressed throughout development in fetal thymus, and if it could be induced by a hormonal factor. To this end, murine fetal thymus lobes were excised at different days of gestation and CaMKIV content determined. As shown in Fig. 1A, lane 4, CaMKIV was not expressed in fetal thymus at E14, nor after culture for several days in minimal culture lacking FCS (lane2), suggesting that a specific factor was needed to induce its expression. This interpretation was confirmed by the observation that expression of CaMKIV was induced after addition of 10% FCS (Fig. 1A, lane 3). Whether induction of CaMKIV was due to the presence of T_3 in FCS, or to a different factor is not clear at present, since serum depletion of thyroid

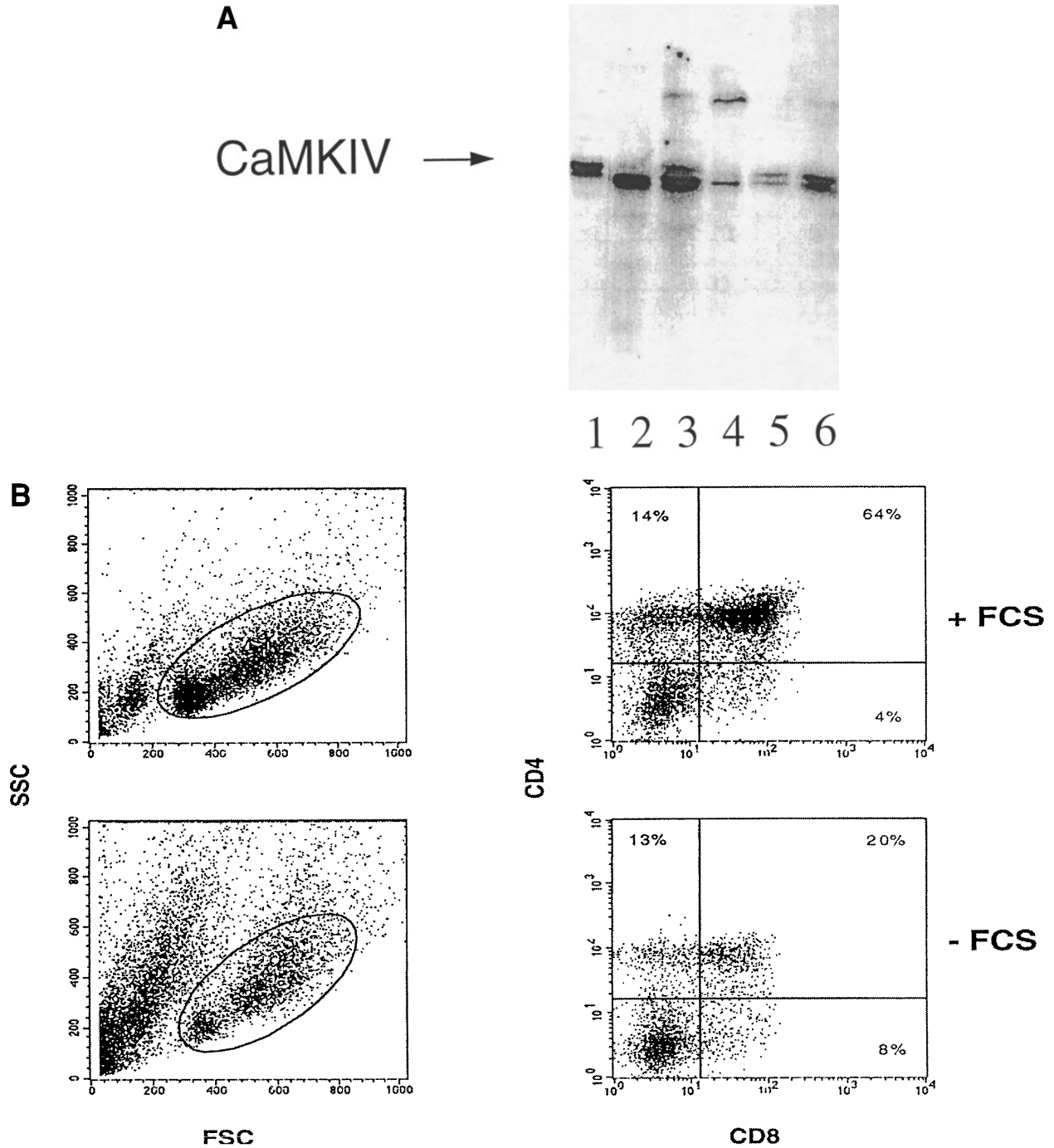

FIG. 1. Expression of CaMKIV in developing thymus *in vivo* and *in vitro*. (A) For the detection of CaMKIV in mice fetal thymus, lobes have been excised at different days of gestation and either cultured for 5 days in minimal medium in the absence (lane 2), or presence (lane 3), of 10% fetal calf serum (FCS), or ex vivo (lanes 4-6) homogenized in lysis buffer, separated by SDS-PAGE, blotted onto nitrocellulose, and CaMKIV was determined by the ^{125}I-CaM overlay technique as described in the methods. 50μl buffer/10^6 cells was used for lysis and extraction and 50μl extract/lane was applied to gels for electrophoresis. Rat thymus postnatal day 7, P7 (lane 1); FTOC of E14 murine fetal thymus after 5 days in culture with (lane 3) or without (lane 2) 10% FCS; murine fetal thymus ex vivo at days E14 (lane 4), E16 (lane 5) and E17 (lane 6). (B) Day 14 embryonic thymi were cultured for 5 days in the presence or absence of FCS, and stained with anti-CD4 and anti-CD8 antibodies for flow-cytometric analysis. Right panels show proportions of CD4/CD8 thymocyte subsets among live cells gated as indicated in the left panels. SSC=side scattering; FSC=forward scattering.

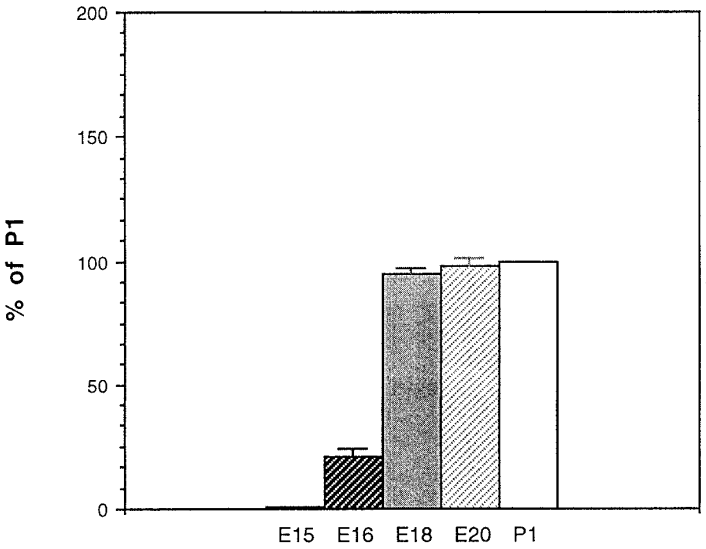

FIG. 2. Determination of CaMKIV levels in rat fetal thymus at different stages of embryonal development. Thymus lobes were excised between E15 and postnatal day 1 (P1), as indicated and CaMKIV determined in cell extracts for the same number of cells as described in [22]. The amount of CaMKIV was determined in double experiments either by using the ^{125}I-CaM overlay technique and quantification by Phospho-Imager as described in [22], or by using CaMKIV specific antibodies on Western blots and quantification by scanning densitometry (Shimadzu Corp., Kyoto, Japan). The same results were obtained with both methods. The amount of CaMKIV in thymus extracts from P1 was set to 100%.

hormones by treatment with an anion-chromatography resin and treatment with charcoal [28] did not prevent its expression (data not shown).

To investigate whether mature T-cells could develop normally in the absence of FCS in FTOC, the surface phenotype of cultured cells was analysed by FACS after 5 days. As shown in Fig. 1B., both immature $CD4^+8^+$ double positive (DP) and mature $CD4^+8^-$ or $CD4^-8^+$ single positive (SP) cells were observed, although viable cell recovery in the absence of FCS was 6 times lower, and the proportion of DP cells was greatly decreased. To determine the timing of CaMKIV expression during fetal development *in vivo,* measurements were made on freshly isolated fetal thymic tissues at various days of gestation. As shown in Fig. 1A., CaMKIV was first detectable in murine fetal thymus at E16, and fully expressed at E18 (Fig. 1A, lanes 4-6). In order to quantitate these observations rat fetal thymus were excised at different days of gestation and the amount of CaMKIV determined at different developmental stages (Fig. 2). At day E15 CaMKIV was practically undetectable, however by E16 CaMKIV amounted to about 20% of the amount expressed at postnatal day P1 (set to 100%) and was practically fully expressed by E18, indicating that the expression of CaMKIV followed a similar time course in the thymus as had been observed in brain [22].

Next, we were interested in investigating whether the level of expression of CaMKIV varied during T-cell development. To this end we determined the amount of CaMKIV in FACS sorted adult thymus subsets representing different stages of maturation (ref. 29). As shown in Fig. 3A, during the early stages of thymocyte differentiation (the most immature DN cells with the surface markers $CD44^+/CD25^-$, $CD44^+/CD25^+$ and $CD44^-/CD25^+$), CaMKIV was practically undetectable. With the appearance of the latest DN subset ($CD44^-/CD25^-$), CaMKIV could be detected at low levels, and remained unchanged in the subsequent $CD8^+/CD4^-/CD3^-$ immature single positive (ISP) stage. However, during the transition from ISP to DP, CaMKIV content increased fivefold indicating that during this late maturation process the presence of CaMKIV is critical. By contrast, in mature SP $CD4^+8^-/CD8^+4^-$ cells expression of CaMKIV was markedly reduced, but still detectable. As already previously reported by Hanissian et al. [7], CaMKIV could be detected in peripheral T-, but not B-cells (Fig. 3A). Analysis of the DP population in more detail after separation into TCR $\alpha\beta^-$, $\alpha\beta^{lo}$ or $\alpha\beta^{hi}$ populations, revealed that the level of expression of CaMKIV remained high until the TCR $\alpha\beta^{lo}$ stage, whereas the TCR $\alpha\beta^{hi}$ subset contained less than 5% CaMKIV as compared to TCR $\alpha\beta^{lo}$ taken as 100% (see Fig. 3B).

DISCUSSION

In this study we have reported the following two major results:

(1) In fetal thymus organ culture the expression of CaMKIV was induced by a hormonal factor

(2) During T-cell development the expression of CaMKIV was tightly regulated

In this, and in previous publications [21,22], we have provided compelling evidence that CaMKIV is induced by hormones in fetal brain or in thymus. In fetal brain we demonstrated that the thyroid hormone T_3 is the hormonal factor inducing CaMKIV during the early stages of embryonal brain development [21,22]. In FTOC, however, we could not conclusively show that T_3 is the inducing factor regulating CaMKIV expression, since stripping of FCS according to published procedures [28], did not succeed in removing the inducing factor. Either T_3 was not removed or CaMKIV was induced by a different factor in fetal thymus. The latter possibility would not be unusual since it was reported recently by Thompson [30], that the gene *hairless* could only be induced by T_3 in the developing rat brain, but not in skin.

The results reported here suggest that CaMKIV plays an important role during T-cell development, most probably due to its involvement in the regulation

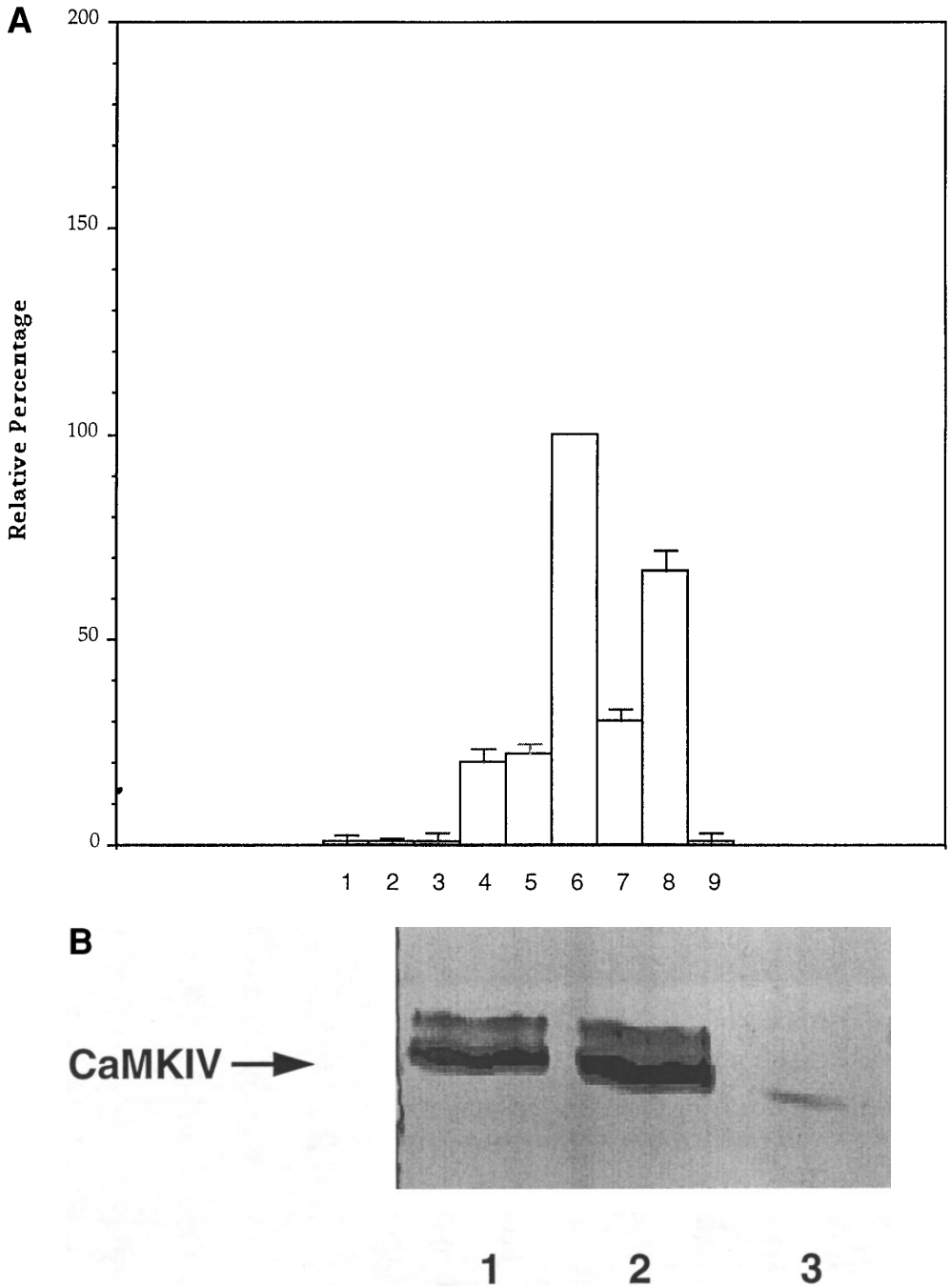

FIG. 3. Determination of CaMKIV during T-cell development. (A) T-cells from different thymus subsets were purified by FACS as described in the methods section. Proteins were extracted and the content of CaMKIV determined as described for Figs. 1A and 2. Lane (1) DN $44^{+}25^{-}$; (2) DN $44^{+}25^{-}$; (3) DN $44^{-}25^{+}$; (4) DN $44^{-}25^{-}$; (5) Immature single positives (ISP); (6) DP; (7) SP; (8) peripheral T-cells; (9) peripheral B-cells. The content of CaMKIV in DP extracts was set to 100%. (B) Expression of CaMKIV in $CD4^{+}8^{+}$ DP subpopulations separated by FACS on the basis of surface expression of TCR $\alpha\beta$. DP TCR α/β^{-}, α/β^{lo} and α/β^{hi} represent 45%, 50% and 5% of total DP thymocytes, respectively. The amount of CaMKIV was determined using a specific antibody as described in the Legend of Fig. 2.

of Ca^{2+}-dependent gene expression (see Ref. 11). It has further been reported by Hanissian et al. [7], that CaMKIV is immediately activated upon T-cell receptor signaling after CD3 or PMA/ionomycin stimulation. In this context, it is of interest that Ho et al. [31], provided evidence that reporter gene constructs driven by AP-1 (i.e. c-Fos/c-Jun) dependent sequences in Jurkat T-cells could be activated by CaMKIV in a Ca^{2+}-dependent

manner. We have reported similar results for a fetal rat brain primary tissue culture system in which we could demonstrate that Ca^{2+}-dependent expression of c-fos was strongly dependent on the presence of CaMKIV [21].

Two different Ca^{2+}-signaling pathways are likely to converge in the regulation of IL-2 gene expression and IL-2 production upon T-cell activation. After activation of T-lymphocytes via antigen/TCR interaction a sustained increase in intracellular Ca^{2+} is obtained [2], which in turn activates two Ca^{2+}/calmodulin dependent enzymes: the kinase CaMKIV [7] described in the present paper and the phosphatase calcineurin [32]. The latter activates the cytoplasmic component of the nuclear factor of activated T-cells ($NFAT_c$) via Ca^{2+}-dependent dephosphorylation, permiting $NFAT_c$ to enter the nucleus and to associate with members of inducible transcription factors such as AP-1 thereby inducing the IL-2 gene [33]. Dephosphorylation of $NFAT_c$ by calcineurin is the step which can be blocked by immunosuppressants such as cyclosporinA or FK506 [34,35], therefore preventing T-cell activation. On the other hand, Ca^{2+}-dependent activation of CaMKIV in activated T-cells most likely leads to phosphorylation of CREB [5,11], an important prerequisite for the activation of immediate early genes (e.g. see Ref. 21), leading to AP-1 transcription. The importance of CREB for T-cell activation and IL-2 production was confirmed by Barton et al. [4], who showed that when a dominant/negative form of CREB which could not be phosphorylated at Ser133 was expressed as a transgene in mice, the expression of immediate early genes and the production of IL-2 were dramatically affected. In addition, Anderson et al. [5], provided evidence that activation of thymic T-cells expressing a catalytically inactive form of CaMKIV, either via CD3 or PMA/ionomycin resulted in a severe reduction in their ability to phosphorylate CREB, to induce immediate early genes and to express IL-2 genes [5]. It was further reported [5], that inactivation of CaMKIV had a severe impact on the size of the thymus of these mice resulting in a significant reduction in thymic cellularity which was however not a result of defective maturation of T-cells. This observation is in line with the results reported here, where we show in fetal thymic organ cultures lacking CaMKIV (due to the absence of FCS), that despite normal phenotypic development of DP and mature SP T cells, there is a 6 fold reduction in cell recovery and a significant decrease in proportion of DP with respect to the DN subset (Fig. 1B).

Maturation of T-cells in the thymus is a complex developmental process taking almost 3 weeks from a pluripotent stem cell to a fully developed mature $CD4^+$ or $CD8^+$ SP cell during which time lineage commitment, gene rearrangement, expression of the T-cell receptor, and selection processes take place (for review see [36]). Anderson et al. [5] recently demonstrated that transgenic mice expressing a catalytically inactive form of CaMKIV not only were hampered in T-cell activation, but also exhibited a significant defect in thymic cellularity indicating that CaMKIV may play a critical role in T-cell maturation. Therefore, the authors raised the possibility that defective CaMKIV expression could prevent the maturation of DN into DP cells. At day E14 in mice, or E15 in rat, CaMKIV is not yet expressed (Fig. 1A, 2), and thymocytes are predominantly $CD4^-8^-$ (DN) $CD25^+$ [37,38]. During adult T-cell development the content of CaMKIV in various subsets changes remarkably (Fig. 3A). In stages corresponding to E14 in mice, (up to DN $CD25^+$), no CaMKIV can be detected, however, after CD25 downregulation, ($CD25^-44^-$DN) and upon upregulation of CD8 at the ISP stage, it becomes readily detectable. This corresponds phenotypically to the developmental stage around E16 where CaMKIV first becomes detectable during fetal thymus development. This is in excellent agreement with the observation that during the maturation process of adult T-cells CaMKIV could not be detected before the DN$CD25^-44^-$ stage (see Fig.3A). Within the next 12 hours maturation to the DP stage occurs [39], coincidident with upregulation of RAG genes [26], and the start of TCRα gene rearrangement and expression [27,40]. During this period, the expression of CaMKIV is increased five fold in agreement with the observation that the enzyme is maximally expressed between E16 and E18 where the same events are occuring during fetal thymic development (Fig. 2). Interestingly, in the DP T-cell population CaMKIV reached the highest level of expression in those cells in which the T-cell receptor started to be expressed, i.e. TCR $\alpha\beta^{lo}$ (see Fig. 3B), whereas in those of the subset TCR $\alpha\beta^{hi}$ in which expression of TCR $\alpha\beta$ is upregulated to the level expressed by mature T-cells CaMKIV could hardly be detected.

This observation of a tight regulation of CaMKIV expression during T-cell development leaves - among others - two important questions to be answered:

(1) What signals are regulating expression of CaMKIV during T-cell development?

(2) Which other processes, besides Ca^{2+}-dependent induction of immediate early genes such as c-fos, are regulated by CaMKIV during T-cell development, e.g. during the conversion of DN to DP cells in which period the expression of CaMKIV is significantly upregulated?

In conclusion, we could demonstrate that the expression of CaMKIV in the thymus is hormonally regulated, and that during T-cell development the level of expression of CaMKIV is under tight regulatory control.

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