Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 622–627, January 1999
Immunology

Costimulatory signals are required for induction of transcription factor Nur77 during negative selection of $CD4^+CD8^+$ thymocytes

DERK AMSEN*, CONCHI REVILLA CALVO*†, BARBARA A. OSBORNE‡, AND ADA M. KRUISBEEK*§

*Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; and ‡Department of Veterinary and Animal Sciences, University of Massachusetts, 304 Paige Lab, Amherst, MA 01003

Edited by Philippa Marrack, National Jewish Medical and Research Center, Denver, CO, and approved November 19, 1998 (received for review September 25, 1998)

ABSTRACT **A major question in end-stage T cell development is how T cell receptor(TCR) ligation on immature $CD4^+CD8^+$ double positive thymocytes is translated into either survival (positive selection) or apoptotic (negative selection) signals. Because different types of antigen-presenting cells (APCs) induce positive or negative selection in the thymus and express different costimulatory molecules, involvement of such costimulatory molecules in determining cell fate of DP thymocytes is considered here. If TCR-generated signals are modulated by APCs, this should be reflected in the activation of distinct biochemical pathways. We here demonstrate that costimulatory signals involved in negative selection also are required for induction of protein expression of Nur77 and its family members. These transcription factors are critically involved in negative but not positive selection. In contrast, the signals that costimulate negative selection are not required for induction of several molecular events associated with positive selection. These include activation of the immediate early gene Egr-1, the mitogen-activated protein kinase ERK2, and surface expression of the CD69 marker. Thus, costimulation for negative selection selectively provides signals for activation of apoptotic mediators. These data provide molecular insights into how TCR-engagement by ligands on different thymic APCs can determine cell fate.**

The random process that creates T cell receptor (TCR) diversity generates a large pool of thymocytes, of which only a few will migrate to the periphery. Within the population of thymocytes that has arrived at the $CD4^+CD8^+$ double positive (DP) stage of development, positive and negative selection processes determine which cells are allowed to develop into mature CD4 or CD8 TCRab cells. Both of these selection processes depend on ligation of the TCR by peptide-loaded MHC molecules present in the thymus but result in diametrically different outcomes—survival and maturation (positive selection) or apoptosis (negative selection) (reviewed in ref. 1).

The critical issue in understanding end-stage T cell development is how TCR occupancy can be perceived in such markedly different ways by DP thymocytes. Whether positive or negative selection is induced appears to depend in part on the affinity/avidity of the interaction between thymocyte TCRs and major histocompatibility complex (MHC)–peptide complexes on presenting cells in the thymus (2, 3). However, a different model of thymocyte selection contends that in fact the type of thymic cell presenting MHC–peptide ligands to developing thymocytes dictates the outcome of selection. Support for this model has been provided by many different experimental systems (4–10) and is difficult to ignore. This differential ability of different types of thymic antigen-presenting cells (APCs) to induce either positive or negative selection may reflect in part their localization in different spatial and temporal compartments. In addition, variables such as MHC expression levels and expression of adhesion molecules may also contribute to the outcome of the interaction between DP thymocytes and thymic APCs. Finally, the differential ability of thymic presenting cells to induce positive or negative selection may reflect cell type-specific expression of costimulatory molecules. Such molecules may, through engagement of their counter-receptors on DP thymocytes, qualitatively influence the signals induced by the TCR, leading to induction of either apoptotic or survival pathways.

In support of a contribution of cell-derived costimulatory signals, clonal deletion of thymocytes was indeed shown to occur only if, next to signals through the TCR, additional APC-derived signals are provided (11–14). Whether thymocytes also use the molecular interactions that mature T cells exploit to generate costimulatory signals has been controversial. On the one hand, several investigators have reported a lack of a phenotype with respect to negative selection in $CD28^{-/-}$ mice (15–17). On the other hand, apoptosis of thymocytes induced in suspension can be costimulated by crosslinking the CD28 receptor, either by using antibodies (12, 18, 19) or by its natural ligands CD80 and CD86 (13, 14, 20). Moreover, these findings have been corroborated in the intact thymus in fetal thymic organ culture and recently also in CD28-deficient mice (13, 21), providing formal support for a role for CD28 in clonal deletion *in vivo*. While acknowledging that other molecules than CD28 can costimulate deletion (refs. 11–17; present study), these findings document that CD28–CD80/86 interactions can serve to provide prototypical costimulatory signals for thymocyte deletion. Supportive evidence for this notion stems from the observation that thymic medullary expression of CD80 is correlated with preferential deletion of self-reactive thymocytes in the medulla (22, 23). Also, CD86 expression in the medullary region of the thymus is substantially reduced in gp39-deficient mice (24), a phenotype correlated with reduced deletion of thymocytes specific for endogenously expressed self-antigens (24).

Thus far, only two molecular signaling modules have specifically been linked to either positive or negative selection. Activation of the mitogen-activated protein kinase (MAPK) cascade is required only for positive selection: transgenically expressed dominant negative mutants of Ras, Raf1, and MEK-1 each reduce positive selection (25–27), whereas negative selection is unaffected by perturbations in the MAPK cascade. By contrast, a specific role in negative selection has

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: EMSA, electrophoretic mobility-shift assay; APC, antigen-presenting cell; DP, double positive; MHC, major histocompatibility complex; GST, glutathione *S*-transferase; TCR, T cell receptor; ECDI, 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide; MAPK, mitogen-activated protein kinase.
†Present address: Centro de Investigaciones Biológicas, Madrid, Spain.
§To whom reprint requests should be addressed. e-mail: akru@nki.nl.

been documented for members of the Nur77 family of orphan steroid receptors. Nur77 is expressed in an immediate early fashion in response to apoptotic TCR triggering in thymocytes (28, 29), and transgenic expression of Nur77 or its relative Nor1 in thymocytes leads to massive apoptosis (30). Moreover, mice expressing a transgene that encodes dominant negative Nur77 lacking its transactivation domain exhibit severely impaired ability to induce clonal deletion (31, 32), whereas positive selection proceeds undisturbed. These findings document that transactivation of as-yet-unidentified pro-apoptotic target genes by Nur77 and closely related factors is a prerequisite step in thymocyte clonal deletion but not in positive selection. The precise identity of these pro-apoptotic target genes is currently unclear. It has been suggested that Nur77 functions upstream from the CD95 ligand–CD95 interaction. Thus, the massive apoptosis occurring in thymuses from Nur77 transgenic mice was reported to be reduced by introduction of the *gld* mutation (30). However, these results were recently contradicted by another study (33) in which such rescue was not detected. As expression of dominant negative Nur77 yields a much more pronounced block in negative selection (31, 32) than mutations in CD95 or its ligand (34), it is likely that the major pathway(s) downstream from Nur77 are independent of CD95 and CD95 ligand.

We report here that events associated with positive selection—such as induction of expression of Egr-1 (35, 36) and CD69 (14, 37)—as well as activation of the MAPK ERK2 (25–27) are not affected by costimulation. In sharp contrast, a strict dependence on costimulation was found for protein expression of the orphan steroid receptor Nur77 and for induction of total DNA-binding activity to the Nur77 binding element. Given the critical dependence of negative selection on transactivation by Nur77 and related factors, these results provide a molecular explanation for the requirement for costimulation during negative selection and further our understanding of how thymic APCs can determine cell fate during thymocyte development.

MATERIALS AND METHODS

Antibodies and Reagents. The following purified antibodies were used: 11–4-1 (anti-K^k) (38), GL1 (anti-CD86) (39), 145.2C11 (anti-CD3) (40), 37.51 (anti-CD28) (41), goat anti-hamster IgG (H+L) (Pierce), and rabbit polyclonal IgG anti-ERK-2 (Santa Cruz Biotechnology). Production and purification of glutathione *S*-transferase (GST)–cJun (1–223) was done as described (42). Myelin basic protein was purchased from Sigma. Pigeon cytochrome C peptide 88–104 (p17) was synthesized at the peptide synthesis facility at the Netherlands Cancer Institute.

Cells and Mice. All cells were cultured in Iscove's modified Dulbecco's medium (GIBCO/BRL) supplemented with 10% fetal calf serum (BioWhittaker), 2×10^{-5} M 2-mercaptoethanol (Merck), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2 in air. Thymocytes from (2B4α × 2B4β) F_1 mice (43) on the 129 MHC II knockout background (44) were used. Mice were between 4 and 6 weeks of age and were bred under specific-pathogen-free conditions. DCEKhi7 is a daughter cell line of DAP3.3C1 (45) transfected with I-E^k (J. Miller and R. H. Germain, unpublished data). LEC is a B cell lymphoma derived from CBA/Ht mice (46).

Thymocyte Purification. Thymocytes were purified by panning on petri dishes coated with 20 mg/ml 53–6 (American type Culture Collection) for 45 min at 4°C followed by 12 to 15 washes to remove unbound cells. After this, vigorous pipetting was used to isolate $CD4^+CD8^+$ thymocytes. Typically, these cells >96% pure $CD4^+CD8^+$ as measured by using flow cytometry. All experiments have been performed both with purified and unfractionated thymocytes and yielded similar results.

***In Vitro* Deletion Assay.** Thymocytes were brought into single-cell suspension in complete medium. Thymocytes ($2–4 \times 10^5$) were cultured in 96-well flat-bottom plates overnight with 1×10^5 APCs. After 14–18 hr, cells were harvested and prepared for flow cytometry analysis. Fixation of DCEK cells was carried out as described (47).

Flow Cytometry. Cells were resuspended in PBS containing 0.5% BSA and 0.02% sodium azide and incubated with antibodies on ice for 30 min. Incubation with biotin-labeled anti-CD69 (PharMingen) was followed by incubation with avidin–fluorescein isothiocyanate (FITC) (Sigma). For measurement of deletion, thymocytes were (after surface staining for CD8 expression) exposed to 1 μg/ml ethidium bromide (Sigma) as described (48). Cells (1×10^4 to 2.5×10^5 per sample) were analyzed on a Becton Dickinson FACSCAN by using LYSIS 2 software. APCs were segregated electronically on the basis of forward scatter/side scatter pattern.

Stimulation of Cells and Separation from APCs. APC lines were grown overnight in the presence of magnetic beads (Biomag, Cambridge, MA). After two rounds of magnetic selection, APCs were either fixed with 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (ECDI) and subjected to another round of magnetic selection or left untreated. Subsequently, APCs were prepulsed for two hr in 15-ml polystyrene tubes with peptide and/or antibodies. Finally, 5×10^6 thymocytes and 1×10^6 APCs were mixed in 24-well plates (Costar) in a total volume of 1 ml of prewarmed Iscoves medium containing 10% fetal calf serum. Plates were immediately centrifuged for 1 min at 1,200 rpm in a Hettich (Tuttlingen, Germany) Rotanta RP centrifuge and put at 37°C. After incubation, 1 ml of ice-cold PBS was squirted into each well, and vigorous pipetting was used to harvest the cells. Thymocytes were separated from the APCs by two rounds of magnetic selection at 4°C and contained typically <1% APC.

Western Blot. Thymocytes were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell equivalents (10^7 per lane) were loaded onto 7.5% SDS/PAGE gels. Nitrocellulose membranes (Schleicher and Schuell) were blocked with PBS containing 10% nonfat milk powder and 0.1% Tween 20 and probed with anti-Nur77 (49) (anti-Nur77 clone 12.14 is available through PharMingen, catalog no. 13471A), followed by biotinylated goat anti-mouse IgG (Pierce) and streptavidin–horseradish peroxidase (Amersham). Alternatively, membranes were probed with anti-Egr-1 (Santa Cruz Biotechnology) followed by biotinylated donkey anti-rabbit Ig (Amersham) and streptavidin–horseradish peroxidase. Antibody reactivity was detected by using Amersham's Enhanced Chemiluminescence kit according to manufacturer's instructions.

Electrophoretic Mobility-Shift Assay (EMSA). Thymocytes isolated from coculture with APCs were lysed in a buffer containing 150 mM NaCl, 10 mM Tris (pH 8.0), 1% Triton X-100, 10 μg/ml leupeptin, and 1 mM PMSF. After clearance, protein concentrations were determined by using Bicinchonic acid (BCA) protein assay (Pierce). Subsequently, 20 μg of protein was tested by using EMSA as described (50). For competition experiments, 1 μl of unlabeled oligonucleotide was added to the mixture together with the labeled oligonucleotide.

ERK2 and JNK Kinase Assays. Purified DP thymocytes were rested at 37°C for 5 hr and subsequently incubated in medium with antibodies (10 μg/ml) for 30 min on ice. Cells were washed twice with cold medium, and goat anti-hamster antibody was added at 10 μg/ml in warm (37°C) medium. After this, cells were incubated for the indicated times at 37°C. The reactions were stopped by addition of ice-cold PBS. Cells were lysed in whole-cell extract lysis buffer (51). Subsequently, 50 μg

of whole-cell lysate per sample was tested for Erk2 and JNK activities by *in vitro* kinase assays after immunoprecipitation of Erk2 or precipitation with GST–c-Jun (1–223) as described (51, 52).

RESULTS

Anti-CD3-Mediated Expression of Nur77 Is Promoted by CD28 Engagement. Negative selection of immature DP thymocytes requires costimulatory signals in addition to high-affinity TCR engagement. It has been shown that the interaction between CD80/CD86 on APCs with CD28 on DP thymocytes can generate such costimulatory signals both in the intact thymus (13, 21) and in *in vitro* suspension cultures (12, 20) (Fig. 1*A*). A critical event in thymocyte negative selection is induction of expression of Nur77 (28, 29). To evaluate whether expression of this protein would be subject to regulation by costimulation, we triggered the TCR–CD3 complex on purified DP thymocytes in the presence or absence of a crosslinking anti-CD28 antibody. As shown in Fig. 1, treatment with a combination of antibodies specific for the TCR–CD3 complex and the CD28 receptor resulted in expression of Nur77 within 2 hr of stimulation. In contrast, engagement of only the antigen receptor or CD28 failed to induce significant expression of Nur77 protein. Expression of Nur77 protein is thus promoted by engagement of CD28.

Development of an APC-Based System for Studying Thymocyte Gene Regulation Associated with Selection. To exclude the possibility that the stimulatory effect on Nur77 described above was an artifact of antibody-mediated engagement of CD28 (20), we examined whether B7 molecules expressed on APCs would also costimulate expression of Nur77. For this, we made use of thymocytes from 2B4 TCR-transgenic mice, that react to the 88–104 COOH-terminal peptide from pigeon cytochrome C (p17) presented on IE^k (43).To avoid complications from the changing requirements of thymocytes at later stages of maturity (19), we used only preselection DP thymocytes: 2B4 TCR transgenic mice were backcrossed to 129 MHC class II-deficient mice, and thymocytes from such crosses are unable to undergo positive selection. Because Nur77 expression also may appear in the APC used, we developed an approach that would allow separation of the thymocytes from the APC. To achieve this, monoclonal transformed APC cell lines were first cultured overnight with magnetic beads (53). These beads are ingested by the APCs such that these cells can be magnetically isolated. This procedure has no effect on the ability of these cells to induce clonal deletion in an overnight deletion assay and allows efficient separation of thymocytes from the APC; typically, contamination was <1% (data not shown).

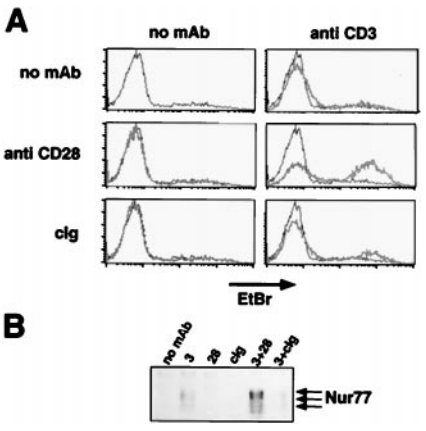

FIG. 1. Anti-CD3 induced expression of Nur77 is promoted by engagement of CD28. DP thymocytes were incubated with plate-bound antibodies (10 μg/ml) to CD3, CD28, or control antibody in different combinations. (*A*) After overnight incubation, cells were harvested and stained with ethidium bromide to assess viability. Shown in each histogram is background ethidium bromide staining from thymocytes incubated without antibody treatment (thin line) or with the treatment indicated in the figure (thick line). (*B*) Alternatively, thymocytes were collected after 2 hr, and whole-cell lysates were analyzed for Nur77 expression by Western blot analysis. Note that Nur77 from thymocytes typically runs at several different sizes, presumably because of hyperphosphorylation.

B7 Molecules Selectively Cooperate with TCR Triggering for Induction of Nur77 but Not Egr-1 Expression. In a previous report, we showed that the B cell lymphoma LEC predominantly uses B7–2 (CD86) for induction of deletion, as an antibody to CD86 inhibits its ability to deliver apoptotic signals to DP thymocytes by >80% (13). We therefore tested whether the CD86 molecules on this APC function to induce Nur77 protein expression in DP thymocytes. When prepulsed with p17, LEC B lymphoma cells efficiently induce Nur77 protein expression in 2B4 DP thymocytes (Fig. 2). However, depriving these cells of costimulatory activity by addition of a blocking antibody against CD86 severely perturbs the ability of these cells to induce the expression of Nur77 protein (Fig. 2). Time course experiments revealed that anti-CD86 caused a similar reduction of Nur77 protein levels after 1, 2, and 4 hr of coculture (data not shown).

To examine the specificity of the regulation of gene expression by CD86-delivered signals, we also tested the effects of CD86 blockade on protein expression of Egr-1. This protein also is encoded by an immediate early gene, which is rapidly transcribed on TCR ligation (28, 35, 36); expression of this immediate early gene is associated with positive selection of a DP thymocyte cell line (36). Furthermore, an instrumental role of this protein in positive selection is suggested by the finding that overexpression of Egr-1 enhances positive selection by low-affinity ligands in transgenic mice (54). In contrast to Nur77, the dose response for Egr-1 induction was entirely unaffected by antibody-mediated blockade of CD86 (Fig. 2). These findings demonstrate that costimulatory interactions selectively cooperate with some, but not all TCR-triggered signaling events in DP thymocytes.

B7-Independent Costimulatory Interactions Exist That Regulate Nur77 Expression. The interaction between B7 molecules and CD28 has been shown in various systems (both *in vitro* and *in vivo*) to be capable of providing costimulatory signals for clonal deletion. It is clear, however, that receptors other than CD28 also can fulfill this function, as illustrated by the lack of an overt phenotype on negative selection in CD28-deficient mice found in several studies (15, 17). Consistent with this observation, we and others identified APC lines whose ability to induce clonal deletion cannot be abro-

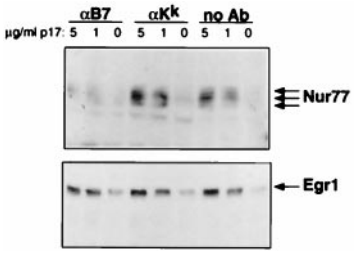

FIG. 2. B cell lymphoma LEC uses CD86 molecules to costimulate expression of Nur77 but not Egr-1 protein. 2B4 thymocytes were cocultured with LEC and different concentrations of p17 (5, 1 or 0 μg/ml). LEC had been preincubated with 5 μg/ml anti-CD86, 10 μg/ml anti-K^k, or no antibody. After 2 hr of coincubation, thymocytes were separated from LEC and whole-cell lysates were analyzed by Western blot analysis using antibodies to Nur77 (*Upper*) or to Egr-1 (*Lower*). Similar results were obtained after 1 and 4 hr of coincubation (data not shown).

gated by B7 blocking. For instance, induction of deletion by the fibroblast cell line DCEKhi7 (DCEK) can only marginally be blocked by anti-B7 (13), indicating that this cell line expresses other as-yet-unidentified costimulatory molecules. The activity of such molecules can be destroyed by chemical fixation of DCEK cells with ECDI. This treatment leads to an inability of these APCs to induce clonal deletion, whereas peptide-presenting ability of such fixed cells remains intact (13). Importantly, fixation with ECDI also destroys the ability of DCEK cells to induce Nur77 expression in DP thymocytes (Fig. 3). This pronounced difference in induction of Nur77 protein expression between thymocytes that recognized p17 with or without costimulation remained at all time points examined over a 1–4 hr time course.

To evaluate whether the lack of Nur77 protein expression was a reflection of reduced TCR-triggering by fixed DCEK, we also examined induction of Egr-1 protein expression. Like antibody-mediated blocking of CD86 (see Fig. 2), fixation of DCEK failed to significantly alter the induction of expression of the Egr-1 protein (Fig. 3*A*). These findings demonstrate the capacity of fixed APCs to present peptide to the thymocytes and the specificity of the effects of destruction of costimulation on induction of Nur77.

Costimulation Is Required for Induction of NUR77/NGFI-B Binding Element (NBRE)-Binding Activity. In mice carrying a targeted disruption of the Nur77 gene, clonal deletion is not impaired (55). In contrast, transgenic expression of a truncated Nur77 protein lacking the transactivation domain but still binding to the Nur77/NBRE leads to inhibition of negative selection (31, 32). These findings indicate that thymocytes can express factors related to Nur77 that can also transactivate expression of pro-apoptotic genes from Nur77-binding elements. Although in mice expressing truncated Nur77 binding to DNA and transactivation by these factors is blocked, such factors can efficiently compensate for its absence in Nur77-deficient mice. Indeed, thymocytes undergoing negative selection express at least one homologue of Nur77 (termed Nor-1) that binds to the same element as Nur77 and shares the capacity to activate transcription from this element (56). Thus, not Nur77 alone, but most likely Nur77 or closely related proteins such as Nor-1, associate with Nur77-binding elements to activate transcription of as-yet-unidentified pro-apoptotic target genes.

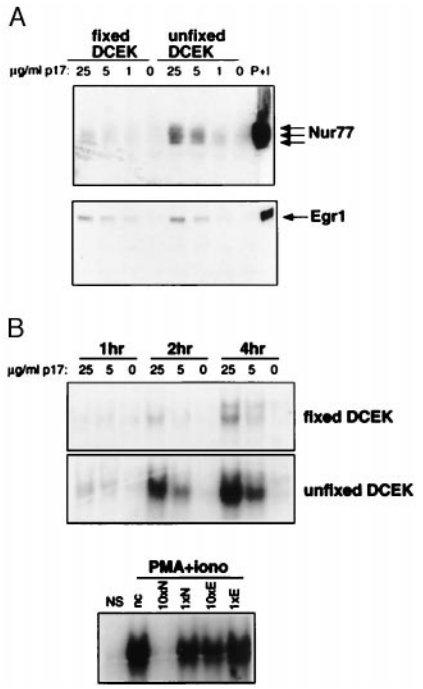

FIG. 3. (*A*) Fixation-sensitive costimulatory molecules coinduce expression of Nur77 but not Egr-1 protein. 2B4 TCR transgenic thymocytes were cocultured for 2 hr with either unfixed or fixed DCEK and different concentrations of p17 (25, 5, 1, or 0 μg/ml). Thymocytes subsequently were separated from DCEK, and whole-cell lysates were analyzed by Western blot analysis using antibodies to Nur77 (*Upper*) or to Egr-1 (*Lower*). Similar results were obtained after 1 and 4 hr of incubation (data not shown). P+I: cells incubated with 1 μM phorbol 12-myristate 13-acetate (PMA) and 0.5 μM ionomycin. (*B*) Induction of NBRE binding activity in thymocytes requires costimulation. Lysates prepared as in (*A*) were tested by EMSA using ^{32}P-labeled NBRE as a probe (*Upper*). To test the specificity of NBRE binding, lysates from thymocytes treated with 1 μM PMA and 0.5 μM ionomycin were incubated with ^{32}P-labeled NBRE in the absence of competitor (nc) or with 10 ng or 1 ng of unlabeled NBRE (10×N and 1×N, respectively) or unrelated E2F probe (10×E and 1×E respectively) (*Lower*). NS, nonstimulated cells.

To investigate the costimulation dependency of all factors that can bind to Nur77-binding elements, we tested thymocyte lysates for NBRE-binding activity in EMSAs. A complex of proteins binding to the NBRE is clearly detectable after 2 hr of thymocyte incubation with DCEK and p17, increasing until after 4 hr of incubation (Fig. 3*B*). The specificity of this complex is documented by its disappearance on addition of unlabeled NBRE oligonucleotide but not on addition of an unrelated probe (Fig. 3*B*). Importantly, at all time points examined, NBRE binding activity induced by fixed DCEK was significantly lower than the activity induced by unfixed DCEK (Fig. 3*B*). We therefore conclude that costimulation provides specific signals that are necessary for efficient induction of the total NBRE binding activity in DP thymocytes.

Events Associated with Positive Selection Do Not Require Costimulation. We next investigated to what extent other TCR-induced responses associated with selection and maturation depend on costimulation. Surface expression of CD69 is induced during positive selection; we therefore analyzed CD69 expression after overnight culture, using fixed or unfixed DCEK as presenting cells. In agreement with earlier reports (11, 13), unfixed DCEK efficiently induce deletion in a p17 concentration-dependent manner, whereas ECDI-fixed DCEK are defective in this respect (Fig. 4*A*). In sharp contrast, presentation of p17 on fixed DCEK efficiently induces surface expression of CD69 on these thymocytes (Fig. 4*B*), demonstrating that this event does not require costimulation.

Transgenic expression of a catalytically inactive form of MEK-1 results in inhibition of positive selection (25), identifying MAPK activation as a requisite step in this process. We therefore tested whether costimulation plays a role in activation of the MAPK Erk2. Because the time required for

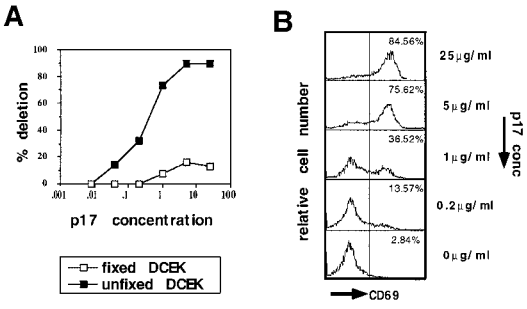

FIG. 4. CD69 surface expression does not require costimulation. (*A*) 2B4 TCR transgenic thymocytes were cultured with either unfixed (■) or ECDI-fixed DCEK (□) and different concentrations of p17. After 14 hr of incubation cells were stained with anti-CD69 and ethidium bromide. Percentage of specific deletion was calculated as follows: 100 × (% EtBr$^-$ cells without anti-CD3 − % EtBr$^-$ cells at experimental anti-CD3 concentration/% EtBr$^-$ cells without anti-CD3). (*B*) CD69 expression on viable (ethidium bromide-negative) thymocytes cultured with fixed DCEK as in *A*.

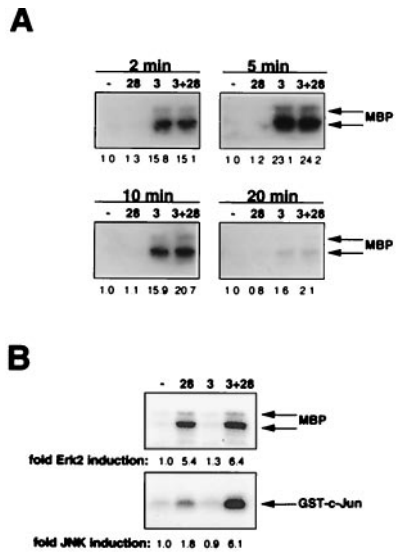

FIG. 5. Activation of Erk2 does not require costimulation. (*A*) DP thymocytes were stimulated with anti-CD3 with or without anti-CD28, and cell lysates were tested after different periods of stimulation for Erk2 activity by *in vitro* i.p. kinase reaction using MBP as a substrate. (*B*) In addition, DP thymocytes were stimulated for 5 min with anti-CD3 with or without anti-CD28 and analyzed for JNK activity in a solid state kinase reaction using GST–c-Jun (1–223) as a substrate (*Upper*) or Erk2 activity as in *A* (*Lower*). The numbers underneath the lanes represent the fold induction compared with samples treated with only crosslinker, calculated from quantification obtained by phosphorimager.

separation of thymocytes from the APC is prohibitively long in comparison to the transient activities of this kinase, we applied antibody-mediated activation for this analysis. As shown in Fig. 5*A*, treatment of DP thymocytes with a TCR trigger alone results in rapid activation of Erk2 (within 1 min), reaching peak levels at 2 min and returning to almost baseline by 10 min. Provision of costimulation by anti-CD28 did not affect the magnitude or the duration of this activity. To evaluate whether anti-CD28 provides any signals to DP thymocytes, we also measured JNK activity in the same samples. Activation of this kinase is strongly promoted by anti-CD28 (Fig. 5*B*), in accordance with previous reports (52, 57, 58). Thus, we conclude that Erk2 activation, like surface expression of CD69, is independent of costimulation. Induction of these early events in positive selection are thus independent of the biochemical cascades promoted by CD28 or fixation-sensitive costimulatory signals. In sharp contrast, one of the prerequisite nuclear signals involved in negative selection is exquisitely dependent on costimulatory signals.

DISCUSSION

It has been known for some time that TCR engagement on immature DP thymocytes can trigger alternate cell fates, with positive signals resulting in further differentiation and negative signals resulting in apoptosis. It also is known that the type of APC providing TCR ligands determines cell fate, with cortical epithelial cells most proficient at eliciting positive responses and bone marrow-derived cells at triggering apoptotic signals. The findings presented here provide molecular insights into how different thymic APC regulate negative vs. positive selection.

We have examined the costimulation dependency of a variety of molecular events associated with both positive and negative selection. The impetus for these experiments is the observation that induction of TCR-triggered negative selection in DP thymocytes requires additional signals from costimulatory receptors (10–14, 19). The present data reveal a strict dependence on costimulation for TCR-induced expression of the immediate early gene Nur77 and its family members. These transcription factors are critically involved in activation of the apoptotic program in thymocytes undergoing negative selection. Induction of molecular events associated with positive selection, by contrast, is triggered efficiently by TCR engagement alone (in the absence of the costimulatory signals explored here). These events include induction of expression of CD69 and activation of the MAPK Erk2. Importantly, whereas delivery of costimulation markedly affects TCR-induced expression of Nur77, the peptide dose-response curves for expression of Egr-1 are comparable in the presence or absence of costimulation. This finding strongly suggests that costimulation provides specific secondary signals that lead to induction of Nur77 protein expression and does not merely serve to enhance all signaling through the TCR.

The present results bear on the crucial issue of how thymocytes make the decision between positive and negative selection and suggest that TCR triggering on DP thymocytes may result in either positive or negative selection signals, depending on which APC presents ligands to the TCR. The core of this proposal is that APC may dictate the outcome of selection by differential expression of accessory molecules that provide critical additional information to the thymocytes about which program should be activated on TCR ligation: apoptosis or maturation. We have shown that costimulatory signals indeed provide such critical information for up-regulation of Nur77 (a protein involved in negative but not positive selection) and hypothesize that it is expression of such costimulatory molecules that allows DC and medullary epithelial cells to induce negative selection. The lack of expression of such molecules on cortical epithelial cells may be the reason that these cells are ineffective at inducing clonal deletion.

Costimulation does not significantly affect induction of various events involved in positive selection such as activation of Erk2 (Fig. 5) and expression of Egr-1 (Figs. 2 and 3). In addition, several other hallmarks of positive selection can be induced by TCR triggering alone independent of costimulation. These include surface expression of CD69 (Fig. 4*B*) and CD5, coreceptor (CD4 or CD8) down-regulation, increased expression of TCRα protein, decreased RAG-1 expression, and up-regulation of Bcl-2 (13, 14, 59, 60). However, when using such systems, we never achieved full functional maturation, documenting that triggering of the TCR alone is not sufficient for induction of positive selection. We therefore consider it likely that positive selection also requires specialized accessory interactions that either promote survival or provide specific additional signals that drive expression of genes required for maturation. Indeed, such a model would explain the superior ability of cortical epithelium and the (relative) inability of bone marrow-derived thymic APC to induce positive selection (7, 61, 62).

It should be noted that an APC-based model of thymocyte selection is not inconsistent with earlier data obtained in the fetal thymic organ cultures that show negative selection by high-affinity peptides and positive selection by low-affinity peptides (63–65). In those experiments, peptide was added exogenously, such that all types of APC can be expected to present the peptide. As a consequence, induction of positive selection by a high-affinity peptide on cortical epithelial cells will be obscured by subsequent negative selection by APC-expressing costimulatory molecules. Only low-affinity peptides may therefore be found to induce positive selection, because these are the only peptides that do not also induce negative selection.

Conclusion. Costimulatory signals, such as those generated by the interaction between CD80/86 on thymic APC and CD28 receptors on immature DP thymocytes, produce signals that shape the TCR response into one generating negative

selection. We report here that both CD80/86-dependent and CD86/86-independent pathways trigger protein expression of the Nur77 transcription factor and its family members. Because dominant negative Nur77 blocks TCR-triggered negative selection, we contend that induction of the Nur77 family of transcription factors represents the critical nuclear event through which costimulation translates TCR engagement on DP thymocytes into negative signals. When triggered without costimulation, TCR engagement on DP thymocytes results in a number of early signaling events associated with positive selection. The finding that fine-tuning of the signals delivered through the TCR can occur through costimulatory signals provides an explanation for the specialized role of different thymic APCs in the determination of cell fate.

We thank Ton Schumacher and Jannie Borst for critically reading the manuscript and Marie Anne van Halem for preparation of the manuscript.

1. Jameson, S. C., Hogquist, K. A. & Bevan, M. J. (1995) *Annu. Rev. Immunol.* **13,** 93–126.
2. Benoist, C. & Mathis, D. (1997) *Curr. Opin. Immunol.* **9,** 245–249.
3. Marrack, P. & Kappler, J. (1997) *Curr. Opin. Immunol.* **9,** 250–255.
4. Sprent, J., Kosak, H., Gao, E.-K., Surh, C. D. & Webb, S. R. (1993) *Immunol. Rev.* **11,** 151–176.
5. Lo, D. & Sprent, J. (1986) *Nature (London)* **329,** 672–675.
6. Benoist, C. & Mathis, D. (1989) *Cell* **58,** 1027–1033.
7. Bill, J. & Palmer, E. (1989) *Nature (London)* **341,** 649–651.
8. Carlow, D. A., Van Oers, N. S. C., Teh, S.-J. & Teh, H.-S. (1992) *J. Immunol.* **148,** 1595–1603.
9. Hoffman, M. W., Heath, W. R., Ruschmeyer, D. & Miller, J. F. (1996) *Proc. Natl. Acad. Sci. USA* **92,** 9851–9855.
10. Laufer, T. M., DeKoning, J., Markowitz, J. S., Lo, D. & Glimcher, L. H. (1996) *Nature (London)* **383,** 81–85.
11. Page, D. M., Kane, L. P., Allison, J. P. & Hedrick, S. M. (1993) *J. Immunol.* **151,** 1868–1880.
12. Punt, J. A., Osborne, B. A., Takaham, Y., Sharrow, S. O. & Singer, A. (1994) *J. Exp. Med.* **179,** 709–713.
13. Amsen, D. & Kruisbeek, A. M. (1996) *Int. Immunol.* **8,** 1927–1936.
14. Kishimoto, H., Cai, Z., Brunmark, A., Jackson, M. R., Peterson, P. A. & Sprent, J. (1996) *J. Exp. Med.* **184,** 531–537.
15. Shahinian, A., Pfeffer, K., Lee, K. P., Kündig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B. & Mak, T. W. (1993) *Science* **261,** 609–612.
16. Lucas, P. J., Negishi, I., Nakayama, K., Fields, L. E. & Loh, D. Y. (1995) *J. Immunol.* **154,** 5757–5768.
17. Walunas, T. L., Sperling, A. I., Khattri, R., Thompson, C. B. & Bluestone, J. A. (1995) *J. Immunol.* **156,** 1006–1014.
18. Punt, J. A., Havran, W., Abe, R., Sarin, A. & Singer, A. (1997) *J. Exp. Med.* **186,** 1911–1922.
19. Kishimoto, H. & Sprent, J. (1997) *J. Exp. Med.* **185,** 263–272.
20. Lesage, S., Charron, J., Winslow, G. & Hugo, P. (1997) *J. Immunol.* **159,** 2078–2081.
21. Noel, P. J., Alegre, M.-L., Reiner, S. L. & Thompson, C. B. (1998) *Cell. Immunol.* **187,** 131–138.
22. Burkly, L. C., Degermann, S., Longley, J., Hagman, J., Brinster, R. L., Lo, D. & Flavell, R. A. (1993) *J. Immunol.* **151,** 3954–3960.
23. Degermann, S., Surh, C. D., Glimcher, L. H., Sprent, J. & Lo, D. (1994) *J. Immunol.* **152,** 3254–3263.
24. Foy, T. M., Page, D. M., Waldschmidt, T. J., Schoneveld, A., Laman, J. D., Masters, S. R., Tygrett, L., Ledbetter, J. A., Aruffo, A., Classen, E., *et al.* (1995) *J. Exp. Med.* **182,** 1377–1388.
25. Alberola-Ila, J., Forbush, K. A., Seger, R., Krebs, E. G. & Perlmutter, R. M. (1995) *Nature (London)* **373,** 620–623.
26. O'Shea, C. C., Crompton, T., Rosewell, I. R., Hayday, A. C. & Owen, M. J. (1996) *Eur. J. Immunol.* **26,** 2350–2355.
27. Swan, K. A., Alberola-Ila, J., Gross, J. A., Appleby, M. W., Forbush, K. A., Thomas, J. F. & Perlmutter, R. M. (1995) *EMBO J.* **14,** 276–285.
28. Liu, Z.-G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M. & Osborne, B. A. (1994) *Nature (London)* **367,** 281–284.
29. Woronicz, J. D., Cainan, B., Ngo, V. & Winoto, A. (1994) *Nature (London)* **367,** 277–281.
30. Weih, F., Ryseck, R.-P., Chen, L. & Bravo, R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5533–5538.
31. Calnan, B. J., Szychowski, S., Chan, K.-M., Cado, D. & Winoto, A. (1995) *Immunity* **3,** 273–282.
32. Zhou, T., Cheng, J., Yang, P., Wang, Z., Liu, C., Siu, X., Bluethmann, H. & Mountz, J. D. (1996) *J. Exp. Med.* **183,** 1879–1892.
33. Chan, F. K.-M., Chen, A. & Winoto, A. (1998) *J. Immunol.* **161,** 4252–4256.
34. Nagata, S. (1997) *Cell* **88,** 355–365.
35. Sukhatme, V. P., Cao, X., Chang, L. C., Tsai-Morris, C.-H., Stamenkovich, D., Ferreira, P. C. P., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., *et al.* (1988) *Cell* **53,** 37–43.
36. Shao, H., Kono, D. H., Chen, L.-Y., Rubin, E. M. & Kaye, J. (1997) *J. Exp. Med.* **185,** 731–744.
37. Yamashita, L., Nagata, T., Tada, T. & Nakayama, T. (1993) *Int. Immunol.* **5,** 110–116.
38. Ozato, K., Mayer, N. & Sachs, D. H. (1980) *J. Immunol.* **124,** 533–541.
39. Hathcock, K. S., Laszlo, G., Dickler, H. B., Bradshaw, J., Linsley, P. & Hodes, R. J. (1993) *Science* **262,** 905–907.
40. Leo, O., Foo, M., Sachs, D. H. & Samelson, L. E. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378.
41. Gross, J. A., Callas, E. & Allison, J. P. (1992) *J. Immunol.* **149,** 380–382.
42. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
43. Berg, L. J., Pullen, A. M., Fazekas de St. Groth, B., Mathis, D., Benoist, C. & Davis, M. M. (1989) *Cell* **58,** 1035–1046.
44. Grusby, M. J., Johnson, R. S., Paparannou, V. E. & Glimcher, L. H. (1991) *Science* **253,** 1417–1420.
45. Germain, R. N. & Quill, H. (1986) *Science* **253,** 1417–1420.
46. Van Pel, A., Vessière, F. & Boon, T. (1983) *J. Exp. Med.* **157,** 1992–2001.
47. Jenkins, M. K. & Schwartz, R. H. (1987) *J. Exp. Med.* **165,** 302–319.
48. Lyons, A. B., Samuel, K., Sanderson, A. & Maddy, A. H. (1992) *Cytometry* **13,** 809–821.
49. Zheng, B., Han, S., Ahu, Q., Goldsby, R. & Kelsoe, G. (1996) *Nature (London)* **384,** 263–266.
50. Woronicz, J. D., Lina, A., Calnan, B. J., Szychowski, S., Cheng, L. & Winoto, A. (1995) *Mol. Cell. Biol.* **15,** 6364–6376.
51. Hibi, M., Lin, A., Smeal, T., Minden, A. & Karin, M. (1993) *Genes Dev.* **7,** 2135–2148.
52. Revilla Calvo, C., Amsen, D. & Kruisbeek, A. M. (1997) *J. Exp. Med.* **186,** 1645–1653.
53. Kang, S.-M., Beverly, B., Tran, A.-C., Brorson, K., Schwartz, R. H. & Lenardo, M. J. (1992) *Science* **257,** 1134–1138.
54. Miyazaki, T. & Lemonnier, F. A. (1998) *J. Exp. Med.* **188,** 715–723.
55. Lee, S. L., Wesselschmidt, R. L., Linette, G. P., Kanagawa, O., Russell, J. H. & Milbrandt, J. (1995) *Science* **269,** 532–535.
56. Cheng, L. E.-C., Chan, F. K.-M., Cado, D. & Winoto, A. (1997) *EMBO J.* **16,** 1865–1875.
57. Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M. & Ben-Neriah, Y. (1994) *Cell* **77,** 727–736.
58. Li, W., Wahley, C. D., Mondino, A. & Mueller, D. L. (1996) *Science* **271,** 1272–1275.
59. Kearse, K. P., Takahama, Y., Punt, J. A., Sharrow, S. O. & Singer, A. (1995) *J. Exp. Med.* **181,** 193–202.
60. Cibotti, R., Punt, J. A., Dash, K. S., Sharrow, S. O. & Singer, A. (1997) *Immunity* **6,** 245–255.
61. Brocker, T., Riedinger, M. & Karjalainen, K. (1997) *J. Exp. Med.* **185,** 541–550.
62. Ernst, B. B., Surh, C. D. & Sprent, J. (1996) *J. Exp. Med.* **183,** 1235–1240.
63. Ashton-Rickardt, P. G., Van Kaer, L., Schumacher, T. N. M., Ploegh, H. L. & Tonegawa, S. (1993) *Cell* **73,** 1041–1049.
64. Sebzda, E., Wallace, V. A., Mayer, J., Yeung, R. S. M., Mak, T. W. & Ohashi, P. S. (1994) *Science* **263,** 1615–1618.
65. Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J. & Carbone, F. R. (1994) *Cell* **76,** 17–27.