Brief Definitive Report

Thymic T Cell Anergy in Autoimmune Nonobese Diabetic Mice Is Mediated by Deficient T Cell Receptor Regulation of the Pathway of $p21^{ras}$ Activation

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Summary

Thymic T cell anergy, as manifested by thymocyte proliferative unresponsiveness to antigens expressed in the thymic environment, is commonly believed to mediate the acquisition of immunological self-tolerance. However, we previously found that thymic T cell anergy may lead to the breakdown of tolerance and predispose to autoimmunity in nonobese diabetic (NOD) mice. Here, we show that NOD thymic T cell anergy, as revealed by proliferative unresponsiveness in vitro after stimulation through the T cell receptor (TCR), is associated with defective TCR-mediated signal transduction along the PKC/$p21^{ras}$/$p42^{mapk}$ pathway of T cell activation. PKC activity is reduced in NOD thymocytes. Activation of $p21^{ras}$ is deficient in quiescent and stimulated NOD T cells, and this is correlated with a significant reduction in the tyrosine phosphorylation of $p42^{mapk}$, a serine/threonine kinase active downstream of $p21^{ras}$. Treatment of NOD T cells with a phorbol ester not only enhances their $p21^{ras}$ activity and $p42^{mapk}$ tyrosine phosphorylation but also restores their proliferative responsiveness. Since $p42^{mapk}$ activity is required for progression through to S phase of the cell cycle, our data suggest that reduced tyrosine phosphorylation of $p42^{mapk}$ in stimulated NOD T cells may abrogate its activity and elicit the proliferative unresponsiveness of these cells.

Functional inactivation or anergy of a T cell is manifested by a long-lasting proliferative unresponsiveness, and may occur as a consequence of an interaction between an Ag or mitogen with the CD3-TCR complex in the absence of a second non-Ag-specific costimulator signal provided by an APC (1). T cell anergy in the thymus and/or periphery generally results in immunological self-tolerance (2–4). In contrast, we have found that beginning at the time of insulitis (7 wk of age), mature $CD4^+8^-$ and $CD4^-8^+$ thymic T cells from prediabetic NOD mice are anergic as assessed by stimulation of proliferation in vitro after TCR cross-linking with either an anti-TCR mAb, anti-CD3 mAb, or Con A. This anergy is not due to the inability of thymic APCs to provide costimulation (5, 6, and Rapoport, M., A. Jaramillo, D. Zipris, A. Lazarus, D. Serreze, E. Leiter, P. Cyopick, and T. Delovitch, manuscript submitted for publication), but rather arises predominantly from the inability of these NOD thymic T cells to be stimulated to produce sufficient amounts of IL-4 to support their proliferation (6, and Rapoport, M., et al., manuscript submitted for publication). IL-4 completely reverses this thymic T cell anergy in vitro and when administered in vivo, prevents the onset of diabetes in NOD mice (Rapoport, M. et al., manuscript submitted for publication). These studies raise the possibility that thymic T cell anergy might influence susceptibility to diabetes in NOD mice. Elucidation of the biochemical mechanisms of this NOD thymic T cell anergy may further unravel the basis of the polygenic control of susceptibility to type I diabetes.

In many types of cells, interactions between membrane receptors and a variety of growth factors leads to the activation of the protooncogene $p21^{ras}$ and subsequent cell proliferation (7–10). Stimulation of T cell proliferation through the TCR very rapidly activates $p21^{ras}$ by converting its inactive GDP-bound form to its active GTP-bound form (11). Previously, we observed that stimulation of NOD thymic T cells with phorbol esters (e.g., phorbol 12-myristate 13-acetate, PMA) restores their proliferative responsiveness upon cross-linking of their TCR (5). Since phorbol ester–induced activation of protein kinase C (PKC) augments $p21^{ras}$ activity in T cells (11), we examined whether deficient activation of the $p21^{ras}$ pathway mediates the proliferative unresponsiveness of NOD thymic T cells. This report provides evidence in support of this notion by demonstrating deficient $p21^{ras}$ activation and $p42^{mapk}$ tyrosine phosphorylation in

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stimulated NOD thymic T cells, and reversal of these defects by treatment with PMA.

Materials and Methods

Reagents and mAbs. Ascites containing the H57-597 anti-TCR-α/β mAb (12) was generously provided by Dr. R. Kubo (National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO). The 145-2C11 anti-CD3ϵ mAb (13) was kindly supplied by Dr. J. Bluestone (University of Chicago, Chicago, IL). The Py-72 anti-phosphotyrosine mAb (14) was kindly provided by Dr. B. M. Sefton (Salk Institute, San Diego, CA). Murine rIL-2 (15) was a kind gift from Dr. G. B. Mills (Toronto General Hospital, Toronto, Ontario, Canada). Recombinant human $p21^{c\text{-}H\text{-}ras}$ and the rabbit anti-GTPase activating protein (GAP) polyclonal antibody (16) were kindly provided by Dr. M. F. Moran (C. H. Best Institute, Toronto, Ontario, Canada).

T Cell Isolation and Activation. Prediabetic male and female inbred 8–12-wk-old NOD/Del mice were used. Insulitis in these mice occurs at 7 wk of age, and diabetes incidence is 50–60% in females and 10% in males by 24 wk of age. Age- and sex-matched BALB/cJ mice were used as controls. Thymic T cells and purified splenic T cells were isolated as described (Rapoport, M. et al., manuscript submitted for publication). Splenic T cells consisted of >95% T cells as estimated by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) with the anti-CD3 mAb. Thymic T cell blasts were generated by stimulation of thymocytes with Con A (2.5 μg/ml; Sigma Immunochemicals, St. Louis, MO) for 48 h in RPMI 1640 medium supplemented with 10% FCS, antibiotics and 50 μM 2-ME (complete RPMI). After washing three times, cells were maintained in exponential growth for 72 h in this medium supplemented with 100 U/ml rIL-2. Cells were deprived of IL-2 and rested for 24 h before use in various functional assays. Alternatively, T cells were activated by PMA (100 ng/ml; Sigma Immunochemicals) before assay.

$p21^{ras}$ Assay. Cells (5×10^7/ml) were permeabilized by addition of 0.4 U/ml of streptolysin O (Wellcome Diagnostics, Greenville, NC) and labeled with α-[^{32}P]GTP (5 μCi; 3,000 Ci/mmol), as described (11). The CHELATE program (17) was used to predict the concentrations of $CaCl_2$ and $MgCl_2$ required to give 100 nM and 5 mM free Ca^{2+} and free Mg^{2+}, respectively, at pH 7.2 and 37°C. After stimulation, cells were lysed in ice-cold 50 mM Hepes buffer, pH 7.4, containing 1% NP-40, 1 mM EGTA, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.5% deoxycholate, and 0.05% SDS. Lysates were precleared for 2 h at 4°C with goat anti-rat IgG coupled to agarose (0.5 ml, 50% solution; Sigma Immunochemicals). Immunoprecipitations (60 min at 4°C) were performed in duplicate using 30 μg/ml of either the Y13-259 rat anti-$p21^{v\text{-}H\text{-}ras}$ mAb (Oncogene Science Inc., Manhasset, NY) or normal rat IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), and then goat anti-rat IgG coupled to agarose. After washing immunoprecipitates 8×, nucleotides were eluted during an incubation for 20 min at 68°C, and separated on polyethyleneimine–cellulose thin layer chromatography plates developed in 1 M KH_2PO_4, pH 3.4 (11). Plates were autoradiographed, and determination of the ras-bound GTP/GTP plus GDP ratio was evaluated by β-scintillation counting.

ras.GAP Assay. Cells were disrupted in lysis buffer B (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 0.5% DTT, 100 mM NaCl, 1 mM $MgCl_2$, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM PMSF, and 0.2 mM Na_3VO_4). Aliquots of lysates were immunoprecipitated with either a polyclonal rabbit anti-*ras*.GAP Ab or a control preimmune rabbit serum and protein A–Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). GAP activity in these immunoprecipitates was assayed, as described (16). GAP activity in nonactivated T cells (calculated as $p21^{ras}$-bound basal cpm) – ($p21^{ras}$-bound cpm in nonactivated cells) was taken to represent maximal (100%) GAP activity. The relative GAP activity in activated T cells was calculated as follows: 100%× [($p21^{ras}$-bound basal cpm) – ($p21^{ras}$-bound cpm in activated cells)/($p21^{ras}$-bound basal cpm) – ($p21^{ras}$-bound cpm in nonactivated cells)].

PKC Assay. Thymocytes (2×10^7/ml) were washed in PBS and lysed by sonication. Intact cells were removed by low speed centrifugation, and the lysates were analyzed for their PKC activity measuring ^{32}P incorporation into histone H1, as described (18).

Western Blots. Quiescent or activated T cells (10^7) were resuspended in suspension buffer (150 mM NaCl, 500 μM Na_3VO_4, 50 μM $ZnCl_2$, 2 mM EDTA, 2 mM PMSF, 50 mM Hepes, pH 7.2), and then boiled for 10 min in SDS sample buffer (16). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) and immunoblotted successively with the Py-72 antiphosphotyrosine mouse mAb (1 μg/ml) or a control mouse mAb (1 μg/ml) for 4 h, rabbit anti–mouse IgG (2 μg/ml, Jackson Immunoresearch Laboratories, Inc.) for 1 h and ^{125}I-labeled protein A (1 μCi/ml, Amersham Corp., Arlington Heights, IL) for 1 h. Membranes were then dried and autoradiographed for 24 h. For detection of $p42^{mapk}$, GAP, and PKC, cell samples were processed and immunoblotted with a polyclonal rabbit anti-$p42^{mapk}$ antibody (1 μg/ml, Upstate Biotechnology, Inc., Lake Placid, NY), polyclonal rabbit anti-GAP antiserum (1:500 dilution), and a pan-reactive rabbit anti-PKC antibody (2 μg/ml, UBI), respectively, or a control rabbit preimmune serum (1:500 dilution).

Results and Discussion

The ability of TCR cross-linking to activate $p21^{ras}$ in thymocytes from 8-wk-old BALB/c (H-2^d) and prediabetic NOD (H-2^{g7}) mice that display high and low proliferative responsiveness (5), respectively, was assessed. Con A induced a four- to fivefold increase in $p21^{ras}$ activity in activated BALB/c thymocytes, whereas it failed to induce $p21^{ras}$ activation in NOD thymocytes (Fig. 1 *A*). Western blots obtained using the pan-reactive Y13-259 anti-*ras* mAb showed no significant difference in the amounts of $p21^{ras}$ present in BALB/c and NOD thymocytes (Lazarus, A. H., unpublished data). To examine whether NOD thymic T cell blasts are also characterized by decreased $p21^{ras}$ activity, we determined the $p21^{ras}$ activity in NOD and BALB/c Con A activated thymic T cell blasts. The relative amount of GTP-bound $p21^{ras}$ in stimulated rested NOD T cell blasts was considerably lower than that observed in similarly treated control BALB/c T cell blasts (Fig. 1 *B*). Con A stimulates a two- to threefold increase in $p21^{ras}$ activity in BALB/c but not NOD spleen T cells (Fig. 1 *C*). Thus, deficient $p21^{ras}$ activation is an inherent signaling defect that is expressed after stimulation of quiescent NOD thymocytes as well as NOD thymic and peripheral T cell blasts.

Activation of $p21^{ras}$ in T cells stimulated via the TCR is regulated by an inhibition of $p21^{ras}$ GTPase activity (11, 19, 20). To examine whether the relatively low amount of GTP-bound $p21^{ras}$ observed in stimulated NOD T cells results from a decrease in the activity of the associated GTPase ac-

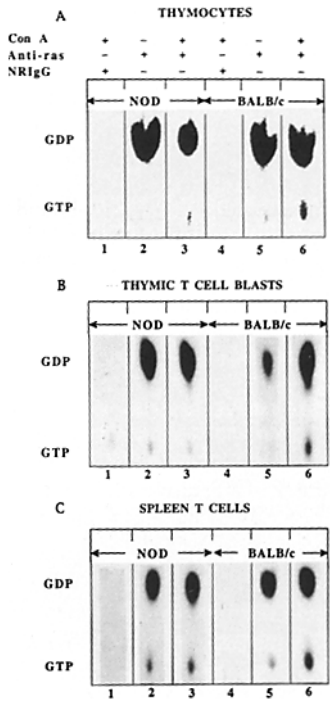

Figure 1. p21[ras] activity is reduced in activated prediabetic NOD T cells. (*A*) NOD (lanes *1–3*) and BALB/c (lanes *4–6*) quiescent thymocytes were isolated and incubated in the absence (lanes *2* and *5*) or presence (lanes *3* and *6*) of Con A (2.5 μg/ml) for 15 min at 37°C in complete RPMI medium. The p21[ras] activities of these cells were then analyzed as indicated. Samples were immunoprecipitated with either the Y13-259 rat anti-v-H p21[ras] mAb (lanes *2*, *3*, *5*, and *6*) or normal rat IgG (lanes *1* and *4*). (*B*) NOD (lanes *1–3*) and BALB/c (lanes *4–6*) thymic T cell blasts were generated by stimulation of thymocytes with Con A for 48 h, maintained in exponential growth for 72 h in the presence of rIL-2 (100 U/ml), and then deprived of IL-2 for 24 h before assay of p21[ras] activity. In this experiment, the relative amount of GTP-bound p21[ras] was greater in unstimulated NOD thymocytes (lane *2*) than in unstimulated BALB/c thymocytes (lane *5*). In general, this was not the case as is evident in Fig. 4 *A*. (*C*) NOD (lanes *1–3*) and BALB/c (lanes *4–6*) splenic T cells were activated by Con A and their p21[ras] activities analyzed as in (*A*) above.

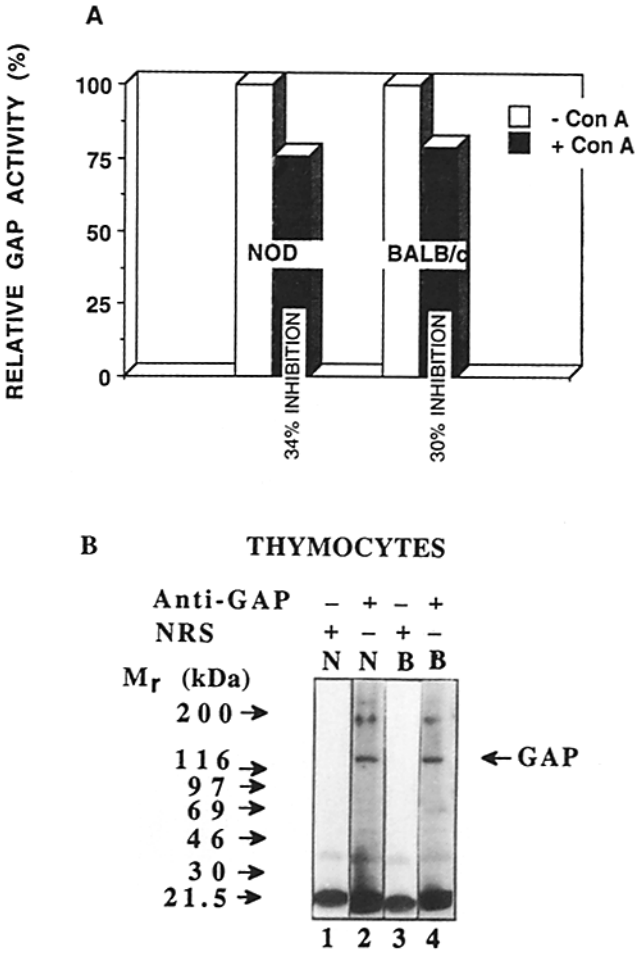

Figure 2. Relative activity and quantity of *ras*.GAP protein is normal in NOD thymocytes. (*A*) Quiescent thymocytes from NOD and BALB/c mice were incubated for 15 min at 37°C in complete RPMI in the absence (*open bars*) or presence (*solid bars*) of Con A (2.5 μg/ml). *ras*.GAP was then immunoprecipitated and tested for its enzymatic activity. The p21[ras]-bound basal cpm value was 93,000. The p21[ras]-bound cpm values in activated NOD and BALB/c thymocytes were about 68,000 and 59,000, respectively. The p21[ras]-bound cpm values for nonactivated NOD and BALB/c thymocytes were about 55,000 and 44,000, respectively. The values for percent inhibition of GAP activity in NOD and BALB/c T cells are shown. (*B*) Western blots of cell lysates from NOD (lanes *1* and *2*) and BALB/c thymocytes (lanes *3* and *4*). Proteins derived from thymic NOD and BALB/c T cells were quantitated for the amount of ras.GAP using an anti-GAP antiserum (lanes *2* and *4*) or a control rabbit preimmune serum (lanes *1* and *3*), as indicated.

tivating protein (*ras*.GAP, M_r 120,000) (11, 21, 22), we assayed both the relative activities and amounts of *ras*.GAP present in stimulated and control unstimulated thymocytes. The level of *ras*.GAP activity was equivalent in stimulated NOD and BALB/c thymocytes (Fig. 2 *A*). Basal *ras*.GAP activity was also comparable in both unstimulated NOD and BALB/c thymocytes. Comparable amouts of p120 *ras*.GAP were present in NOD and BALB/c thymocytes (Fig. 2 *B*). Thus, the relative activities and amounts of *ras*.GAP were equivalent in activated NOD and BALB/c thymocytes, and a decrease in activity or amount of *ras*.GAP does not account for reduced GTP-bound p21[ras] in activated NOD thymocytes. Deficient activation of p21[ras] in NOD T cells is therefore regulated by either another GAP protein, e.g., the NF1 GAP protein (23, 24) or a guanine nucleotide releasing factor(s) (11, 19, 20).

Stimulation of T cells via the TCR or by phorbol esters (e.g., PMA) results not only in p21[ras] activation but also tyrosine phosphorylation and activation of a family of serine/threonine protein kinases known as MAP kinases (MAPK) (25). Two forms of MAPK of M_r 42,000 (termed p42[mapk]) and 44,000 (termed p44[mapk]) exist. These MAPK participate in the control of the G_0-G_1 transition of the cell cycle and the passage of cells through either meiosis or mitosis (26). Active GTP-bound p21[ras] is an upstream effector of MAPK activation by various growth factors in many cell types (26). However, a direct link between p21[ras] and MAPK activities has not yet been demonstrated in T cells. If such a link exists, reduced p21[ras] activity should be associated with diminished MAPK tyrosine phosphorylation in stimulated NOD thymocytes, since tyrosine phosphorylation of MAPK is required

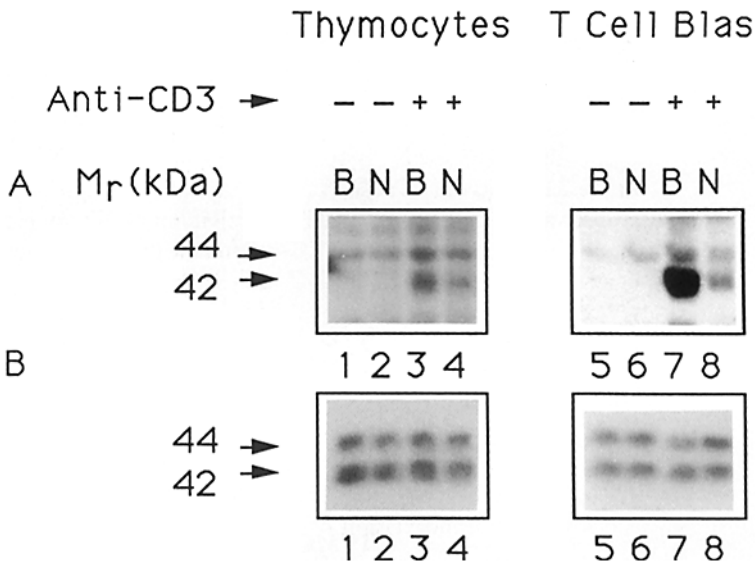

Figure 3. Tyrosine phosphorylation of MAPK is decreased in activated NOD thymocytes. (*A*) NOD (*N*; lanes *2*, *4*, *6*, and *8*) and BALB/c (*B*; lanes *1*, *3*, *5*, and *7*) quiescent thymocytes (10^7) (lanes *1–4*) or rested thymic T cell blasts (lanes *5–8*) were each activated for 15 min at 37°C by the 145-2C11 anti-CD3 mAb (12.5% [vol/vol] solution of hybridoma supernatant). Cell samples were then analyzed by immunoblotting using the Py-72 mouse antiphosphotyrosine mAb. (*B*) Western blots in (*A*) were stripped of bound antibody and reimmunoblotted with a polyclonal rabbit anti-p42^{mapk} affinity-purified antibody. When these blots were overlayed on their respective antiphosphotyrosine blots, the position of p42^{mapk} was superimposable on the 42-kD tyrosine phosphorylated band in (*A*).

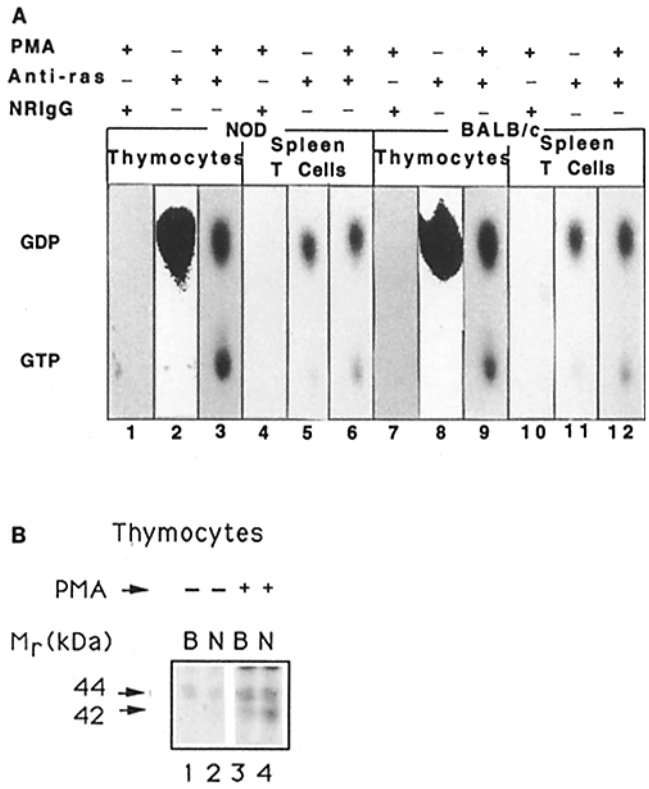

Figure 4. Effect of PMA on NOD T cell activation. (*A*) PMA activates p21^{ras} in NOD T cells. Cells were either unstimulated (lanes *1*, *4*, *7*, and *10*) or stimulated for 15 min with PMA (100 ng/ml) (lanes *2*, *3*, *5*, *6*, *8*, *9*, *11*, and *12*). The Y13-259 anti-p21^{ras} mAb was used for the immunoprecipitation of samples in lanes *3*, *6*, *9*, and *12*, and normal rat IgG was used to treat samples in lanes *1*, *2*, *4*, *5*, *7*, *8*, *10*, and *11*. Thin layer chromatograms of p21^{ras}-bound nucleotides eluted from unstimulated and stimulated NOD thymocytes (lanes *1–3*) and splenic T cells (lanes *4–6*) and from unstimulated and stimulated BALB/c thymocytes (lanes *7–9*) and splenic T cells (lanes *10–12*) are displayed. (*B*) PMA stimulates tyrosine phosphorylation of p42^{mapk} and p44^{mapk} in NOD T cells. BALB/c (lanes *1* and *3*) and NOD (lanes *2* and *4*) quiescent thymocytes were either not activated (lanes *1* and *2*) or activated (lanes *3* and *4*) by PMA for 5 min at 37°C, lysed, and analyzed by Western blot analysis with the Py-72 antiphosphotyrosine mAb, as indicated.

for its activity (25). Anti-CD3 induced substantially less tyrosine phosphorylation of a 42-kD protein in NOD thymocytes in comparison to BALB/c thymocytes (Fig. 3 *A*). A lesser increase in the induced tyrosine phosphorylation of a 44–45-kD protein was also observed in activated NOD vs. BALB/c thymocytes. Similar results were obtained with NOD and BALB/c restimulated thymic T cell blasts. Reactivity with a polyclonal rabbit anti-MAPK antibody identified the 42- and 44–45-kD proteins to be p42^{mapk} and p44^{mapk}, respectively, and to be present in similar amount in activated NOD and BALB/c thymocytes (Fig. 3 *B*). These findings suggest that a defect in TCR-mediated activation of p21^{ras} may result in reduced tyrosine phosphorylation and activation of p42^{mapk} and p44^{mapk}, which subsequently may inhibit the progression through cell cycle and proliferation of NOD thymocytes.

Direct activation of PKC by PMA in combination with either the calcium ionophore ionomycin or Con A corrects the proliferative defect of NOD thymocytes (5). PMA-induced activation of PKC also augments p21^{ras} activity in T cells (11). We therefore examined whether activation of PKC by PMA is able to overcome deficient p21^{ras} activation in stimulated NOD thymic T cells. PMA elicited similar levels of p21^{ras} activation in NOD and BALB/c thymocytes and splenic T cells (Fig. 4 *A*). Tyrosine phosphorylation of p42^{mapk} and p44^{mapk} is also comparable in both NOD and BALB/c PMA stimulated quiescent thymocytes (Fig. 4 *B*). Thus, PMA stimulation of PKC results in normal p21^{ras} activation and p42^{mapk} and p44^{mapk} tyrosine phosphorylation.

Two distinct signaling pathways activated through the TCR, a PKC-dependent and a non-PKC–dependent pathway, regulate p21^{ras} activity in T cells (19, 20). Our previous observations (5, and Rapoport, M. et al., manuscript submitted for publication) that the basal levels of expression of many cell surface molecules, including the TCR, CD3, CD4, CD8, and the IL-2R are comparable in NOD and BALB/c thymic T cells, raised the possibility that basal levels of activity of certain signal transduction intermediates, such as PKC, may differ between T cells from these two mouse strains. PKC

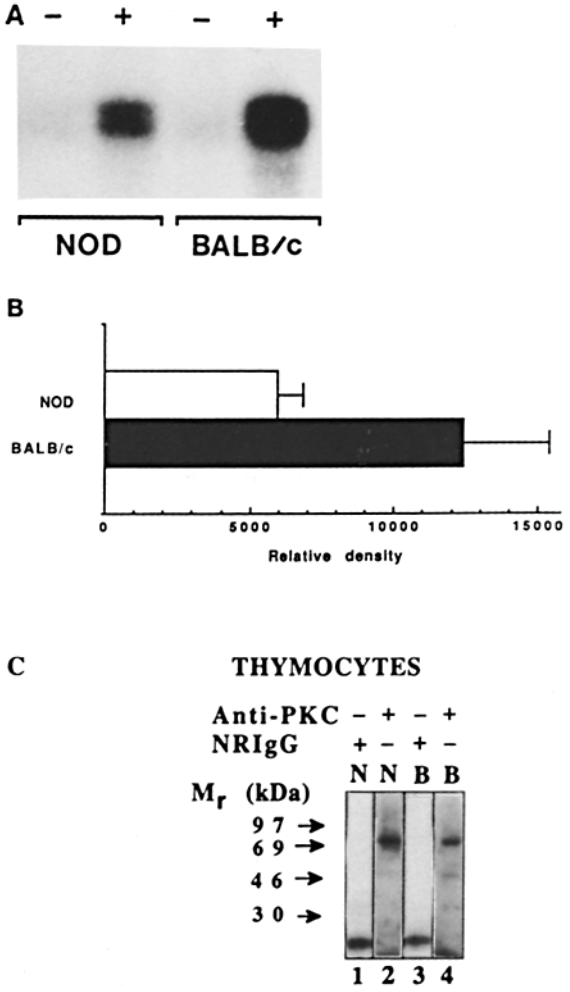

Figure 5. Basal PKC activity is reduced in NOD thymocytes. (*A*) Specific PKC activity was quantitated as described in the text. (*B*) The results shown represent the mean values ± SEM obtained from densitometric scans of samples from nine NOD mice. (*C*) Western blots of cell lysates from NOD (lanes *1* and *2*) and BALB/c thymocytes (lanes *3* and *4*) probed with either 2 μg/ml of a pan-reactive rabbit anti-PKC antibody (lanes *2* and *4*) or normal rabbit IgG (lanes *1* and *3*) and ^{125}I-protein A.

activation is required for the PKC-dependent pathway of $p21^{ras}$ activation (11). To examine whether the low $p21^{ras}$ activity in NOD T cells may be linked to decreased levels of PKC activity, we measured the basal levels of PKC enzymatic activity in vitro in NOD and BALB/c thymic T cell lysates. The basal activity of PKC in quiescent NOD thymocytes was reduced about twofold compared with that observed in control BALB/c thymocytes (Figs. 5, *A* and *B*). Comparable amounts of PKC protein were detected by a polyclonal rabbit anti-mouse PKC antibody that recognizes all PKC isoforms in Western blots of NOD and BALB/c thymocytes (Fig. 5 *C*). Conceivably, the deficient basal PKC activity in NOD thymic T cells may give rise via the PKC-dependent pathway to decreased $p21^{ras}$ activity in these T cells.

Thus, NOD thymic and peripheral T cell anergy may result from a defect(s) along the PKC/$p21^{ras}$/$p42^{mapk}$ pathway of T cell activation. This possibility is supported by our findings presented here that: (*a*) PKC activity is reduced in NOD thymocytes; (*b*) $p21^{ras}$ activation is deficient in quiescent and stimulated NOD T cells; (*c*) a direct link exists between $p21^{ras}$ and MAPK activation in T cells; (*d*) the level of tyrosine phosphorylation of $p42^{mapk}$ is diminished in activated NOD T cells; (*e*) PMA corrects the proliferative defect of NOD T cells; and (*f*) PMA enhances both $p21^{ras}$ activity and MAPK tyrosine phosphorylation in activated NOD T cells. Since MAPK activity is required for gene activation, progression through the cell cycle and cell proliferation, reduced tyrosine phosphorylation of $p42^{mapk}$ in stimulated NOD T cells likely abrogates its activity and elicits the proliferative unresponsiveness of these cells.

Peripheral T cell anergy has been documented both in NOD mice (5, 27, 28) and in prediabetic patients (29), and in the latter case has been implicated as an indicator of progression to overt disease. Our observation that $p21^{ras}$ activation is deficient in stimulated NOD peripheral T cells therefore raises the possibility that analysis of $p21^{ras}$ activity in human peripheral blood T cells may be used to detect diabetes onset in prediabetic individuals at high risk. It also remains to be determined whether our findings of decreased $p21^{ras}$ activity are restricted to anergic prediabetic NOD T cells or can also be applied to anergic T cells in other autoimmune diseases. The latter scenario would provide additional support for the possibility raised herein that an important association exists between a defect(s) in TCR-linked signal transduction and susceptibility to T cell–mediated autoimmune disease.

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