A 50-kDa ERK-like protein is up-regulated by a dual altered peptide ligand that suppresses myasthenia gravis-associated responses

Hava Ben-David*, Badiga Venkata Aruna*, Rony Seger†, Michael Sela*‡, and Edna Mozes*

Departments of *Immunology and †Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel

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Myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG), are T cell-dependent antibody-mediated autoimmune diseases. A dual altered peptide ligand (APL) that is composed of the tandemly arranged two single amino acid analogues of two myasthenogenic peptides, p195–212 and p259–271, down-regulated *in vitro* and *in vivo* MG-associated autoreactive responses. The dual APL was shown to exert its beneficial effects by up-regulating ERK1,2 in $CD4^+CD25^+$ regulatory cells. In this study, we investigated a novel 50-kDa ERK-like protein (ERK-50) that is up-regulated significantly in addition to ERK1,2 after treatment with the dual APL. We report here that ERK-50 was up-regulated in LN cells and in LN-derived T cells of mice that were immunized with the myasthenogenic peptides and treated with the dual APL. Moreover, ERK-50 was up-regulated in dual-APL-treated mice that were immunized with the *Torpedo* acetylcholine receptor. ERK-50 was demonstrated to be recognized by antibodies directed against the C and N termini of ERK1, against the C terminus of ERK2, and against general ERK. The 50-kDa ERK was shown to be stimulated by Con A, and inhibition of MEK1 down-regulated the 50-kDa ERK as was shown for ERK1,2. However, 4β-phorbol 12-myristate 13-acetate (TPA) did not stimulate ERK-50. Finally, the activated ERK-50 was up-regulated in the dual-APL-induced $CD4^+CD25^+$ regulatory cells. Thus, ERK-50 is suggested to be a novel ERK isoform, being up-regulated in response to treatment with the dual APL.

autoimmunity | immunomodulation | kinase

Myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG), are immune disorders that are characterized by circulating antibodies and lymphocyte autoreactivity to the nicotinic acetylcholine receptor (AChR), leading to reduced numbers of AChR molecules at the postsynaptic end plates. Weakness and fatigability of voluntary muscles characterize the disease (1, 2). Two peptides representing sequences of the human AChR α-subunit, namely p195–212 and p259–271, were able to stimulate peripheral blood lymphocytes of patients with MG and were shown to be immunodominant T cell epitopes of SJL and BALB/c mice, respectively (3, 4). A dual altered peptide ligand (APL) composed of the tandemly arranged two single amino acid analogs of the two peptides mentioned above was demonstrated to inhibit efficiently the *in vivo* priming of lymph node (LN) cells to either myasthenogenic peptide (p195–212 or p259–271) and to down-regulate the clinical manifestations of an ongoing experimental autoimmune MG (5, 6). The suppression activity of the dual APL could be adoptively transferred by splenocytes of dual-APL-treated mice (7). Furthermore, the $CD4^+CD25^+$ regulatory T cells that are known to play a critical role in the maintenance of peripheral tolerance (8) were found to be functionally involved in the suppressive action of the dual APL (9, 10).

Another mechanism by which the dual APL might act is alteration of the signal transduction pathways via the T cell receptor (TCR). TCR engagement by a ligand may activate multiple pathways of which the MAPK cascades lead to cell fate commitment (11). We have previously shown that the JNK activity was up-regulated in T cells of dual-APL-treated mice, an event that was correlated with an elevation in Fas/FasL in these cells (9, 12).

ERK1 and ERK2, which are 44-kDa and 42-kDa molecules, respectively, are key signaling enzymes that are activated by a large number of extracellular stimuli and play an important role in proliferation, differentiation, and development (13, 14). ERK1,2 activation requires phosphorylation of two regulatory residues, threonine and tyrosine, that reside in a TEY phosphorylation motif (15). This phosphorylation is mediated by their upstream activator MEK, which phosphorylates both regulatory residues of ERK (16). However, the activity of ERK is regulated not only by MEK but also by the action of various phosphatases, which remove phosphates from the Thr alone, Tyr alone, or both residues to render the ERK inactive (17). Activated ERK1,2 regulate gene expression by phosphorylating multiple targets, including nuclear transcription factors such as c-Jun, Elk-1, c-fos, and signal transducer and activator of transcription (STAT) proteins (14, 18). Besides ERK1 and ERK2, alternatively spliced forms (such as the rodent ERK1b and the primate ERK1c) have been reported to influence the specificity of the ERK cascade (19).

Administration of the dual APL was demonstrated to up-regulate ERK1,2 activation in the induced $CD4^+CD25^+$ regulatory cells and inhibition of ERK1,2 in dual-APL-pretreated $CD4^+CD25^+$ cells, and abrogated their ability to suppress MG-associated responses. Furthermore, inhibition of ERK1,2 in the dual-APL-pretreated $CD4^+CD25^+$ cells was accompanied by a down-regulation of the Foxp3 gene expression, indicating the importance of ERK1,2 in the function of $CD4^+CD25^+$ cells after treatment with the dual APL (H.B.-D., B.V.A., M.S., and E.M., unpublished work).

The present study was undertaken to investigate a 50-kDa ERK that was detected after treatment with the dual APL. The major 50-kDa band of ERK was shown to react with various antibodies directed to ERK1,2 and to be inhibited by MEK1 inhibitor. ERK-50 was up-regulated after Con A stimulation; however, it was not affected by 4β-phorbol 12-myristate 13-acetate (TPA). Furthermore, the 50-kDa ERK was up-regulated in the dual-APL-induced $CD4^+CD25^+$ regulatory cell population. Thus, the 50-kDa ERK is suggested to be a novel ERK isoform that responds to specific TCR activation and is up-regulated by the dual APL.

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The authors declare no conflict of interest.

Abbreviations: APL, altered peptide ligand; CFA, complete Freund's adjuvant; dp-ERK, dual phosphorylated ERK; MG, myasthenia gravis; TPA, 4β-phorbol 12-myristate 13-acetate; U0126, 4-bis (2-aminophenylthio) butadiene.

‡To whom correspondence should be addressed. E-mail: michael.sela@weizmann.ac.il.

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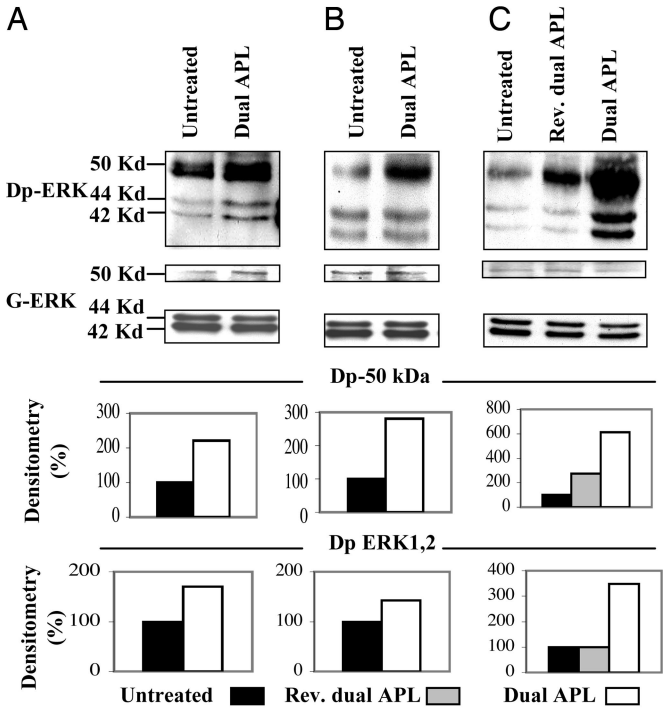

Fig. 1. Phosphorylation of a 50-kDa ERK in cells of p195–212-immunized SJL mice and in a p195–212-specific T cell line. (*A* and *B*) SJL mice were immunized with p195–212 (10 μg per mouse in CFA) and concomitantly injected s.c. with the dual APL (200 μg per mouse in PBS). Popliteal LN cells were harvested 10 days later, and whole cell (*A*) or LN-derived T cell (*B*) lysates were prepared, separated on SDS/PAGE, and transferred to a nitrocellulose membrane that was blotted with anti-dp-ERK1,2 and with its respective anti-G-ERK. (*C*) Cells of a p195–212-specific T cell line were incubated in the presence of p195–212 (5 μg/ml) and medium, the reversed dual APL, or the dual APL (500 μg/ml) for 24 h. Cell lysates were prepared and separated on SDS/PAGE, as described above. The bar graphs show results of densitometry expressed as percentage compared with the untreated groups (considered as 100%). Results were calculated based on the respective totals. *P* values for the 50-kDa ERK in the dual-APL-treated cells: $P = 0.002$ for LN cells, $P = 0.01$ for T cells, and $P = 0.001$ for p195–212-specific T cell line. Shown is one experiment of three performed.

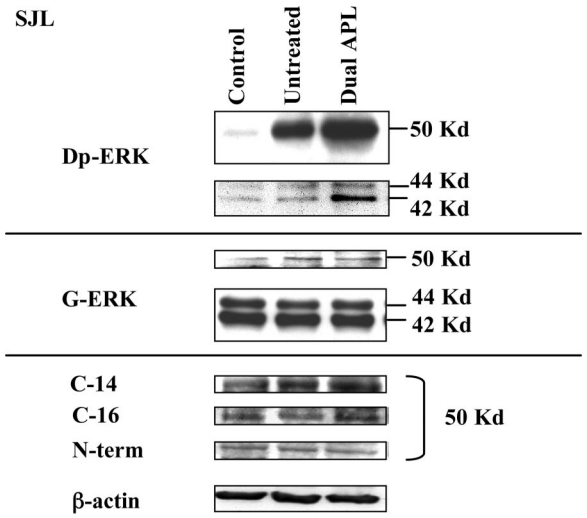

Fig. 2. The recognition of the 50-kDa ERK by ERK1,2-specific antibodies. SJL mice were immunized with p195–212 (10 μg per mouse in CFA) and concomitantly treated with the dual APL (200 μg per mouse in PBS). Ten days later, popliteal LN cells were harvested, and cell lysates were prepared, separated on SDS/PAGE and transferred to a nitrocellulose membrane that was blotted with anti-dp-ERK1,2, with anti-G-ERK, with anti-C terminus of ERK-Ab (C14 and C16), and with anti-N terminus of ERK antibodies. In addition, protein load was also determined by anti-β actin antibody. Shown are representative experiments of three performed.

Results

Phosphorylation of the 50-kDa ERK in T Cells Specific to p195–212. To find out the effect of the dual APL on the activation of ERK, we first immunized SJL mice with p195–212 alone or treated them concomitantly with the dual APL. Ten days after immunization and treatment, LN cells were harvested, and whole-cell or LN-derived T cell lysates were prepared and assayed for activated ERK42 and ERK44, using the anti-dual-phosphorylated ERK (dp-ERK) antibody, which specifically recognizes the TEY motif in the activation loop of ERKs. Fig. 1 shows ERK1,2 activities and the general (G)-ERK-reacted membranes of one representative experiment of three as well as the respective bar graphs that are based on densitometry. As can be seen in the blots and the respective graphs of the whole LN-cell lysates (Fig. 1*A*), the dp-ERK antibody detected, in addition to the expected 42-kDa and 44-kDa bands, a wide band of 50 kDa that was significantly up-regulated after treatment with the dual APL ($P = 0.002$ for the dual-APL-treated cells, as compared with cells of untreated mice considered as 100%). To examine the origin of the cells that express this protein, we isolated T cells of the whole lymph nodes and tested them for dp-ERK. Fig. 1*B* shows that the 50-kDa band was significantly up-regulated in LN-derived T cells after treatment with the dual APL ($P = 0.01$ for the dual-APL-treated cells, as compared with cells of untreated mice considered as 100%). This was further confirmed by using a specific p195–212 T cell line (that consists of 96% $CD4^+$ cells) in which the dp-ERK antibody detected the 50-kDa band most profoundly in the presence of the dual APL (Fig. 1*C*) ($P = 0.001$ for the dual-APL-treated cells, as compared with untreated cells of the line considered as 100%). The reversed dual APL, which serves as a control, stimulated the 50-kDa protein but to a lesser extent than the dual APL (Fig. 1*C*) ($P = 0.01$ for the dual APL as compared with the reversed dual-APL-treated cells of the line). The intensity of the up-regulated 50-kDa protein suggests that the latter might have a central function in dual-APL-treated T cells.

As can be seen in the G-ERK-reacted membranes, the anti-G-ERK antibody could also detect the band, although weakly, of 50 kDa in addition to the expected 42-kDa and 44-kDa bands. Thus, the phosphorylated 50-kDa band is detected after immunization with p195–212 and in the specific p195–212 T cell line, and is up-regulated after treatment with the dual APL.

Specificity of the 50-kDa ERK. To confirm that the detected 50-kDa band is an ERK protein, we tested whether this band is recognized by antibodies against the C and N termini of ERK1,2. To this end, SJL mice were immunized with p195–212 alone or were concomitantly treated with the dual APL. Cells of mice immunized with PBS in complete Freund's adjuvant (CFA) served as control. Ten days later, LN cells were harvested, and whole-cell lysates were prepared and assayed for the 50-kDa band, using antibodies against the C terminus of ERK1,2, N terminus of ERK1, G-ERK, and dp-ERK1,2. As can be seen in Fig. 2, the 50-kDa ERK was recognized by antibodies against the C terminus of ERK1 and ERK2 and by the antibody against the N terminus of ERK1. The 50-kDa band was weakly recognized by anti-G-ERK, and therefore the protein load was determined also by antibody against the β-actin for confirmation. Thus, the 50-kDa band is recognized by specific antibodies that react with the ERK1,2 proteins.

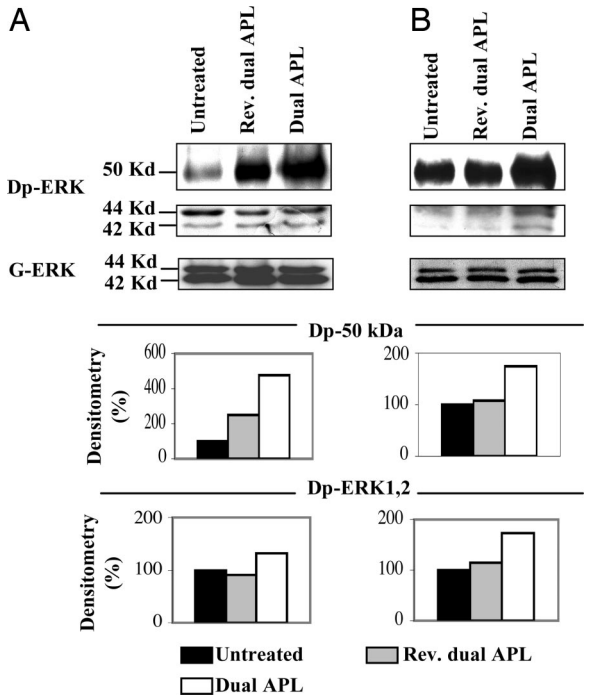

Fig. 3. Up-regulation of the 50-kDa ERK in BALB/c (*A*) and C57BL/6 (*B*) mice immunized with TAChR and treated with the dual APL. BALB/c and C57BL/6 mice were immunized with TAChR (10 μg per mouse in CFA) and concomitantly treated with the reversed or the dual APL (200 μg per mouse in PBS, s.c.). Popliteal LN cells were harvested 10 days later, and LN-cell lysates were prepared, separated on SDS/PAGE, and transferred to a nitrocellulose membrane that was blotted with anti-dp-ERK1,2 and with its respective anti-G-ERK. The bar graphs show results of densitometry expressed as percentage compared with the untreated groups (considered as 100%). Results were calculated based on the respective totals. $P = 0.002$ for the dual-APL-treated cells of BALB/c mice, $P = 0.01$ for the dual-APL-treated cells of C57BL/6 mice, as compared with cells of untreated mice. Shown is one representative experiment of three performed.

Up-Regulation of the 50-kDa ERK After Immunization with the TAChR and Treatment with the Dual APL. It was of interest to find out whether immunization of mice with the multideterminant antigen TAChR followed by treatment with the dual APL will lead to up-regulation of the 50-kDa band as well. To this end, BALB/c and C57BL/6 mice were immunized with TAChR alone or were concomitantly treated with the dual or the reversed (control) dual APL. Ten days after immunization and treatment, LN cells were harvested, lysed, and assayed for dp-ERK. As can be seen in Fig. 3 *A* and *B*, the 50-kDa band was observed in LN cells of TAChR immunized untreated BALB/c and C57BL/6 mice and up-regulated after treatment with the dual APL ($P = 0.002$ for the dual-APL-treated cells of BALB/c mice, $P = 0.01$ for the dual-APL-treated cells of C57BL/6 mice, as compared with cells of untreated mice considered as 100%). Treatment with the control reversed dual APL up-regulated the 50-kDa fraction, however, to a lesser extent. Thus, immunization with the whole macromolecule of TAChR resulted in a 50-kDa band that was up-regulated after treatment with the dual APL. This suggests that the apparently new 50-kDa ERK-like protein may have a major function in MG-associated responses that is initiated by immunization with either the myasthenogenic peptides or the TAChR. Furthermore, the 50-kDa ERK may play a role in the dual-APL mechanism of action because it is up-regulated after treatment with the dual APL.

Phosphorylation of the 50-kDa ERK upon Con A Stimulation. We wanted to further test whether the 50-kDa protein is up-regulated in response to stimulation with Con A, a nonspecific mitogenic stimulator of T cells that is known to trigger ERK1,2 proteins. To this end, SJL mice were immunized with p195–212, and 10 days later their LN cells were harvested and incubated with Con A for 12 and 24 h. Extracts of the cells were prepared and assayed for the dp-ERK. Fig. 4 demonstrates the anti-dp-ERK1,2 and anti-G-ERK-reacted membranes of one representative experiment of three performed as well as its respective bar graph. It can be seen that stimulation with Con A for 12 h slightly decreased the activity of the 50-kDa ERK; however, after 24 h of incubation with Con A, its activity was significantly up-regulated ($P = 0.003$ after 24 h of stimulation, as compared with cells at time 0, considered as 100%). Thus, Con A is capable of stimulating the 50-kDa ERK.

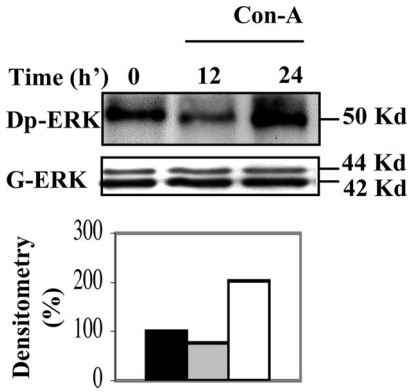

Fig. 4. Con A up-regulates the 50-kDa ERK. SJL mice were immunized with p195–212 (10 μg per mouse in CFA). Ten days later, popliteal LN cells were harvested and incubated with Con A (1 μg/ml at 37°C) for the indicated number of hours. Cells were washed, and lysates were prepared, separated on SDS/PAGE, and transferred to a nitrocellulose membrane that was blotted with anti-dp-ERK1,2 and with anti-G-ERK. The bar graph shows results of densitometry expressed as percentage in compared with time 0′ (considered as 100%). Results were calculated based on the respective totals. $P = 0.003$ for the 50-kDa ERK after 24 h of stimulation. Shown is one representative experiment of three performed.

Biochemical Regulation of the 50-kDa ERK. To further characterize the 50-kDa ERK in comparison with dp-ERK1,2, we studied its regulation after incubation with activators and suppressors of ERK1,2. To this end, SJL mice were immunized with the myasthenogenic peptide p195–212 alone or were concomitantly treated with the dual APL. Ten days later, LN cells of these mice were harvested, lysed, and assayed for dp-ERK. Fig. 5 demonstrates the dp-ERK and the G-ERK-reacted membranes of one representative experiment of three as well as the respective bar graphs of ERK1,2 and the 50-kDa ERK. Stimulation of LN cells with TPA resulted in an expected enhanced staining of the 42-kDa and 44-kDa bands, especially in the dual-APL-treated cells ($P \leq 0.002$ for cells treated with the dual APL and incubated with TPA, as compared with cells treated with the dual APL). However, incubation with TPA did not stimulate the 50-kDa ERK, even in the dual-APL-treated cells. Stimulation of LN cells with TPA followed by treatment with the MEK1 inhibitor 4-bis (2-aminophenylthio) butadiene (U0126) resulted in the down-regulation of the 50-kDa ERK to levels that were even lower than those seen in control (nontreated) cells [*P* values: $P \leq 0.01$ (50-kDa ERK), $P \leq 0.02$ (ERK1,2), for cells treated with the dual APL and incubated with U0126 plus TPA, as compared with cells treated with the dual APL]. Results are shown as blots and bar graphs based on densitometry, as compared with cells of peptide-immunized, nontreated mice, considered as 100%.

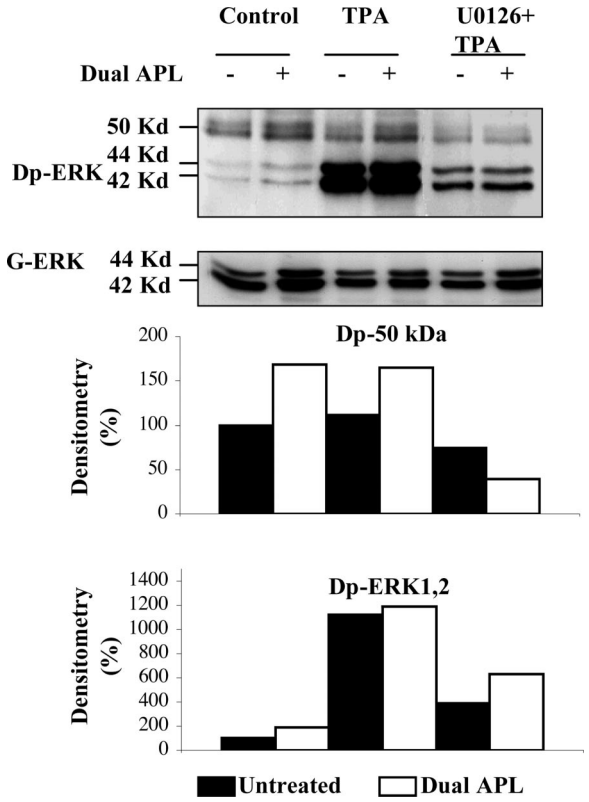

Fig. 5. The effect of TPA and U0126 on the phosphorylation of the 50-kDa ERK. SJL mice were immunized with p195–212 (10 μg per mouse in CFA) and concomitantly treated with the dual APL (200 μg per mouse in PBS). Ten days later, popliteal LN cells were harvested, and part of them were incubated with TPA (200 nM, 15 min at 37°C) alone or with U0126 (5 μM, 20 min at 37°C) and then with TPA. Cells were washed, and lysates were prepared, separated on SDS/PAGE, and transferred to a nitrocellulose membrane that was blotted with anti-dp-ERK1,2 and with anti-G-ERK. The bar graphs show results of densitometric units expressed in percentage as compared with control (non-treated) considered as 100%. *P* value for the 50-kDa ERK is $P \leq 0.01$ for cells that were incubated with U0126+TPA, as compared with cells treated with the dual APL only. P values for ERK1,2 are: $P \leq 0.002$ for cells that were incubated with TPA and $P \leq 0.02$ for cells that were incubated with U0126+TPA, as compared with cells treated with the dual APL only. Shown is one representative experiment of three performed.

The 50-kDa ERK Is Up-Regulated in the Dual-APL-Induced CD4$^+$CD25$^+$ Cell Population. CD4$^+$CD25$^+$ cells were previously reported to play a role in the mechanism of action by which the dual APL down-regulates MG and MG-associated responses (9, 12, 20). Because we have previously demonstrated that activated ERK1,2 are up-regulated in CD4$^+$CD25$^+$ cells, it was of interest to test the activation of the 50-kDa ERK in this subpopulation. BALB/c mice were first tested for the activation of the 50-kDa ERK after immunization with the myasthenogenic peptide p259–271 alone or after treatment with the dual APL. Ten days later, LN cells of these mice were harvested, lysed, and assayed for dp-ERK. As can be seen in Fig. 6*A*, the 50-kDa ERK was significantly up-regulated after treatment with the dual APL. Therefore, we attempted the testing of the 50-kDa ERK in an enriched CD4$^+$CD25$^+$ population of dual-APL-treated mice. To this end, we pretreated BALB/c mice with PBS or with the dual APL. Splenocytes of these mice were harvested and stained for CD4 and CD25 markers. Splenocytes obtained from the dual-APL-treated mice were either depleted of or enriched with CD25$^+$ cells. The dot plots of one representative experiment are shown in Fig. 6*B1*. An up-regulation (from 3.5% to 5.5%) in the induced CD4$^+$CD25$^+$ population was observed after treatment

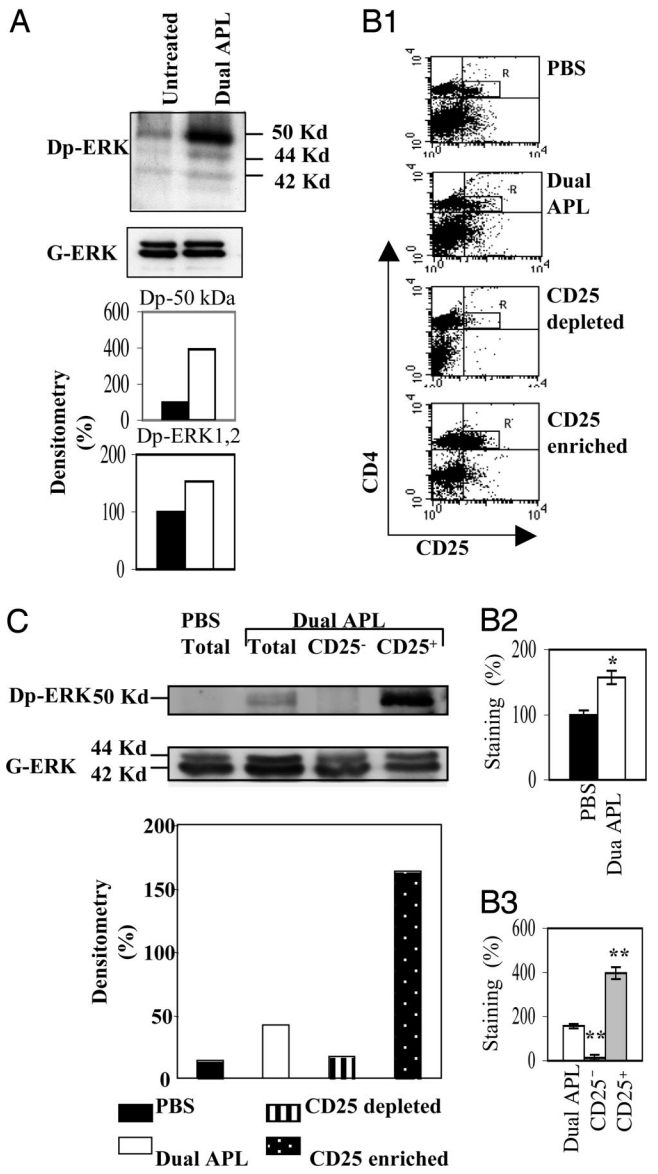

Fig. 6. The 50-kDa ERK is up-regulated in the dual-APL-induced CD4$^+$CD25$^+$ regulatory cells. BALB/c mice were either immunized with p259–271 (10 μg per mouse in CFA) and concomitantly treated with the dual APL (200 μg per mouse in PBS) (*A*) or pretreated s.c. with PBS or with the dual APL (200 μg per mouse in PBS) three times at 2-day intervals (*B1–B3* and *C*). Splenocytes obtained from the dual-APL-pretreated mice were depleted of or enriched with the CD4$^+$CD25$^+$ cells and stained with anti-CD4-PE, anti-CD25-FITC Abs, and their matched isotype controls (*B1*). (*B2*) Mean percent of staining determined in three experiments ($\pm$SD) as compared with PBS-pretreated cells (considered as 100%) (*, $P \leq 0.008$). (*B3*) Mean percent of staining determined in three experiments ($\pm$SD) as compared with dual-APL-pretreated cells (considered as 100%) (**, $P < 0.006$). LN cells obtained from the immunized mice (*A*) and splenocytes of the pretreated mice before and after CD4$^+$CD25$^+$ enrichment (*B1–B3*) were lysed, separated on SDS/PAGE, and transferred to a nitrocellulose membrane, and the blots were reacted with anti-dp-ERK1,2 or with anti-G-ERK (*C*). Results of the densitometry are expressed in percentage compared with cells of PBS-pretreated mice (considered as 100%), with *P* values of $P \leq 0.02$ for the dual APL and $P \leq 0.001$ for CD4$^+$CD25$^+$ enriched cell populations, as compared with cells of PBS-pretreated mice. Shown is one representative experiment of three performed.

with the dual APL in comparison with PBS-treated cells. Mean results of three experiments performed are presented in Fig. 6*B2*. The efficacy of the depletion and the enrichment of CD4$^+$CD25$^+$ cells is shown in the dot plots in Fig. 6*B1*, and the

mean results of three experiments are summarized in Fig. 6*B3*. Cell lysates were prepared from cell populations treated with PBS or with the dual APL, as well as from dual APL depleted of and enriched with $CD4^+CD25^+$ cells and tested for the presence of the 50-kDa ERK. Fig. 6*C* demonstrates one representative experiment of three performed in which the 50-kDa ERK was up-regulated in the dual APL-pretreated splenocytes in comparison with PBS-pretreated splenocytes. Depletion of $CD4^+CD25^+$ cells diminished this up-regulation, whereas enrichment of the induced $CD4^+CD25^+$ population markedly up-regulated the 50-kDa ERK expression. Thus, the 50-kDa ERK is up-regulated in the dual-APL-induced $CD4^+CD25^+$ cell population. Results of densitometry are shown as well ($P \leq 0.02$ for the dual APL and $P \leq 0.001$ for $CD4^+CD25^+$ enriched cell populations, as compared with cells of PBS-untreated mice).

Discussion

The main findings of the present study are that a novel band of 50-kDa ERK (in addition to ERK1,2) was up-regulated after treatment with the dual APL in SJL, BALB/c, and C57BL/6 mice that were immunized with either the myasthenogenic peptides or the TAChR. The 50-kDa ERK was expressed in T cells and specifically in the regulatory $CD4^+CD25^+$ T cells. The 50-kDa ERK was recognized by specific antibodies that react with ERK1,2 and shared some characteristics with its counterpart kinases ERK1,2. This clearly indicates a 50-kDa ERK that is up-regulated by a dual APL that was shown to ameliorate MG manifestations.

The ERK cascade is involved in diverse physiological processes that result in different outcomes. Thus, elucidation of the components by which different signals can be transmitted by similar signaling cascades is important. To date, there are two ERK genes (ERK1,2), and those encode two main proteins, p44 and p42 (19), and a few alternatively spliced forms such as the rodent ERK1b (21) and the primate ERK1c (22). We suggest that the major 50-kDa ERK that was observed in different mouse strains (SJL, BALB/c, and C57BL/6) after immunization with the myasthenogenic peptides or the TAChR and that was up-regulated by the dual APL may be an alternative spliced form of ERK. The findings that the 50-kDa band was detected by the dp-ERK antibody that specifically recognizes the dual-phosphorylated TEY motif and by antibodies against the C and the N termini of ERK, as well as by the anti-G-ERK antibody, supports this suggestion. Furthermore, the 50-kDa ERK was inhibited by the MEK1 inhibitor U0126, suggesting that it is regulated by MEK1, the upstream activator of ERK1,2. One of the most important regulatory steps in the cascade of ERK is the activation of ERKs by MEKs. MEK1 exclusively phosphorylates both Tyr and Thr residues in the Thr-Glu-Tyr motif in the activation loop (Thr-183, Tyr-185 in human ERK2) of ERKs (14). This process seems to be responsible for the specificity of the cascade and for its impressive cooperativity (23).

The 50-kDa ERK was stimulated by Con A (Fig. 4). The activation was detected after 24 h of incubation with Con A. It has been reported that ERK1,2 activation by Con A occurs within shorter incubation period (minutes to 1 h) (24). It is possible that the activation state of the 50-kDa ERK is maintained for a longer period, and this might account for the observed differences between the 50-kDa ERK and ERK1,2 after Con A stimulation. On the other hand, TPA, which significantly stimulates ERK1,2, could not stimulate the 50-kDa ERK. One reason that might explain this is an up-regulation of one or more phosphatases after TPA stimulation. Thus, although the mode of regulation of ERK1,2 and the 50-kDa ERK by MEK1 was similar, the different mode of activation by the above stimulators may be due to differences either in the phosphorylation or in the dephosphorylation processes, which may both culminate in the differential phosphorylation observed under some conditions. The duration and strength of activation of ERK1,2 and the 50-kDa ERK may also account for the detected changes after incubation with Con A or TPA. In neuronal PC12 cells, for example, transient activation of ERK results in proliferation, whereas a sustained activation causes differentiation (25).

The 50-kDa ERK was demonstrated to be expressed mainly in T cells either isolated from LN cells of mice or in a T cell line specific to the myasthenogenic peptide p195–212, after incubation with the dual APL (Fig. 1). One of the first pathways identified as important for positive selection of T cells was the Ras/ERK pathway. Triggering of the TCR activates the Ras/ERK cascade, leading to differentiation processes (26). Accumulating data show that interference with the Ras/ERK pathway using dominant negative Ras and catalytically inactive MEK1 transgenes inhibited positive selection (11). In addition, T cell clones rendered anergic and unable to produce IL-2 were shown to have defective MEK signaling (27).

The 50-kDa ERK that was up-regulated after treatment with the dual APL was found to be expressed in the dual-APL-induced $CD4^+CD25^+$ cell population (Fig. 6) rather than in the majority of $CD4^+CD25^-$ cells. $CD4^+CD25^+$ regulatory cells represent a well characterized population of cells that play crucial roles in immunological tolerance and in autoimmunity (28–30). The role of $CD4^+CD25^+$ cells in the control of autoimmune diseases was clearly demonstrated in experimental models in which elimination of $CD25^+$ T cells resulted in acceleration of disease progression including inflammatory bowel disease, experimental autoimmune encephalomyelitis, autoimmune diabetes (31), and lupus (32). In previous studies performed in our laboratory, we demonstrated that inhibition of ERK1,2 in $CD4^+CD25^+$ cells abrogated their ability to suppress IFN-γ secretion by LN cells of immunized mice (H.B.-D., B.V.A., M.S., and E.M., unpublished work). The 50-kDa ERK may also play a functional role in the $CD4^+CD25^+$ population of cells that is induced by the dual APL.

In summary, we demonstrated a major dual-phosphorylated, 50-kDa ERK that is expressed in T cells and is up-regulated after treatment- with the dual APL. Because the dual APL was shown to down-regulate MG-associated autoimmune responses and to ameliorate experimental autoimmune MG, the 50-kDa ERK might play a role in the immunomodulation of MG and possibly in other diseases as well.

Materials and Methods

Mice. Female mice of the inbred strains SJL (The Jackson Laboratory), BALB/c (Harlan), and C57BL/6 (Harlan) were used at the age of 8–12 weeks. The study was approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

TAChR, Synthetic Myasthenogenic Peptides, and Peptide Analog. AChR was purified from *Torpedo californica* as described (33) and was used for immunization. Peptides p259–271 (VIVELIPSTSSAV) and p195–212 (DTPYLDITYHFVMQRLPL) were synthesized and characterized as described (5). The dual APL (VIVKLIPSTSSAVDTPYLDITYHFVAQRLPL) (262Lys-207Ala) was designed as described (5) and synthesized (97% purity) by UCB Bioproducts. A peptide synthesized in the reversed order (reversed peptide) (LPLRQAVFHYTIDLYPTDVASSTSPILKVIV) of the dual APL was used as a control.

Immunization and Treatment of Mice. SJL, BALB/c, or C57BL/6 mice were immunized with 10 μg per mouse of p195–212, p259–271, and *Torpedo* AChR (TAChR), respectively, in CFA (Difco) and concomitantly treated with the dual or the reversed dual APL (200 μg per mouse, s.c.) in PBS. For pretreatment,

BALB/c mice were injected s.c. with the dual APL (200 μg per mouse in PBS) or with PBS three times at 2-day intervals.

Establishment of a p195–212-Specific T Cell Line. LN cells were harvested from SJL mice 10 days after their immunization with p195–212 (10 μg per mouse in CFA). The cells ($n = 5 \times 10^7$) were cultured in 25-ml flasks in 5 ml of enriched RPMI medium 1640 supplemented with 1% normal mouse serum and 5 μg/ml p195–212. After 4 days of incubation, cells were washed and resuspended in 5 ml of enriched RPMI medium 1640 supplemented with 10% FCS and 2 ng/ml recombinant murine IL-2 (Peprotech). Cells were exposed to the stimulating peptide (5 μg/ml) presented on irradiated (3,000 rad) syngeneic spleen cells every 14 days (34) and were maintained between stimulations in enriched medium containing IL-2. For all experiments, cells of the line were used 7 days after antigenic stimulation.

Isolation of T Cells. Petri dishes were coated with 5 ml of goat anti-mouse Ig (15 μg/ml in PBS) overnight at 4°C and washed three times. Cell suspensions of popliteal lymph nodes or spleens (50×10^6 per 5 ml in RPMI/10% FCS) obtained from the various mouse groups were incubated on the coated plates for 70 min at 4°C. The nonadherent cells, which were mainly T cells (95% as assessed by FACS analysis), were collected, washed in RPMI, counted, and used.

Preparation of Cell Lysates. LN cells, splenocytes, purified T cells, or p195–212-specific cells of the line were incubated for 10 min on ice in the presence of cold lysis buffer (50×10^6 per ml) containing: 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na-orthovanadate, 30 mM Na-pyrophosphate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.2.

Western Blot Analysis. Lysates were boiled in the presence of sample buffer. Equal amounts of proteins were separated on SDS/PAGE by using 10% polyacrylamide and transferred to nitrocellulose membrane. After blocking, the membrane was reacted with the relevant antibodies. The membrane was further incubated with the second antibody coupled to HRP. Detection was carried out by enhanced chemiluminescence method. Protein expression was determined by photodensitometry using the NIH Image program. The results were calculated based on the respective totals.

Reagents and Antibodies. Con A was obtained from MP Biomedicals, U0126 was obtained from Calbiochem, and TPA was obtained from Sigma (Rehovot, Israel). Antibodies directed to the anti-C terminus of ERK-Ab (C-16), anti-ERK2-Ab (C-14), and anti-Actin-Ab (C-2) were obtained from Santa Cruz Biotechnology. Anti-dp-ERK, anti-G-ERK, and anti-N terminus of ERK1-Ab were obtained from Sigma.

Depletion of $CD4^+CD25^+$ T Cells and Fluorescence Staining. Depletion of the $CD25^+$ T cells was performed by using the StemSep system (StemCell Technologies). Briefly, LN cells obtained from dual-APL-pretreated mice were incubated with an anti-CD25-biotinylated antibody (clone 7D4; Southern Biotechnology Associates), washed, and incubated with 10% normal mouse serum for 1 h. The cells were further incubated with an anti-biotin tetrameric complex (StemCell Technologies), washed again, and incubated with magnetic beads (StemCell Technologies). The cells were run through a column (StemCell Technologies), and the eluted cells were collected, used for Western blot and fluorescence staining with anti-CD25/IL-2R-FITC antibody (clone 7D4), anti-CD4-PE antibody (clone GK1.5), and their matched isotype controls (Southern Biotechnology Associates), and analyzed by FACS.

Statistical Analysis. To evaluate the differences between groups, Student's unpaired t test was used. Values of $P \leq 0.05$ were considered significant.

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