0026-895X/07/7101-314–322$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2007 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 71:314–322, 2007

Vol. 71, No. 1
28639/3167258
Printed in U.S.A.

RasGRP1 Confers the Phorbol Ester-Sensitive Phenotype to EL4 Lymphoma Cells

Shujie Han, Stewart M. Knoepp, Mark A. Hallman, and Kathryn E. Meier

Department of Pharmaceutical Sciences, Washington State University, Pullman, Washington (S.H., K.E.M.); and Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, South Carolina (S.M.K., M.A.H., K.E.M.)

Received July 6, 2006; accepted October 20, 2006

ABSTRACT

The murine EL4 lymphoma cell line exists in variants that are either sensitive or resistant to the tumor promoter phorbol 12-myristate 13-acetate (PMA). In sensitive EL4 cells, PMA causes robust Erk mitogen-activated protein kinase activation that results in growth arrest. In resistant cells, PMA induces minimal Erk activation, without growth arrest. PMA stimulates IL-2 production in sensitive, but not resistant, cells. The role of RasGRP1, a PMA-activated guanine nucleotide exchange factor for Ras, in EL4 phenotype was examined. Endogenous RasGRP1 protein is expressed at much higher levels in sensitive than in resistant cells. PMA-induced Ras activation is observed in sensitive cells but not in resistant cells lacking RasGRP1. PMA induces down-regulation of RasGRP1 protein in sensitive cells but increases RasGRP1 in resistant cells. Transfection of RasGRP1 into resistant cells enhances PMA-induced Erk activation. In the reverse experiment, introduction of small interfering RNA (siRNA) for RasGRP1 suppresses PMA-induced Ras and Erk activations in sensitive cells. Sensitive cells incubated with siRNA for RasGRP1 exhibit the PMA-resistant phenotype, in that they are able to proliferate in the presence of PMA and do not secrete IL-2 when stimulated with PMA. These studies indicate that the PMA-sensitive phenotype, as previously defined for the EL4 cell line, is conferred by endogenous expression of RasGRP1 protein.

EL4, a cell line originated from a carcinogen-induced murine thymoma, provides a unique model system for the study of phorbol ester response and resistance. Responses of sensitive "wild-type" (WT) EL4 cells to phorbol 12-myristate 13-acetate (PMA) include protein kinase C (PKC) activation (Kramer and Sando, 1986; Meier et al., 1991; Baier-Bitterlich et al., 1996; Sansbury et al., 1997), tyrosine phosphorylation (Richardson and Sando, 1995; Luo and Sando, 1997), Erk mitogen-activated protein kinase activation (Meier et al., 1991; Gause et al., 1993; Sansbury et al., 1997), adhesion (Resnick et al., 1997), IL-2 production (Farrar et al., 1980; Sando et al., 1982; Pearlstein et al., 1983; Harrison et al., 1987; Rayter et al., 1992), and growth arrest (Sando et al., 1982; Harrison et al., 1987; Desrivieres et al., 1997; Sansbury et al., 1997). To study the mechanisms by which PMA elicits these responses, we and other investigators have characterized PMA-resistant EL4 cells, which by definition proliferate in the presence of PMA (Resnick et al., 1997; Sansbury et al., 1997; Ku and Meier, 2000). Resistant EL4 cells activate PKCs in response to PMA but show minimal activation of Ras (Rayter et al., 1992), Erks (Meier et al., 1991), MEK (Gause et al., 1993), pp90^{RSK} (Meier et al., 1991), or JNK (Bradshaw et al., 1996). Erk activation is required for PMA-induced growth arrest in sensitive cells (Sansbury et al., 1997). This situation mimics that seen in thymocytes, in which rapid and robust Erk activation leads to negative selection (McNeil et al., 2005). Some PMA-resistant lines have been developed via selection for growth in the presence of PMA. However, in the absence of selective pressure, this phenotype exists as a natural variant in the EL4 cell population (Sansbury et al., 1997). Despite extensive characterization of PMA-sensitive and -resistant EL4 cell lines, the molecular basis for the phenotypes has not been fully established.

It is now clear that PKC isozymes are not the only receptors for tumor-promoting phorbol esters. Studies by our lab and others have indicated that differences in expression of PKC isoforms between sensitive and resistant EL4 cells are

This work was supported by the National Institutes of Health (grants CA58640 and CA94144), National Science Foundation (grant EPS-9630167), US Department of Defense (grants DAMD17-98-8524 and DAMD17-01-1–0730), the University Research Committee of the Medical University of South Carolina, and the Medical Scientist Training Program at the Medical University of South Carolina.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.028639.

ABBREVIATIONS: WT, wild-type; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; Erk, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA; IL, interleukin.

not responsible for the major differences in PMA sensitivity (Resnick et al., 1997; Sansbury et al., 1997). Other proteins, such as chimaerins and RasGRP1 (Kazanietz, 2002), contain the diglyceride/phorbol ester binding sites (C1 domains) present in PKC isoforms and thus bind PMA with affinity similar to that of PKCs (Tognon et al., 1998; Lorenzo et al., 2001). RasGRP1 is a guanine nucleotide exchange factor for Ras that binds phorbol esters in a calcium-independent manner (Lorenzo et al., 2000). This protein, which is highly expressed in T lymphocytes (Ebinu et al., 2000), is essential for thymocyte differentiation (Dower et al., 2000). RasGRP1 mediates PMA- and diglyceride-induced activation of Ras in T-cells (Ebinu et al., 2000; Jones et al., 2002) and in some other cell types (Lorenzo et al., 2001). Recent studies have established a physiologic role for RasGRP1 in mediating diglyceride signals in lymphoid cells (Sanjuan et al., 2003; Zheng et al., 2005). RasGRP1 has been shown to play a critical role in T cell differentiation (Priatel et al., 2006). There are three other members of the RasGRP family, each with distinct patterns of tissue expression (Ebinu et al., 1998; Reuther et al., 2002; Yang et al., 2002; Li et al., 2003). All, except for a mouse form of RasGRP4 (Li et al., 2003), are regulated by phorbol ester and diglyceride.

In this report, we further delineate pathways responsible for PMA sensitivity in EL4 thymoma cells. In particular, we show that RasGRP1 plays a critical role in the phorbol ester responsive phenotype.

Materials and Methods

Cell Culture. EL4 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, or Summit Biotechnology, Fort Collins, CO), nonessential amino acids, and penicillin/streptomycin. The original WT (PMA-sensitive) and variant (PMA-resistant) EL4 cell lines used in our lab were provided by Dr. David Morris (University of Washington, Seattle, WA). The derivation of clonal EL4 cell lines by our group was described previously (Ku and Meier, 2000). WT-derived (PMA-sensitive) clonal cell lines were maintained in suspension culture dishes (Corning) to discourage selection for adherent cells; variant clones were maintained in standard tissue culture flasks.

Cell Proliferation Assays. For cell proliferation assays, growing cells (≥95% viability) were seeded in 24-well tissue culture plates at 2×10^5 cells/well with 2 ml of complete medium (including serum). Cells were incubated with 100 nM PMA or 0.1% ethanol (vehicle) at 37°C for varying times, in complete medium. Cell number was determined by mixing cells with 0.02% trypan blue in phosphate-buffered saline (PBS) and counting dye-excluding cells using a hemocytometer.

Immunoblotting and Immunoprecipitation. Antibodies were obtained from the following sources: phospho-Erk, Promega (Madison, WI); Erk-1, Santa Cruz Biotechnology (Santa Cruz, CA); phospho-Raf (Ser338), Cell Signaling Technology Inc. (Danvers, MA); Ras, Santa Cruz Biotechnology or Chemicon (Temcula, CA); and RasGRP, Santa Cruz Biotechnology.

EL4 cells were treated with and without PMA as described previously (Ku and Meier, 2000). After treatment, cells were collected by centrifugation at 1200*g*. Adherent cells were harvested using a cell scraper before the centrifugation. Cells were lysed in a buffer containing 20 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Extracts were sedimented at 100,000*g* for 10 min at 4°C to remove insoluble material. Samples equalized for protein (100 μg), as determined by Coomassie Protein Assay (Pierce), were separated by SDS-PAGE on 7.5% Laemmli gels, transferred to polyvinylidene difluoride paper, incubated with antibodies, and developed using enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were imaged by densitometry and quantified using NIH Image software (http://rsb.info.nih.gov/nih-image/).

Transfections. The full-length cDNA for human RasGRP1, generously provided by Dr. Jim Stone (University of Alberta, Edmonton, AB, Canada), was subcloned into the pcDNA3 expression vector and transiently transfected into EL4 V5 cells. Empty vector pcDNA3 (Invitrogen, Carlsbad, CA) was used as a negative control. Actively growing cells were washed once with RPMI 1640 medium. Cells (2×10^7) were then mixed with 10 μg of the vector. The cell suspension (0.4 ml) was electroporated using a BTX Electro Cell Manipulator with parameters of 140 V, 720 Ω, and 3175 μF across a BTX cuvette with a 2-mm electrode gap. Pulse lengths varied between 25 and 28 ms. These conditions, optimized in our lab for EL4 cells, yielded a transfection efficiency of ~10% as assessed using a green fluorescent protein vector. Cells were incubated at room temperature for 15 min, then transferred to complete medium and returned to the cell culture incubator. Twenty-four hours later, cells were collected for further analysis.

Ras Activation Assay. Raf-1 RBD agarose (Upstate Biotechnology, Lake Placid, NY), which specifically binds to GTP-bound Ras, was used to pull down active Ras. Whole-cell extracts (1000 μg in 1 ml) were added to 10 μg of Raf-1 RBD agarose for 1 h at 4°C. Precipitates were washed three times with 1 ml of ice-cold lysis buffer, and then resuspended in 4× (Laemmli sample buffer for protein separation by SDS-PAGE. Immunoblotting was performed using an anti-Ras monoclonal antibody (Chemicon).

siRNA Experiments. RasGRP1 siRNA, control siRNA, RasGRP1 primer, siRNA transfection reagent and transfection medium, and RasGRP antibody were obtained from Santa Cruz Biotechnology. Cells, grown to 60 to 80% confluence, were incubated with RasGRP1 siRNA or control siRNA for 5 to 7 h in the absence of serum. FBS was then added to a final concentration of 10%; cells were incubated for an additional 18 to 24 h under cell culture conditions. The medium was then changed to RPMI with 10% FBS, and the cells were incubated for an additional 48 h before incubation with and without 100 nM PMA. Protein levels for RasGRP, phospho-Erk, and actin (immunoblotting), and activated Ras (pulldown assay) were assessed in cell extracts using the methods described above. Levels of RasGRP1 mRNA were assessed by RT-PCR, using the following protocol. Total RNA was extracted from harvested cells using TRIzol solution (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using ThermoScript RT-PCR System (Invitrogen) in a reaction volume of 20 μl under the conditions recommended by the manufacturer. Total RNA (3 μg) was used as a template for cDNA synthesis. The resulting cDNA was used as a template for PCR. PCR was performed in a 50-μl reaction volume with a buffer consisting of 10× PCR buffer without Mg, 50 mM $MgCl_2$, 10 mM dNTP mix, Platinum *Taq* DNA polymerase, and 1 μl of each primer. PCR was performed by initial denaturation at 94°C for 2 min followed by 40 cycles consisting of denaturation at 94°C for 30 s, annealing for 30 s at 55°C, and extension at 72°C for 30 s. RT-PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV illumination.

IL-2 Production Assays. WT2 and V7 cells were incubated in duplicate with or without 100 nM PMA for varying times. Supernatants (medium) were obtained by centrifugation for 3 min using a microcentrifuge. Enzyme-linked immunosorbent assay plates were coated with IL-2 antibody (BD Pharmingen, San Diego, CA) in binding solution (0.1 M Na_2HPO_4/NaH_2PO_4, pH 9.0) overnight at 4°. Plates were washed three times with 0.05% Tween 20 in PBS, then blocked for 1 h with 200 μl of 1% bovine serum albumin in PBS at room temperature. Duplicate samples of culture supernatant (100 μl/well) were added and incubated overnight at 4°. Biotinylated IL-2

(BD Pharmingen, San Diego, CA) was then added. After 1 h, streptavidin-horseradish peroxidase was added. After 30 min, the reaction was developed using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) for 20 min. A microplate reader (Bio-Rad Laboratories, Hercules, CA) was used to quantify the results at 450 nm. Statistical significance was analyzed using InStat (GraphPad Software, San Diego CA).

Results

Characterization of Clonal EL4 Cell Lines. To further explore the differences in PMA responsiveness between sensitive and resistant EL4 cells, we used a panel of clonal EL4 cell lines developed previously in our laboratory (Ku and Meier, 2000). The characteristics of these cells can be briefly summarized as follows. WT-derived cells (WT2, WT3, and WT5) do not proliferate in the presence of PMA. Studies by another group have established that the mechanism involves PMA-induced growth arrest in sensitive cells (Desrivieres et al., 1997). In contrast, most clones derived from a "variant" (V) cell line (V5, V7, V9, and V11) proliferate at a normal rate in the presence of PMA. Erk2 is robustly activated by PMA in all WT-derived clones but is activated to only a minor extent in the variant clones listed above. Two variant clones of "intermediate" phenotype, V3 and V10, are exceptions in that they show moderate Erk activation in response to PMA and are partially sensitive to PMA-induced growth inhibition. Clones WT2 and V7 have been routinely used in our lab as representative PMA-sensitive and -resistant cell lines, respectively. Erk activation in V7 cells, which is never as extensive as that seen in WT2 cells, requires higher doses of PMA and longer incubation times than for WT-derived cells (Ku and Meier, 2000). Incubation with 100 nM PMA for 15 min elicits the respective maximal responses in either cell type.

Ras Activation in Clonal EL4 Cell Lines. We first examined the ability of PMA to induce Ras activation in clonal EL4 cell lines. A Ras pulldown assay, in which GTP-bound Ras is detected, was used to examine the activation state of Ras. Initial experiments examined Ras and Erk activation at a single time point after PMA addition (15 min). As shown in Fig. 1A and quantified in Fig. 1B, the extent of Ras activation was much higher in WT2 (PMA-sensitive) cells than in V7 (PMA-resistant) cells. As previously reported (Ku and Meier, 2000), the extent of Erk activation was also higher in WT2 than in V7 (Fig. 1A).

Time course experiments were performed with several EL4 cell lines to further examine the extent of PMA-induced Ras and Erk activation (Fig. 2). As shown in Fig. 2A, PMA induces Ras activation in WT2 cells within 5 min. The activation persists for at least 60 min. The time course of Erk

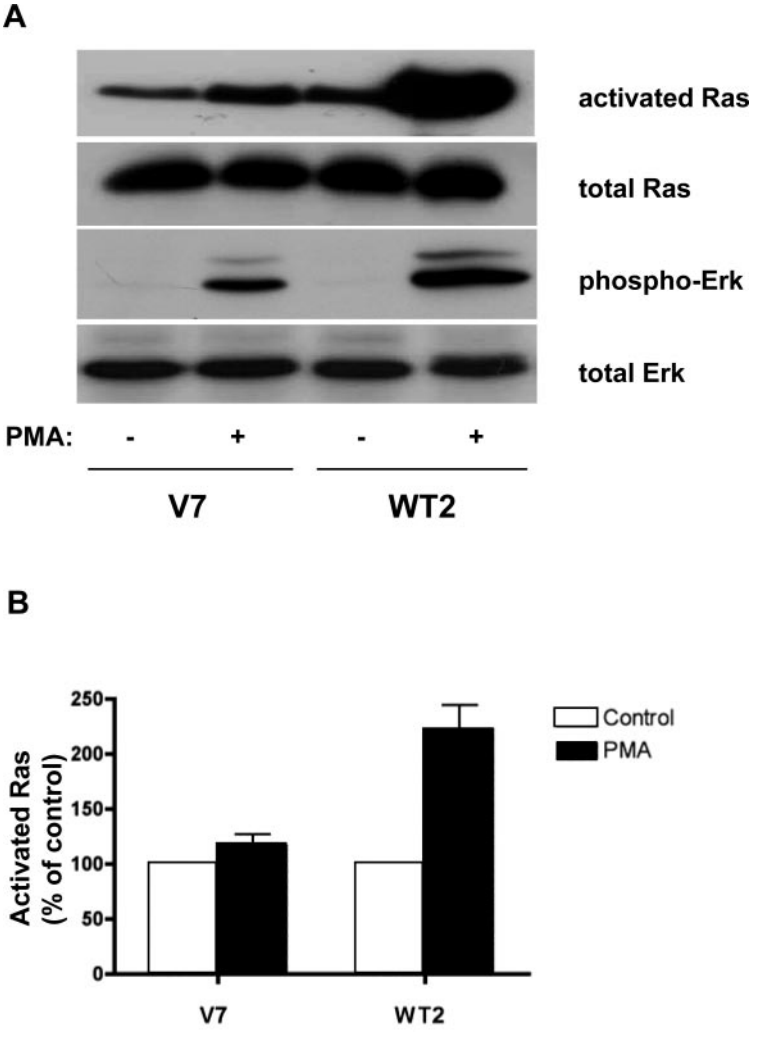

Fig. 1. Effects of PMA on Ras and Erk activation in clonal El4 cell lines. WT2 and V7 cells were treated with or without 100 nM PMA for 15 min. GTP-bound (activated) Ras was precipitated in a pulldown assay, as described under *Materials and Methods*, and detected by immunoblotting for Ras. Immunoblots were performed in the same experiment on whole-cell lysates for total Ras, phospho-Erk, and total Erk. A representative immunoblot is shown in A. Quantified results for Ras activation, normalized to total Ras, are shown in B. Data are expressed as percentage of the level of active Ras observed in each cell line without PMA treatment. Each data point represents mean ± S.E.M. from three separate experiments.

activation, as detected using phospho-Erk antibody, was similar to that of Ras activation. In contrast, in V7 cells, PMA-induced Ras activation can only be detected (to a minor extent) after 15 min. Erk activation is detected in V7 cells by 5 min (Fig. 2B) and subsequently declines. Thus, in resistant cells, the time course of Erk activation seen in V7 does not correlate well with the time course of Ras activation. The magnitude of both Ras and Erk activation is severely blunted in V7 compared with WT2. V3 and V10 cell lines have the intermediate phenotype. In these cell lines (Fig. 2, C and D), PMA-induced Ras activation is minimal or nonexistent, even though there is a moderate level of Erk activation. In both V3 and V10 cell lines, Erk activation is less than that seen in WT2 cells and declines after 30 min. Taken together, these data suggest that PMA-induced Ras activation is correlated with the fulminate Erk activation observed in PMA-sensitive EL4 cells. In resistant and intermediate phenotype cells, there is little or no Ras activation in response to PMA, and Erk activation is comparatively weak.

Expression of RasGRP in Clonal EL4 Cell Lines. We next examined the expression of RasGRP in clonal EL4 cell lines. As shown in Fig. 3A, RasGRP protein (migrating as a doublet at ~85–90 kDa) is expressed at much higher levels in WT-derived than in variant-derived clones. The antibody used recognizes several isoforms of RasGRP, but the protein detected is presumed to be RasGRP1 because of its molecular size (RasGRP is 90 kDa; other members of the family are 69–75 kDa) and because this isoform is highly expressed in T lymphocytes (Ebinu et al., 2000). Further validation is provided in Fig. 6. The effects of PMA on activation of Ras and Erk were tested in the same experiment. An incubation time of 15 min was used, based on the data obtained in Fig. 2. The results presented in Fig. 3A demonstrate that Ras was activated by PMA treatment in all of the cell lines expressing RasGRP1 (i.e., WT-derived cells). The basal level of active Ras was higher in RasGRP1-expressing (WT-derived) cells than in cells lacking RasGRP1. We were surprised to find that PMA-induced Ras activation was absent in intermediate clones V3 and V10, despite the fact that a modest level of Erk activation was evident in these cell lines. Thus, these data show that expression of RasGRP is positively correlated with PMA sensitivity in all clones except V3 and V10. These intermediate phenotype cell lines do not express RasGRP (Fig. 3A), but nonetheless activate Erks to a greater extent than resistant cells in response to PMA. An antibody recognizing Ser338 of Raf-1 was used to screen for Raf activation. Phosphorylation of this residue is important in the activation of Raf-1 by growth factors but is not sufficient for activation (Mason et al., 1999). The results showed that Raf was phosphorylated to some extent in untreated WT-derived cells (Fig. 3A). After addition of PMA to sensitive cells, the phospho-Raf band shifted upward on the gel in all WT cell lines. The latter effect is typically indicative of phosphorylation of a protein on additional sites. These complex results are consistent with the fact that Raf-1 is regulated by phosphorylation on multiple sites by multiple kinases, including positive regulation by Erks (Balan et al., 2006). In contrast, in PMA-resistant and intermediate cells, Raf was not phosphorylated under basal conditions. Addition of PMA induced Raf phosphorylation on Ser338 in these cells, but the extent of phosphorylation and the mobility shifts were not as obvious as in WT-derived cells. The intensity of the phosphorylated band

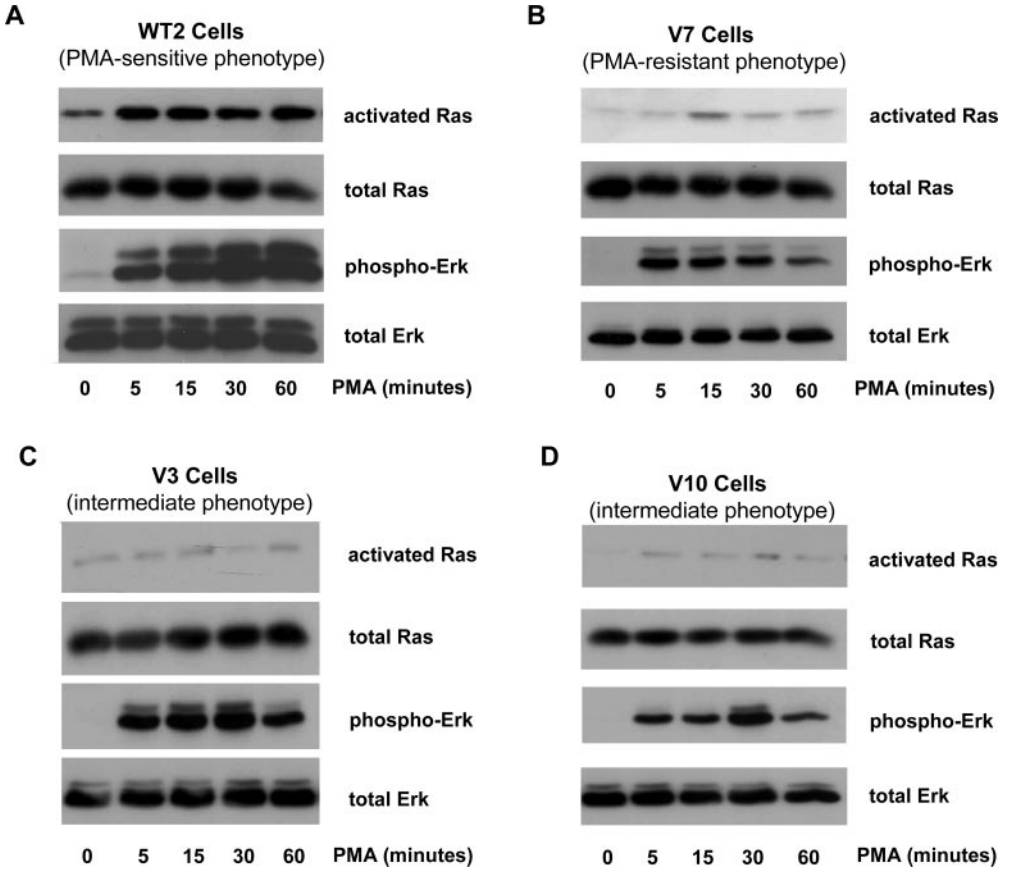

Fig. 2. Time course of the effects of PMA on Ras and Erk activation in clonal EL4 cell lines. WT2 (A), V7 (B), V3 (C), and V10 (D) cells were treated with or without 100 nM PMA for the indicated times. GTP-bound (activated) Ras was precipitated in a pull-down assay, as described under *Materials and Methods*, and detected by immunoblotting for Ras. Immunoblots were performed in the same experiment on whole-cell lysates for total Ras, phospho-Erk, and total Erk.

was greatest in the intermediate cells (V3 and V10), consistent with the greater extent of Erk activation observed in these cells. Finally, the results confirm that PMA-induced Erk activation is minimal in all of the resistant cell lines that lack RasGRP1 (V5, V7, V9, V11). In summary, the data presented in Fig. 3A establish that maximal PMA-induced activations of Ras, Raf, and Erk were only observed in EL4 cells expressing RasGRP1.

We next examined, using semiquantitative RT-PCR, whether the differences in RasGRP1 protein expression between sensitive and resistant cells reflected differences in mRNA expression levels. As shown in Fig. 3B, similar levels of mRNA for RasGRP1 were expressed in WT2 and V7 cells. Thus, the differences in protein expression apparently resulted from differences in post-transcriptional events between EL4 cell lines.

Regulation of RasGRP1 Expression. RasGRP isoforms, like PKC isoforms, can be down-regulated in response to PMA (Rambaratsingh et al., 2003; Tuthill et al., 2006). We therefore tested for long-term effects of PMA on RasGRP1 expression and signaling in EL4 cells (Fig. 4). As shown previously, PMA induced a high level of Erk activation in WT2 cells (Fig. 4A). This activation persisted for 24 h and then declined. RasGRP1 protein levels began to decline after 4 h, indicating down-regulation. It is noteworthy that a transient spike in phospho-Raf was observed at 3 to 8 h. This event was not correlated with a further increase in phospho-Erk levels. The phospho-Raf bands seen at 3 to 8 h exhibited progressive upward gel mobility shifts, suggestive of additional phosphorylation events. In V7 cells (Fig. 4B), effects of PMA on Erk activation were minimal, as shown previously. It is noteworthy that a transient spike in Erk activation was observed 4 h after PMA addition. This event was correlated with a concomitant increase in phospho-Raf. Thus, PMA induces effects on Raf phosphorylation in both sensitive and resistant EL4 cells after several hours. RasGRP1 protein levels unexpectedly increased in V7 cells at 4 h and were maintained at this level for at least 48 h. Despite the increase, the level of RasGRP1 protein was always much lower than that seen in untreated WT2 cells. These data establish that PMA causes down-regulation of RasGRP1 in sensitive EL4 cells, and up-regulation in resistant EL4 cells. The role of RasGRP1 in acute Erk activation was further explored.

Manipulation of RasGRP1 Levels in EL4 Cells. Based on the data presented above, we tested whether RasGRP confers PMA-induced Erk activation to resistant EL4 cells. V5 cells were used for these experiments. Cells transfected with empty vector were used as a negative control. As shown in Fig. 5, transient transfection was successful in increasing expression of RasGRP1 protein. PMA-induced Erk activation was enhanced in the RasGRP1-transfected cells. This conclusion was confirmed in additional experiments in which PMA-induced Erk phosphorylation was quantified and normalized to total Erk levels (data not shown). However, because of the poor transfection efficiency observed for full-length constructs in EL4 cells, alternative experimental strategies were employed as described below.

To further address the role of RasGRP1 in phorbol ester

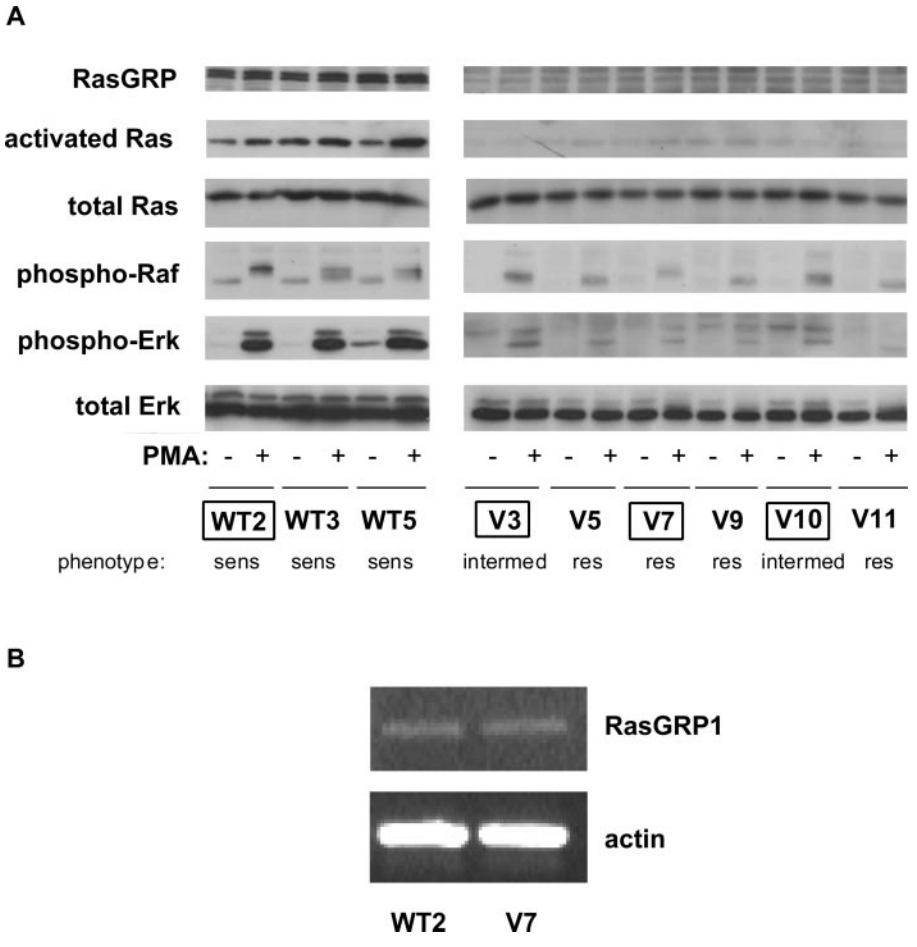

Fig. 3. Expression of RasGRP in clonal EL4 cell lines. In A, the indicated cell lines were incubated in the absence and presence of 100 nM PMA for 15 min. Whole-cell extracts, equalized for protein, were immunoblotted for RasGRP, total Ras, phospho-Raf, phospho-Erk, and total Erk. In addition, a Ras pulldown assay was performed to detect GTP-bound activated Ras, as described under *Materials and Methods*. All incubations were done in the same experiment; blots for the two cell lines were exposed in parallel. The cell lines that are "boxed" are the ones used in Fig. 1; the other cell lines serve as replicates within each phenotype ("sens," PMA-sensitive; "res," PMA-resistant; "intermed," intermediate phenotype. In B, semiquantitative RT-PCR was performed for untreated WT2 and V7 cells, using primers for murine RasGRP1. Actin was also amplified as a control for loading. The products were imaged under UV light on an ethidium bromide gel.

response, we used the opposite strategy of using small interfering RNA (siRNA) to reduce RasGRP1 levels in PMA-sensitive cells expressing endogenous RasGRP1. As shown in Fig. 6A, siRNA against RasGRP1 was very effective in reducing RasGRP1 mRNA levels in WT2 cells. Accordingly, RasGRP1 protein levels were severely reduced after siRNA treatment (Fig. 6B). PMA-induced Ras and Erk activations are blocked in cells lacking RasGRP1. These data confirm that RasGRP1 is largely responsible for conferring PMA-induced Ras and Erk activation to sensitive EL4 cells.

Effects of RasGRP Knockdown on Proliferation. As mentioned earlier, PMA-induced growth arrest is one of the hallmarks of the PMA-sensitive phenotype. PMA-sensitive cells (e.g., WT2) are unable to proliferate in the presence of PMA, whereas resistant cells (e.g., V7) continue to proliferate. Previous work in our laboratory showed that the cytostatic response of sensitive cells can be blocked by a pharmacologic inhibitor of MEK/Erk activation (Sansbury et al., 1997). We therefore tested whether knockdown of RasGRP1 would have a similar effect. WT2 cells were incubated with and without siRNA for RasGRP1. Proliferation was analyzed over two days, in the absence and presence of 100 nM PMA. As shown in Fig. 7A, control WT2 cells were unable to proliferate in the presence of PMA. In contrast, cells treated with siRNA for RasGRP1 proliferate at similar rates with or without PMA (Fig. 7B). A control siRNA had no effect on PMA-induced growth arrest. Immunoblots revealed that RasGRP1 knockdown was maintained for at least 48 h after treatment with RasGRP siRNA (data not shown). Together, these results demonstrate that RasGRP1 was required for PMA-induced growth arrest.

Effects of RasGRP1 Knockdown on IL-2 Production. Another hallmark of the PMA-sensitive phenotype is produc-

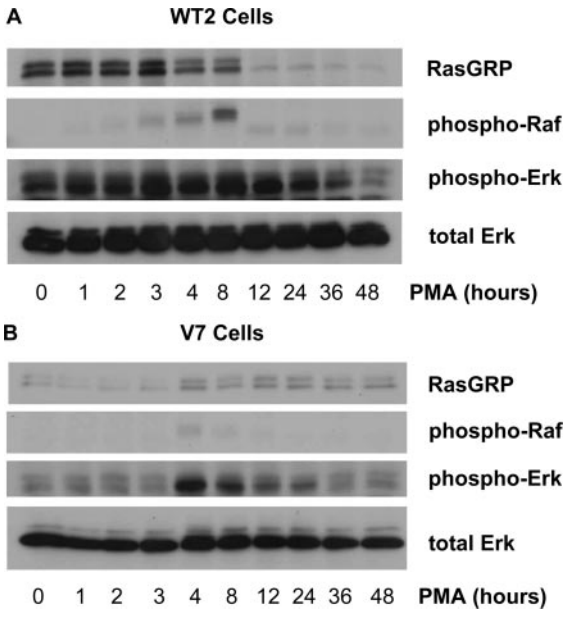

Fig. 4. Long-term effects of PMA on RasGRP levels and on Raf and Erk activation. WT2 (A) and V7 (B) cells were incubated for the indicated times with 100 nM PMA. Whole-cell extracts, equalized for protein, were immunoblotted for RasGRP, phospho-Raf, phospho-Erk, and total Erk. All incubations were done in the same experiment; the blots were exposed in parallel.

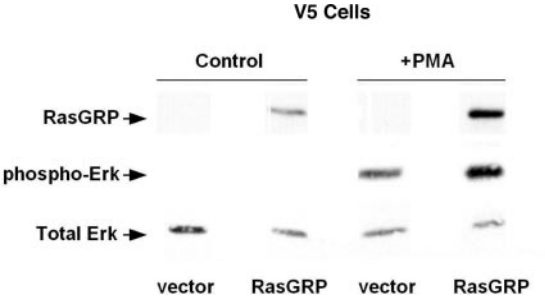

Fig. 5. Effects of RasGRP1 expression on PMA-induced Erk activation. EL4 V5 cells were transiently transfected with empty vector or RasGRP1. After 24 h, suspended cells were incubated with 100 nM PMA for 15 min. Whole-cell extracts, equalized for protein, were immunoblotted for RasGRP, phospho-Erk, and total Erk. All samples were from the same SDS-PAGE gel and were immunoblotted in parallel. The order of the lanes was rearranged for the purposes of presentation.

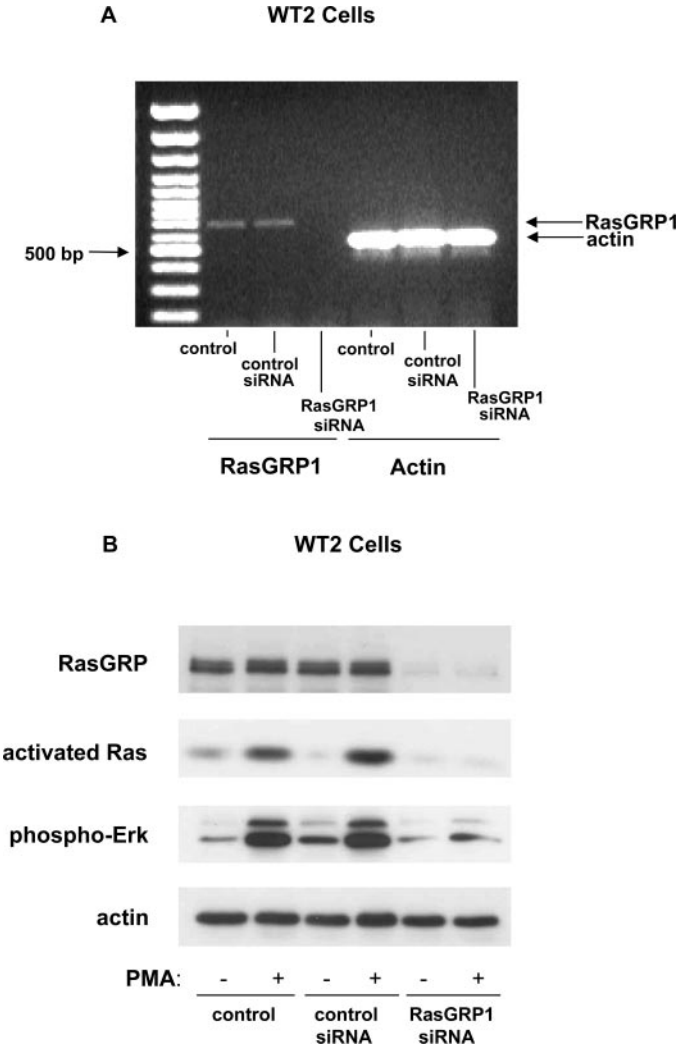

Fig. 6. Effects of RasGRP1 knockdown on PMA-induced Ras and Erk activation. In A, WT2 cells (expressing endogenous RasGRP1) were incubated in the absence of siRNA (control) or in the presence of either a control siRNA or RasGRP1 siRNA, as described under *Materials and Methods*. Message levels for RasGRP1 and actin (loading control) were assessed by semiquantitative RT-PCR. The ethidium bromide gel is shown, imaged under UV light. In B, WT2 cells were treated as in A, then incubated in the absence and presence of 100 nM PMA for 15 min. Levels of RasGRP, activated Erk (phospho-Erk), and actin (loading control) were assessed by immunoblotting of whole-cell extracts, equalized for protein. Activation of Ras (pulldown of GTP-bound Ras) was tested in the same experiment.

tion of IL-2 in response to PMA (Sansbury et al., 1997). Therefore, the effects of RasGRP1 knockdown on this response were tested. The time course of IL-2 production is shown in Fig. 8A. WT2 cells secreted IL-2 when incubated with 100 nM PMA; IL-2 levels in the medium increased from 8 to 24 h after PMA addition. In contrast, V7 cells did not secrete IL-2 in response to PMA. A similar lack of response was observed in intermediate clones V3 and V10 (data not shown). Basal levels of IL-2 are higher in WT2 cells than in V7 cells (170 versus 44 pg/ml; $p < 0.004$ by two-tailed t test). As shown in Fig. 8B, the effects of RasGRP1 siRNA on PMA-induced IL-2 production were analyzed at 24 h in WT2 cells. Treatment with RasGRP1 siRNA blockd the ability of PMA to induce IL-2 production in these cells. In addition, the siRNA significantly reduced basal levels of IL-2 in WT2 cells ($p < 0.004$ by two-tailed t test). A control siRNA had no effect on IL-2 production. These data show that RasGRP1 was required for PMA-induced IL-2 production in EL4 cells.

Discussion

In this study, we further explored the differences in phenotype between EL4 cells that are PMA-sensitive and PMA-resistant. We demonstrated that RasGRP1 protein was much more highly expressed in PMA-sensitive cells. Using overexpression and knockdown strategies, we showed that RasGRP1, a phorbol ester and diglyceride receptor, was responsible for the "classic" features of the PMA-sensitive phenotype (IL-2 production and growth arrest), as well as for the high level of Erk activation seen in sensitive cells upon PMA treatment.

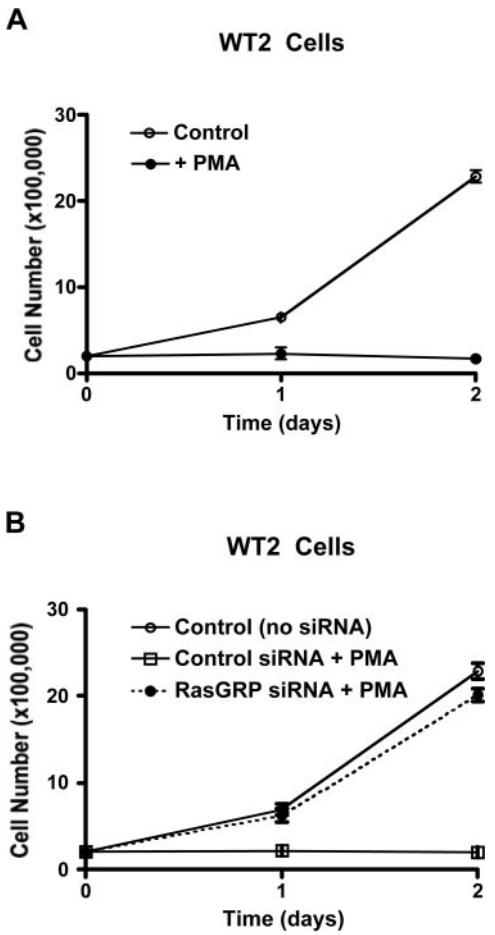

Fig. 7. Effects of RasGRP1 knockdown on PMA-induced growth arrest. WT2 cells were incubated without additions (A) and with either a control siRNA or RasGRP siRNA (B), as described under *Materials and Methods*. Cell proliferation was then assessed, as described under *Materials and Methods*, in the absence and presence of 100 nM PMA. Each data point represents the mean ± S.D. of values from triplicate samples of cells.

Our data establish that RasGRP1 played a key role in all phases of PMA response in EL4 cells. First, we demonstrated that PMA induced Ras activation most efficaciously in cells expressing RasGRP1. A previous study showed that Ras is activated in response to PMA treatment of sensitive EL4 cells (Rayter et al., 1992). Our data extend this observation, using a newer technique to assess Ras activation. Furthermore, using additional EL4 cell lines, we correlated the time course of Ras activation with that of Erk activation. Next, we demonstrated that overexpression of RasGRP1 in PMA-resistant cells enhanced PMA-induced Erk activation. In contrast, knockdown of RasGRP1 in PMA-sensitive cells resulted in loss of PMA-induced Ras activation, Erk activation, and IL-2 production. Thus, our data established that the signaling events that have historically defined "PMA sensitivity" in EL4 cells were all conferred by RasGRP1 expression.

RasGRP1 has been previously implicated in the IL-2 induction observed when primary T-lymphocytes are stimulated with PMA and calcium ionophore (Ebinu et al., 2000). This previous study used an overexpression approach. Our study, using a knockdown approach, definitively established a requirement for RasGRP1 in PMA-induced IL-2 secretion in EL4 cells. It should be noted that EL4 cells differ from primary T cells in that they can achieve substantial IL-2 production in the presence of PMA alone (Sansbury et al., 1997). However, the PMA-induced pathways (Ras, Erk, AP-1) seem to be the same in each case. IL-2 production is not believed to be responsible for PMA-induced cell arrest in EL4 cells; the magnitude and duration of Erk activation seem to induce this adverse effect via other downstream events (Desrivieres et al., 1997; Werlen et al., 2003).

It is noteworthy that Erks were activated to some extent in response to PMA in resistant and intermediate phenotype cells, in which PMA-induced Ras activation was barely detectable. Our results, therefore, suggest that there were alternative (non-Ras) pathways for PMA-induced Erk activation in these cells. The differences observed in Raf-1 phosphorylation status between the two cell lines, along with the effects of PMA on Raf-1 phosphorylation, might provide some insight into such pathways. Thus, these cell lines continue to present an intriguing paradox.

Although both sensitive and resistant EL4 cells express multiple PKC isoforms (Sansbury et al., 1997; Resnick et al., 1998), our data show that RasGRP1 was most critical for PMA-induced Ras activation. These data are consistent with the results of studies establishing that RasGRP isoforms, which are guanine nucleotide exchange factors, act as important receptors for phorbol esters and diglycerides in intact cells (Brose and Rosenmund, 2002; Roose et al., 2005). This did not exclude a role for PKC isoforms in other PMA-mediated responses, including the low-level Erk activation seen in EL4 cells lacking RasGRP1. The recent demonstration of cross-talk between PKCs and RasGRP3 (Roose et al., 2005; Zheng et al., 2005) suggests further aspects for future study.

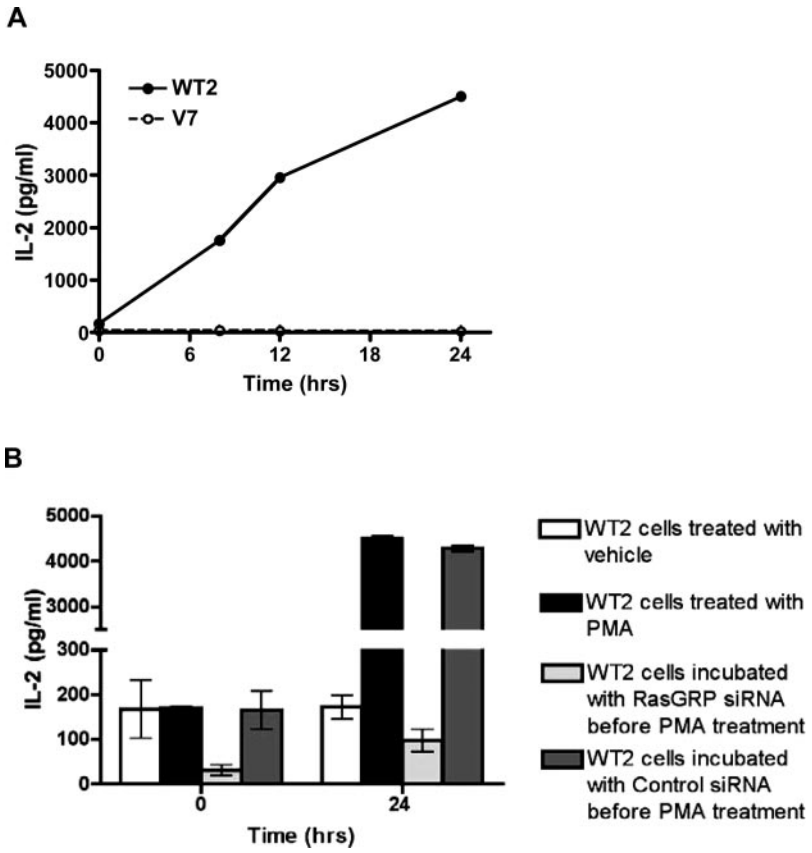

Fig. 8. Effects of RasGRP1 knockdown on PMA-induced IL-2 production. In A, the time course of PMA-induced IL-2 production was assessed in WT2 and V7 cells. Cells were incubated with 100 nM PMA for the indicated times. IL-2 in the medium was quantified by enzyme-linked immunosorbent assay, as described under *Materials and Methods*. Each data point indicates the mean ± S.D. of values from duplicate wells of cells; the error bars are included within the data points. In B, WT2 cells were treated with or without control siRNA or siRNA for RasGRP1 as described under *Materials and Methods*. Cells were then incubated with and without 100 nM PMA for 24 h. "Control" refers to incubation with the vehicle (0.1% ethanol) for PMA addition. Each value represents the mean ± S.D. from duplicate wells of cells.

In particular, the latter study showed that both RasGRP and PKCs were required for maximal Ras/Erk activation in T-lymphocyte cell lines (Roose et al., 2005). It should be emphasized that, because RasGRP isoforms are selectively expressed in certain cells and tissues (Ebinu et al., 1998; Tognon et al., 1998), these proteins are not likely to be responsible for mediating PMA response in all cell types.

The mechanisms underlying the differences in RasGRP1 protein expression between EL4 cell lines remain to be elucidated. Our data indicate that mRNA transcripts for RasGRP1 were expressed at similar levels in both WT2 and V7 cells. Expression of RasGRP isoforms can be regulated at the post-transcriptional level. In particular, post-transcriptional events have been shown to disrupt expression of hRasGRP4 protein in some cell lines and human patient samples (Yang et al., 2002). It is important to note that the observed loss of RasGRP1 expression in the PMA-resistant EL4 cell lines studied here did not occur in response to a genetic manipulation or a known selective pressure but was found as a naturally occurring variation within the "wild-type" (PMA-sensitive) stock in cell culture. This suggests the intriguing potential for gain or loss of RasGRP1 under physiologic conditions. It is also interesting to note that although PMA causes the expected down-regulation of RasGRP protein in sensitive EL4 cells, there is a modest up-regulation in resistant cells (Fig. 4). The mechanism underlying this phenomenon, and its significance, remain to be explored. Another novel observation made in this experiment, which used a much longer time course than was used previously (Ku and Meier, 2000), concerns the delayed effects of PMA on Raf phosphorylation at 4 to 8 h. The pathway(s) responsible for these effects has not yet been determined.

Although PKC isozymes and RasGRP possess similar C1 domains, there are differences in their ligand recognition properties (Lorenzo et al., 2000; Shao et al., 2001; Reuther et al., 2002; Rong et al., 2002; Madani et al., 2004; Pu et al., 2005). These differences may be responsible for the distinct dose-response profiles observed for PMA-induced Erk activation in sensitive versus resistant EL4 cells (Ku and Meier, 2000), as well as for the differential effects of various PKC activators on other signaling events in these cells (Sansbury et al., 1997). EL4 cell lines continue to provide a unique model system in which to examine the multiple signaling proteins involved in phorbol ester response. In particular, the differences in endogenous RasGRP1 expression between EL4 cell lines lend themselves to further studies of the role of this signaling protein in phorbol ester and diglyceride responses.

Acknowledgments

We thank Dr. Jim Stone for the RasGRP1 expression vector and insightful suggestions, Dr. Paige Lawrence for assistance with the IL-2 assay, and Manpreet Chahal for helpful comments.

References

Baier-Bitterlich G, Uberall F, Bauer B, Fresser F, Wachter H, Grunicke H, Utermann G, Altman A, and Baier G (1996) Protein kinase C-theta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol Cell Biol* **16:**1842–1850.

Balan V, Leicht DT, Zhu J, Balan K, Kaplun A, Singh-Gupta V, Qin J, Ruan H, Comb MJ, and Tzivion G (2006) Identification of novel in vito Raf-2 phosphorylation sites mediating positive feedback Raf-1 regulation by extracellular signal-regulated kinase. *Mol Biol Cell* **17:**1141–1153.

Bradshaw CD, Ella KM, Qi C, Sansbury HM, Wisehart-Johnson AE, and Meier KE (1996) Effects of phorbol ester on phospholipase D and mitogen-activated protein kinase activities in T-lymphocyte cell lines. *Immunol Letts* **53:**69–76.

Brose N and Rosenmund C (2002) Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *J Cell Sci* **115:** 4399–4411.

Desrivieres S, Volarevic S, Mercep L, and Ferrari S (1997) Evidence for different mechanisms of growth inhibition of T-cell lymphoma by phorbol esters and concanavalin A. *J Biol Chem* **272:**2470–2476.
Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, and Stone JC (2000) RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nature (Lond) Immunol* **1:**317–321.
Ebinu JO, Bottorff DA, Chan EYW, Stang SL, Dunn RJ, and Stone JC (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science (Wash DC)* **280:**1082–1086.
Ebinu JO, Stang SL, Teixeira C, Bottorff DA, Hooton J, Blumberg PM, Bleakley BM, Ostergaard HL, and Stone JC (2000) RasGRP links T-cell receptor signaling to Ras. *Blood* **95:**3199–3203.
Farrar JJ, Fuller-Farrar J, Simon PL, Hilfiker ML, Stadler BM, and Farrar WL (1980) Thymoma production of T cell growth factor (interleukin 2). *J Immunol* **125:**2555–2558.
Gause KC, Homma MK, Licciardi KA, Seger R, Ahn NG, Peterson MJ, Krebs EG, and Meier KE (1993) Effects of phorbol ester on mitogen-activated protein kinase kinase activity in wild-type and phorbol ester-resistant EL4 thymoma cells. *J Biol Chem* **268:**16124–16129.
Harrison JR, Lynch KR, and Sando JJ (1987) Phorbol esters induce interleukin 2 mRNA in sensitive but not in resistant EL4 cells. *J Biol Chem* **262:**234–238.
Jones DR, Sanjuan MA, Stone JC, and Merida I (2002) Expression of a catalytically inactive form of diacylglycerol kinase α induces sustained signaling through RasGRP. *FASEB J* **16:**595–597.
Kazanietz MG (2002) Novel "nonkinase" phorbol ester receptors: the C1 domain connection [published erratum appears in *Mol Pharmacol* **61:**1265, 2002]. *Mol Pharmacol* **61:**759–767.
Kramer CM and Sando JJ (1986) Substrates for protein kinase C in cytosol of EL4 mouse thymoma cells. *Canc Res* **46:**3040–3045.
Ku H and Meier KE (2000) Phosphorylaton of paxillin via the ERK mitogen-activated protein kinase cascade in EL4 thymoma cells. *J Biol Chem* **275:**11333–11340.
Li L, Yang Y, Wong GW, and Stevens RL (2003) Mast cells in airway hyporesponsive C3H/HeJ mice express a unique isoform of the signaling protien Ras guanine nucleotide releasing protein 4 that is unresponsive to diacylglycerol and phorbol esters. *J Immunol* **171:**390–397.
Lorenzo PS, Beheshti M, Pettit GR, Stone JC, and Blumberg PM (2000) The guanine nucleotide exchange factor RasGRP is a high-affinity target for diacylglycerol and phorbol esters. *Mol Pharmacol* **57:**840–846.
Lorenzo PS, Kung JW, Bottorff DA, Garfield SH, Stone JC, and Blumberg PM (2001) Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res* **61:**943–949.
Luo X and Sando JJ (1997) Deficient tyrosine phosphorylation of c-Cbl and associated proteins in phorbol ester-resistant EL4 mouse thymoma cells. *J Biol Chem* **272:**12221–12228.
Madani S, Hichami A, Charkaoui-Malki M, and Khan NA (2004) Diacylglycerols containing Omega 3 and Omega 6 fatty acids bind to RasGRP and modulate MAP kinase activation [published erratum appears in *J Biol Chem* **279:**23846, 2004]. *J Biol Chem* **279:**1176–1183.
Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, and Maris R (1999) Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO (Eur Mol Biol Organ) J* **18:**2137–2148.
McNeil LK, Starr TK, and Hogquist KA (2005) A requirement for sustained ERK signaling during thymocyte positive selection in vivo. *Proc Natl Acad Sci USA* **102:**13574–13579.
Meier KE, Licciardi KA, Haystead TAJ, and Krebs EG (1991) Activation of messenger-independent protein kinases in wild-type and phorbol ester-resistant EL4 thymoma cells. *J Biol Chem* **256:**1914–1920.
Pearlstein KT, Staiano-Coico L, Miller RA, Pelus LM, Kirch ME, Stutman O, and Palladino MA (1983) Multiple lymphokine production by a phorbol ester-stimulated mouse thymoma: relationship to cell cycle events. *J Natl Canc Inst* **71:**583–590.
Priatel JJ, Chen X, Chanji S, Abraham N, and Teh HS (2006) RasGRP1 transmits prodifferentiation TCR signaling that is crucial for CD4 T cell development. *J Immunol* **177:**1470–1480.
Pu Y, Perry NA, Yang D, Lewin NE, Kedei N, Braun DC, Choi SH, Blumberg PM, Garfield SH, Stone JC, et al. (2005) A novel diacylglycerol-lactone shows marked selectivity in vitro among C1 domains of protein kinase C (PKC) isoforms α and δ as well as selectivity for RasGRP compared with PKCα. *J Biol Chem* **280**:27329–27338.
Rambaratsingh RA, Stone JC, Blumberg PM, and Lorenzo PS (2003) RasGRP1 represents a novel non-protein kinase C phorbol ester signaling pathway in mouse epidermal keratinocytes. *J Biol Chem* **278:**52792–52801.
Rayter SI, Woodrow M, Lucas SC, Cantrell DA, and Downward J (1992) p21ras mediates control of IL-2 gene promoter function in T cell activation. *EMBO (Eur Mol Biol Organ) J* **11:**4549–4556.
Resnick MS, Kang BS, Luu D, Wickham JT, Sando JJ, and Hahn CS (1998) Differential downstream functions of protein kinase Cη and -θ in EL4 mouse thymoma cells. *J Biol Chem* **273:**27654–27661.
Resnick MS, Luo X, Vinton EG, and Sando JJ (1997) Selective up-regulation of protein kinase C eta in phorbol ester-sensitive versus -resistant EL4 mouse thymoma cells. *Cancer Res* **57:**2209–2215.
Reuther GW, Lambert QT, Rebhun JF, Califiuri MA, Quilliam LA, and Der CJ (2002) RasGRP4 is a novel Ras activator isolated from acute myeloid leukemia. *J Biol Chem* **277:**30508–30514.
Richardson AF and Sando JJ (1995) Rapid tyrosine phosphorylation of an 85,000 M(r) protein after phorbol ester stimulation of EL4 thymoma cells. *Cell Signal* **7:**17–30.
Rong SB, Enyedy IJ, Qiao L, Zhao L, Ma D, Pearce LL, Lorenzo PS, Stone JC, Blumberg PM, Wang S, et al. (2002) Structural basis of RasGRP binding to high-affinity PKC ligands. *J Med Chem* **45:**853–860.
Roose JP, Mollenauer M, Gupta VA, Stone J, and Weiss A (2005) A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Mol Cell Biol* **25:**4426–4441.
Sando JJ, Hilfiker ML, Piacentini MJ, and Laufer TM (1982) Identification of phorbol ester receptors in T-cell growth factor-producing and -nonproducing EL4 mouse thymoma cells. *Canc Res* **42:**1676–1680.
Sanjuan MA, Pradet-Balade B, Jones DR, Martinez-A, C., Stone JC, Garcia-Sanz JA, and Merida I (2003) T cell activation in vivo targets diacylglycerol kinase α to the membrane: a novel mechanism for Ras attenuation. *J Immunol* **170:**2877–2883.
Sansbury HM, Wisehart-Johnson AE, Qi C, Fulwood S, and Meier KE (1997) Effects of protein kinase C activators on phorbol ester-sensitive and -resistant EL4 thymoma cells. *Carcinogenesis* **18:**1817–1824.
Shao L, Lewin NE, Lorenzo PS, Hu Z, Enyedy IJ, Garfield SH, Stone JC, Marner FJ, Blumberg PM, and Wang S (2001) Iridals are a novel class of ligands for phorbol ester receptors with modest selectivity for the RasGRP receptor subfamily. *J Med Chem* **44:**3872–3880.
Tognon CE, Kirk HE, Passmore LA, Whitehead IP, Der CJ, and Kay RJ (1998) Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol Cell Biol* **18:**6995–7008.
Tuthill MC, Oki CE, and Lorenzo PS (2006) Differential effects of bryostatin 1 and 12-*O*-tetradecanoylphorbol-13-acetate on the regulation and activation of RasGRP1 in mouse epidermal keratinocytes *Mol Canc Ther* **5:**602–610.
Werlen G, Hausmann B, Naeher D, and Palmer E (2003) Signaling life and death in the thymus: timing is everything. *Science (Wash DC)* **299:**1859–1862.
Yang Y, Li L, Wong GW, Krilis SA, Madhusadhan MS, Sali A, and Stevens RL (2002) RasGRP4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function. *J Biol Chem* **277:**25756–25774.
Zheng Y, Liu H, Coughlin J, Zheng J, Li L, and Stone JC (2005) Phosphorylation of RasGRP3 on threonine 133 provides a mechanistic link between PKC and Ras signaling systems in B cells. *Blood* **105:**3648–3654.

Address correspondence to: Kathryn E. Meier, Department of Pharmaceutical Sciences, Washington State University, Pullman, WA 99164-6534. E-mail: kmeier@wsu.edu