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Protein Kinase A Regulates GATA-3-Dependent Activation of IL-5 Gene Expression in Th2 Cells

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Treatment of Th cells with compounds that elevate cAMP levels augments Th2-type lymphokine expression, in particular the synthesis of IL-5. Using primary murine CD4+ T lymphocytes, we show in this study that inhibition of protein kinase A (PKA) activity in Th2 effector cells impairs IL-5 synthesis, whereas the expression of PKA catalytic subunit α enhances IL-5 synthesis in Th0 cells. In addition, we observed by coexpression of PKA catalytic subunit and GATA-3 in Th1 cells that the stimulatory effect of PKA is dependent on GATA-3 activity. These data demonstrate that activation of PKA in Th effector cells induces the IL-5 gene expression in a GATA-3-dependent manner. The Journal of Immunology, 2003, 170: 2956–2961.

As on the synthesis of lymphokine subsets, effector Th cells have been subdivided into Th1 and Th2 cells that produce predominantly either IFN-γ, IL-2, and lymphotoxin, or IL-4, IL-5, IL-10, and IL-13, respectively (1). Whereas Th1-type lymphokines help to eradicate intracellular pathogens and, when overproduced, can cause autoimmunity, Th2 lymphokines suppress infections with extracellular pathogens, such as parasitic worms, and when overproduced, promote the development of allergic diseases, such as atopic asthma, hay fever, and eczemas (2).

Numerous studies have been performed to elucidate the molecular mechanisms that control the development of naive CD4+ lymphocytes to effector Th1 and Th2 cells (3–5). In addition to the lymphokine IL-4 and the IL-4-induced factor STAT6, the transcription factor GATA-3 has been described to play an important role in the differentiation and function of Th2 cells (6, 7). GATA factors were shown to control numerous developmentally regulated genes (8). In peripheral T effector cells, GATA-3 is highly expressed in Th2, but not in Th1, cells, and previous studies demonstrated that GATA-3 can commit naive Th cells to differentiate toward Th2 cells. In addition, the positive effect of ectopic expression of GATA-3 on the induction of Th2-type lymphokines indicates that GATA-3 also controls the effector phase of Th2 cells (6, 7). Multiple GATA binding sites are located within the promoter region of the human and murine IL-5 genes, and in combination with NF-AT, Ets-, AP-1-like, and further transcription factors, GATA-3 contributes to the inducible IL-5 expression in Th2 cells (6, 9, 10) and mast cells (11). Although no obvious GATA-binding motifs are part of the murine IL-4 promoter, several regions around the murine chromosomal IL-4 gene, including a downstream enhancer element (12), are bound by GATA-3 and contribute to IL-4 gene regulation (13). Expression of a dominant-negative mutant of GATA-3 in T cells of transgenic mice led to a marked reduction in levels of all Th2-like lymphokines and attenuation of Th2 responses, such as of IgE synthesis and airway eosinophilia (14), underlining the important role of GATA-3 in both Th2 commitment and effector function.

Stimulation of adenylylcyclase/cAMP cascade exerts a dual effect on T lymphocytes. Whereas in peripheral T cells and Th1 effector cells elevated cAMP levels inhibit TCR-mediated cellular activation, stop proliferation, and induce apoptosis, they increase effector functions in Th2 cells (15). These contrasting effects on Th1 and Th2 cell functions are reflected in the induction of lymphokine synthesis: while elevated cAMP levels block IL-2 and IFN-γ production, they stimulate the synthesis of IL-4, IL-5, and further Th2-type lymphokines (16–19).

In this study, we focused our attention on the effect of elevated cAMP levels and protein kinase A (PKA) activity on Th2 responses in primary murine Th cells. We show that in Th2 cells, selective inhibition of PKA activity by H89 or overexpression of a mutant version of regulatory subunit I of PKA, unable to bind cAMP, impairs IL-5 expression. In contrast, in Th0 cells, enhanced PKA activity leads to a significant increase in IL-5 production. Because coexpression of the catalytic PKA subunit with GATA-3 in Th1 cells induced both IL-4 and IL-5 synthesis, we conclude that PKA functions as an important regulator of GATA-3-dependent Th2 responses.

Materials and Methods

Cells, DNA transfection, and retroviral infection

Murine EL-4 thymoma cells were grown in RPMI medium containing 5% FCS and 293 human embryonic kidney (HEK) cells in DMEM containing 10% FCS. The 293 HEK cells were transfected using Superfect according to the protocol of Qiagen (Valencia, CA). Stably transduced EL-4 cells were selected with zeocin (250 μg/ml; Invitrogen, San Diego, CA) for 7 days.

Abbreviations used in this paper: PKA, protein kinase A; AKAP, A-kinase anchor protein; F, forskolin; GFF, green fluorescent protein; EG2, enhanced GFP zeocin; EY2, enhanced yellow fluorescence protein zeocin; HEK, human embryonic kidney; I, ionomycin; LN, lymph node; PKAα; catalytic PKA subunit α; PKAαβδ, mutated regulatory PKA subunit Iα; tg, transgenic; TPA or T, 12-O-tetradecanoylphorbol-13-acetate.
days, and the green or yellow fluorescence was checked by FACS. If not stated otherwise, the cells were treated with 10 ng/ml 12-O-tetradecanoyl-phorbol-13-acetate (TPA (T)), 0.5 μM ionomycin (I), and/or 50 μM forskolin (F), or, instead of F, with 1 mM dibutyryl-cAMP, as indicated.

The proximal murine IL-5 promoter spans the nucleotides from position 11001 to 11020; the longer promoter version includes the nucleotides up to 11050. Both were cloned as HindIII/XhoI fragments in front of the TATA-luciferase vector (20). The Gm-120 IL-5 promoter (Fig. 4)

FIGURE 1. F enhances T/I-mediated induction of IL-4 and IL-5 expression in EL-4 thymoma cells and IL-5 expression in primary CD4+ Th0 cells. A, Detection of IL-4 and IL-5 synthesis by intracellular staining and ELISA, respectively. Murine EL-4 thymoma cells were induced for 12 h with TPA (10 ng/ml) and I (0.5 μM) (T/I), F (50 μM), or T/I/F, or left untreated (–). One-half of the cultures were treated with brefeldin (10 μg/ml), and 4 h later IL-4-producing cells were determined by intracellular staining. IL-5 concentration was measured 48 h after activation by ELISA in the cell-free supernatant of the other half of the cultures. B, ELISA of IL-5 secretion by primary CD4+ T cells. CD4+ T cells from LN of DO11.10 TCR tg mice were maintained after primary stimulation for 5 days under Th0, Th1, or Th2 priming conditions. IL-5 production by unstimulated (–) cells and cells restimulated for 48 h by T/I, F, or T/I/F was determined by ELISA. Relative values to T/I/F-treated Th2 cells of two independent experiments (100% equals to 500 or 2500 U/ml) are shown. C, ELISA of IL-5 secretion by unstimulated CD4+ Th0 cells (–) or Th0 cells restimulated for 48 h, as indicated. Relative values of two independent experiments (100% equals to 80 or 30 U/ml) are shown.

FIGURE 2. Inhibition of PKA activity by H89 or a mutant of the PKA regulatory subunit I (PKA RM) impairs IL-5 promoter activity and IL-5 expression in Th2 cells. A, H89 inhibits IL-5 synthesis and PKA activity, but not p38 kinase activation in T/I-induced Th2 cells. CD4+ T cells from LN of DO11.10 TCR tg mice were cultured under Th2-skewing conditions for 5 days and restimulated by T/I in the absence or presence of 2 or 10 μM H89 for 48 h, and IL-5 secretion was measured by ELISA. Relative values to control cells (100% equals to 130 or 75 U/ml) of two independent experiments are shown. PKA activity and p38 activation were measured in whole cell protein extracts 60 and 30 min postrestimulation, respectively. Results of one representative experiment are shown here. B, Inhibitory effect of PKA RM on IL-5 promoter activity. EL-4 cells were cotransfected with a −507 IL-5 promoter-driven luciferase plasmid and vectors expressing PKA C or mutant PKA RM that is unable to bind cAMP. Luciferase activity was measured from cells that were either left untreated (–), or induced with T/I or T/I/F for 20 h. Luciferase values were normalized using a cotransfected Rous sarcoma virus promoter-driven β-galactosidase reporter gene. Mean values of three independent experiments are shown. C, Inhibition of IL-5 production by PKA RM in Th2 cells. One day after primary stimulation, CD4+ T cells from LN of DO11.10 TCR tg mice were infected with recombinant retroviruses transducing PKA RM or a control virus and maintained under Th2 conditions. Two days postinfection, cells were sorted by FACS into EGZ-infected cells (Vec.) and PKA RM-infected cells, as well as noninfected cells (–). Three days later, cells were restimulated for 48 h with T/I, and IL-5 secretion was determined by ELISA. Relative values to uninfected cells of two independent experiments (100% equals to 60 or 30 U/ml) are shown. D, PKA RM expression does not affect IL-9 secretion. ELISAs for IL-9 secretion were performed in supernatants from C.
bears a CTATC to GCATT mutation within its unique GATA binding site (around position −72), rendering it unable to be bound by GATA-3.

DO11.10 TCR transgenic (tg) BALB/c mice (21) were sacrificed at the age of 5–6 wk. Lymph node (LN) CD4+ T cells were isolated by passing them over CD4 T cell recovery columns (Cedarlane, Hornby, Ontario, Canada), according to the manufacturer’s instructions. Naive CD4+ CD62Lhigh cells were enriched by MACS (Miltenyi Biotec, Auburn, CA) to >95% purity. The cells were cultured at 5 × 10^6/ml in X-VIVO15 (BioWhittaker, Walkersville, MD) supplemented with 5% FCS, glutamine, nonessential amino acids, pyruvate (all 2 mM), antibiotics (penicillin, streptomycin), and 50 μM 2-ME. Dendritic cells were prepared from spleens, irradiated, and used as APCs. T cells were activated by the cognate OVA peptide (323–339) presented on APCs and cultured in the presence of respective lymphokines for Th1 and Th2 differentiation (22). Th0 cells were obtained by culturing CD4+ T cells in the presence of IL-2 (50 U/ml), αIL-4 Ab (10 μg/ml), and αIFN-γ Ab (10 μg/ml). After 24 h, cells were infected with retroviruses, as described previously (23).

**Retroviral constructs**

The bicistronic retroviral vectors pEGZ/MCS (enhanced GFP zeocin/multiple cloning site) and pEYZ/MCS (enhanced yellow fluorescent protein zeocin/multiple cloning site) are based on the vector pczCFG2 ICB8 IEYZ in which the CMV enhancer replaces the U3 region of the 5′ long terminal repeat of murine leukemia virus (see Ref. 23 for further details). The retroviral vector expressing the murine catalytic PKA subunit α, PKAα, contains its cDNA of 1.3 kb (24). PCR cloned as a Clal/BamHI fragment in frame with an hemagglutinin tag sequence at its N terminus into the EcoRI site of pEGZ/MCS. In Fig. 3A, a PKAα vector was used that contains a modified hormone-binding domain of the estrogen receptor. The retroviral vector expressing a mutated version of the regulatory PKA subunit Iα, PKAα, which is unable to bind cAMP and, therefore, acts as a repressor of PKA activity (25), contains a PCR-cloned Clal/BamHI dDNA fragment of 1.2 kb inserted into pEYZ/MCS. The retroviral vector expressing murine GATA-3 (7) was constructed by cloning a PCR EcoRI/BamHI fragment of 1.2 kb with a 5′ flag-encoding sequence into the EYZ/MCS vector.

**PKA activity and Western blot assays**

To determine PKA activity, whole cellular protein lysates were used in the PKA assay kit (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer’s instructions. Specific PKA activity was calculated by subtracting the background kinase activity in the presence of a PKA inhibitor peptide provided by the manufacturer.

The Ab detecting phospho-Thr286/Tyr282 p38 (9211) was purchased from Cell Signaling Technology (Beverly, MA), and the Ab raised against p38 (C-20; sc-535) from Santa Cruz Biotechnology (Santa Cruz, CA). In Western blots, whole cellular protein lysate from 5 × 10^6 cells was fractionated by 10% SDS-PAGE and electroblotted onto nitrocellulose membrane. For detection of proteins, appropriate peroxidase-coupled secondary Ab (10 μg/ml) were used along with a standard ECL system (Amersham, Arlington Heights, IL).

**RNase protection assay and ELISA**

For RNase protection assay, total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). The RNA was processed according to RiboQuant protocol of BD Biosciences (San Diego, CA) using the cytokine mCK-1 multiprobe template set. ELISAs were performed for detection of IL-4, IL-5, and IFN-γ secretion using mAbs of BD Biosciences, according to the manufacturer’s instructions.

**Results**

Elevation of cAMP levels in T cells by stimulation of adenyllyl-cyclase with F exerts a strong stimulatory effect on the induction of Th2 responses. This is demonstrated in Fig. 1 for EL-4 thymoma cells and primary effector Th cells. The treatment of EL-4 cells with the phorbolester TPA and the Ca2+ ionophore I (T/I), being potent inducers of IL-2 and IFN-γ RNA synthesis (see, for example, Fig. 3A), hardly affected IL-4 and IL-5 expression, whereas triple treatment of cells by T/I and F exerted a strong synergistic effect on the expression of both IL-4 and IL-5 (Fig. 1A). A similar strong effect of T/I/F treatment on IL-5 synthesis was detected in CD4+ Th0 cells, which, otherwise, synthesize only low amounts of IL-5 (Fig. 1B). Although treatment of these cells by either T, I, or F alone failed to induce IL-5, a combination of two of these inducers, such as T/I or T/F, led to a relatively weak IL-5 synthesis of not more than 30% of T/I/F treatment. Thus, T/I/F appears to be sufficient to activate all signaling pathways necessary for an optimal IL-5 gene expression in Th0 cells (Fig. 1C). As expected, Th1 cells did not secrete any IL-5, irrespective of their treatment (Fig. 1B). In contrast, Th2 cells secrete IL-5 upon T/I stimulation alone, and additional F treatment did not further enhance IL-5 production. In our view, this might be due to the relatively high levels of active cAMP in Th2 cells (26), which facilitate the T/I-mediated IL-5 induction by stimulating PKA activity in these cells.

Therefore, we reasoned that the cAMP/PKA signaling cascade might play an important role in the activation of IL-5 and, possibly, other lymphokines in Th2 cells. To test this hypothesis, we
induced Th2 cells with T/I in the presence or absence of 2 or 10 μM H89, a protein kinase inhibitor that inhibits specifically PKA activity at these low concentrations (27). This treatment, which resulted in a distinct decrease of inducible PKA activity, but not of p38 kinase activation by T/I, led to a significant decrease in IL-5 production up to almost 50% (Fig. 2A). In another approach, we investigated the effect of a more physiological PKA inhibitor, i.e., a mutant version of the regulatory type I subunit of PKA (PKARM), which is unable to bind cAMP (25). In transient transfection experiments, expression of PKARM in EL-4 cells abrogated the T/I/F-mediated activation of the IL-5 promoter (Fig. 2B).

For the inhibition of PKA activity in primary Th2 cells, we constructed a PKAREG-encoding retroviral vector that, due to the existence of an internal ribosomal entry site, expresses also a green fluorescence protein (GFP) and zeocin for the selection of positively transduced cells. CD4+ T cells from LNts of DO11.10 tg mice expressing an OVA-specific TCR (21) were infected with recombinant retroviruses 1 day after primary stimulation with the OVA peptide and maintained under Th2 conditions. Two days postinfection, cells were sorted by FACS into noninfected (-) and cells infected with EGZ (Vec.) and PKA RM virus, and 5 days after primary activation, they were restimulated by T/I for 48 h. Stimulation of PKA RM-transduced cells led to almost 50% reduction in IL-5 secretion compared with control cells (Fig. 2C). In contrast, the secretion of IL-9 remained unaffected by the ectopic expression of PKARM. These observations support our assumption that, in Th2 cells, high levels of activated PKA contribute to IL-5 production.

Whereas T/I treatment was sufficient for optimal IL-5 production in Th2 cells, a third signal was required for full IL-5 synthesis in EL-4 and Th0 cells. Because we observed that additional F treatment led to a strong IL-5 increase in these cells (Fig. 1), we asked whether the missing signal is provided by active PKA. To this end, we established EL-4 cells stably transduced with retrovirus expressing PKA C or control retrovirus (Vec.). As shown in Fig. 3A, expression of PKAc is sufficient to replace the F signal in the T/I-mediated induction of IL-4 and IL-5 RNA synthesis (compare lanes 4 and 6). Interestingly, in contrast to F treatment, the ectopic expression of PKAc failed to impair IL-2 and IFN-γ RNA synthesis in EL-4 cells (compare lanes 4 and 6 in Fig. 3A).

To investigate whether active PKAc plays a similar stimulatory role in IL-5 synthesis in primary T cells, CD4+ T cells from DO11.10 tg mice were infected with recombinant retroviruses. As shown in Fig. 3B, F enhanced the T/I-mediated increase in IL-5 secretion by at least 3-fold, and the ectopic expression of PKAc was sufficient to enhance the T/I-mediated IL-5 induction to a very similar level. On the same hand, ectopic expression of PKAc led to a strong increase in the αCD3/αCD28-Ab-mediated induction of IL-5 synthesis, which was further enhanced by F treatment (Fig. 3C). This indicates that TCR-mediated signals are able to collaborate with the cAMP/PKA signaling cascade in the induction of IL-5 expression.

The stimulatory effect that active PKA exerts on the IL-5 promoter (28) (Fig. 2B) requires GATA-3 activity. This is shown by transient transfection of vectors expressing PKAc or GATA-3 with a luciferase construct driven by the proximal IL-5 promoter into 293 HEK cells. Cotransfection of GATA-3 and PKAc-expressing vectors resulted in a synergistic activation of the proximal IL-5 promoter (Fig. 4A), whereas none or very weak activation was observed for a mutated proximal IL-5 promoter (Gm) to which GATA-3 is unable to bind.

In Th1 effector cells, which express only minor amounts of GATA-3, T/I/F treatment did not induce IL-5 production (Fig. 1B), suggesting an important role of GATA-3 in the PKA-mediated increase in IL-5 synthesis. To investigate the functional cooperation between PKA and GATA-3, we double-transduced Th cells, maintained under Th1 conditions, with retroviruses expressing the PKAc or GATA-3. In this experiment, a GATA-3-expressing retrovirus was constructed that contains a yellow fluorescence protein.

**FIGURE 4.** PKA and GATA-3 cooperate in the activation of IL-5 promoter and of IL-5 and IL-4 expression in Th1 cells. A, Synergistic activation of proximal IL-5 promoter activity by PKAc and GATA-3 in 293 HEK cells. The 293 cells were cotransfected either with a ~120 IL-5 promoter-driven luciferase plasmid or a promoter construct mutated in its proximal GATA-3 binding site (Gm), and vectors expressing PKAc or GATA-3 (G-3), as indicated. Luciferase activity was measured from cells that were either left untreated (−) or treated with T/I or T/I/F for 20 h. Mean values of three independent experiments are shown. B, PKAc and GATA-3 cooperate in the activation of IL-5 secretion in Th1 cells. One day after primary stimulation, CD4+ T cells from LNts of DO11.10 TCR tg mice were infected with recombinant retroviruses transducing EYZ GATA-3 (G-3), EGZ PKAc, or both, and maintained under Th1 conditions. Two days postinfection, cells were sorted into infected and noninfected (−) populations. Two days later, the cells were restimulated for 48 h with T/I or T/I + dibutyryl-cAMP, and IL-5 production was determined by ELISA. C and D, Effect of PKAc and GATA-3 on IL-4 and IFN-γ synthesis in Th1 cells. Supernatants from experiment B were assayed for IL-4 secretion (C) or IFN-γ secretion (D) by ELISAs.
molecules that, bound through so-called A-kinase anchor proteins (79). In cardiac myocytes, it has been shown that a rise of cAMP in such microdomains (32). In cardiac myocytes, an increase in T cell proliferation. Because, moreover, the disruption of AKAP-PKA contacts rendered T cells insensitive to cAMP elevation (34), one may assume that in Th cells active PKA might also be localized in membrane-bound microdomains. It is likely that Th1 and Th2 cells that contain similar F-inducible PKA activities (S. Klein-Hessling, unpublished data) differ in the level of their PKA molecules localized in the distinct cAMP-containing microdomains. The dual effect of increased cAMP levels on T cells appears to be executed by different signaling cascades, and is dependent on cell type. Although F treatment of EL-4 cells led both to an activation of Th2 and inhibition of Th1 lymphokines, ectopic expression of PKAα stimulated Th2, but failed to inhibit Th1 lymphokine expression. Thus, the activation of PKA is selectively involved in the stimulation of Th2 lymphokine expression, whereas other cAMP-dependent pathway(s) appears to down-regulate Th1 lymphokines.

Investigations on the role of increased cAMP levels on Th2 lymphokines in D10 cells, a Th2-type cell line, led to the conclusion that elevated cAMP concentrations do not stimulate PKA, but p38 kinase activity, which, through phosphorylation of GATA-3, appeared to induce IL-5 and IL-13 expression (18). In Th2 lymphocytes, we did not observe a remarkable increase in p38 activation upon F treatment (Fig. 2A). But it is possible that in addition to PKA, other signaling pathways like the p38 kinase cascade could also be regulated by cAMP levels, which, finally, enhance IL-5 expression. Expression of the catalytic subunit α of PKA was found to induce the αCD3/αCD28-mediated IL-5 expression, which was further enhanced by F in primary Th0 cells. Nevertheless, down-regulation of IL-5 production in primary Th2 cells by the ectopic expression of a negatively acting version of regulatory PKA subunit I demonstrates that active PKA plays an important role in IL-5 gene regulation. These observations led us to conclude that the adenyllylcyclase/cAMP/PKA signaling pathway plays an important role in establishing Th2 effector function.

References


