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Specific CD3ε Association of a Phosphodiesterase 4B Isoform Determines Its Selective Tyrosine Phosphorylation After CD3 Ligation

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CAMP-specific phosphodiesterases (PDE) comprise an extensive family of enzymes that control intracellular levels of CAMP and thus regulate T cell responses. It is not known how the function of these enzymes is altered by TCR engagement. We have examined this issue by studying one of the PDE isozymes (PDE4B). PDE4B RNA and protein were detected in resting PBLs, and the levels of PDE4B protein increased with cell cycling. In peripheral blood T cells, two previously reported PDE4B isoforms could be detected: one was 75–80 kDa (PDE4B1) and the other was 65–67 kDa (PDE4B2). These two isoforms differed in their N-terminal sequence, with the presence of four potential myristylation sites in the PDE4B2 that are absent in PDE4B1. Consequently, only PDE4B2 was found in association with the CD3ε chain of the TCR. In addition, although both isoforms were phosphorylated in tyrosines in pervanadate-stimulated T cells, only the TCR-associated PDE4B2 was tyrosine-phosphorylated following CD3 ligation. The kinetics of phosphorylation of TCR-associated PDE4B2 correlated with changes in CAMP levels, suggesting that tyrosine phosphorylation of the TCR-associated PDE4B isoform upon engagement of this receptor may be an important regulatory step in PDE4B function. Our results reveal that selectivity of PDE4B activation can be achieved by differential receptor association and phosphorylation of the alternatively spliced forms of this PDE. The Journal of Immunology, 1999, 162: 2016–2023.

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3 Abbreviations used in this paper: PDE, phosphodiesterase; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.
associated with an increase in PDE catalytic activity. In addition, an alternatively spliced form of PDE4B (PDE4B2B) can be serine phosphorylated in vitro by mitogen-activated protein kinase (MAPK) (25). The biological relevance of this phosphorylation is not clear. Phosphorylation at tyrosine residues or serine/threonine residues may play an important role in the regulation of PDE activity by inducing conformational changes required for enzymatic activation (26, 27). Alternatively, phosphorylation may determine specific intracellular redistribution of a given PDE isof orm and facilitate specific protein-protein interaction.

We hypothesized that the link between TCR-mediated signaling and the associated transient increase in cAMP content could involve PDE regulation in the form of activation-dependent phosphorylation of a TCR-related compartment of PDE. To examine this hypothesis, we looked at changes in the association and phosphorylation of one of the PDE4 subtypes (PDE4B) in PBMC and in cycling T cells following TCR/CD3 ligation. Here, we report that PDE4B protein is expressed in peripheral blood T cells in at least two different variants: one of 75–80 kDa, and another of 65–67 kDa. However, only the 65- to 67-kDa variant is associated with CD3ε, and this translates into its unique tyrosine phosphorylation after TCR/CD3 ligation. Changes in phosphorylation of TCR-associated PDE4B correlated with changes in cAMP levels. Our data reveal that the link between TCR-mediated signaling and the resulting down-regulation of cAMP levels may involve selective tyrosine phosphorylation-dependent activation of a TCR-associated PDE pool.

Materials and Methods

Cells

PBMC were isolated from heparinized blood on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient. Cells were resuspended at 1 x 10^6/ml after three washings in complete culture RPMI 1640 medium containing 2 mM l-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate, 10 mM HEPES, and 10% FBS (Life Technologies, Grand Island, NY). T cell blasts were generated by culturing PBMC with PHA (5 μg/ml) (Sigma, St. Louis, MO) and IL-2 (10 U/ml) (Boehringer Mannheim, Laval, Quebec, Canada) for 72 h at 37°C, 5% CO₂. In some experiments, pure T cells were used after monocyte depletion by incubation in plastic petri dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) at 37°C for 1 h, followed by passing through T cell enrichment columns (R&D Systems, Minneapolis, MN). The resulting population contained >95% CD3^+ cells. The U937 promyelocytic cell line and the EBV-transformed B cell line (GM4672) were cultured in complete culture medium at 37°C, 5% CO₂.

Reagents

The following mAbs were used in these experiments: 12F6, a mouse IgG2a Ab against the human CD3ε chain, kindly provided by Dr. A. Lazarovits (Robarts Research Institute, London, Ontario, Canada) (28); 64.1 (mouse IgG2a) against the human CD3ε chain (obtained from Oncogen Science, Uniondale, NY); UCHT-1 (mouse IgG1) and an irrelevant mouse IgG2a mAb (purchased from Pharmingen, San Diego, CA); 4G10, a mouse IgG2b mAb against phosphotyrosine (kindly provided by Dr. B. Druker, Oregon Health Sciences University, Portland, OR) (29); 387, a rabbit antiserum to mAb against phosphotyrosine (kindly provided by Dr. B. Druker, Oregon Uniondale, NY); UCHT-1 (mouse IgG1) and an irrelevant mouse IgG2a culture medium at 37°C, 5% CO₂.

Detection of PDE4B proteins was performed by SDS-PAGE of T cell lysates (600,000 cell equivalents per group) on a 10% gel transferred to polyvinylidene difluoride membranes, and immunoblotted with the rabbit antiserum against PDE4B. Signal detection was performed by chemiluminescence (Boehringer Mannheim). For blocked Ab experiments, the anti-serum against PDE4B was preabsorbed with the peptide (100 μg/ml) used as immunogen. Specific protein immunoprecipitations (from 10 x 10^6 cells/group) were performed using Abs against PDE4B, CD3ε, or TCR β as described previously (32). Immunoprecipitates were then immunoblotted for phosphotyrosine or PDE4B. Signal detection was performed by chemiluminescence, and intensity was quantitated using an imaging densitometer (model GS 700, Bio-Rad, Hercules, CA) and the molecular Analyist Software (version 1.0, 1994, Bio-Rad).

Reverse transcription and PCR amplification

RT-PCR (33) was carried out using a kit according to the manufacturer’s instructions (Perkin Elmer, Foster City, CA). First-strand cDNA was generated from 1 μg total RNA using oligo(dT)16 to prime the reverse transcription and was directly amplified by PCR after the addition of specific primers and AmpliTaq DNA polymerase. Oligonucleotide primers were as follows (31): PDE4A, 5'-AACAGCTGAAACAGCTAC-3' and 3'-TCAGATGCCCCAAATAAC-5', defining a 907-bp product containing a Sall (34) site; PDE4B, 5'-GCCTCTGACCCGATAAGG-3' and 3'-CGTGGATGCTTCTACCAATA-5', defining a 625-bp product containing a Sall (35, 36) site; PDE4C, 5'-CTTGGCCAGCTGCTGCA-3' and 3'-CCAGGACCTGTTCACAGG-5', defining a 315-bp product containing an AvlII (34) site; PDE4D, 5'-CCGGAGATCTCTAGTGGAC-3' and 3'-CGTGGTGGAAAAGCTCCCTGC-5', defining a 641-bp product containing a Stul (34, 37) site; and PDE7, 5'-ATAATGGACAGCCAAATGT-3' and 3'-CGACTTATATCCGTGACCT-5', defining a 936-bp product containing a Sall (38) site. Normalization of mRNA was achieved by RT-PCR of a constitutive marker, human glyceraldehyde 3-phosphate dehydrogenase, using a commercially available primer set (Clontech, Palo Alto, CA). For each RNA sample, controls lacking reverse transcriptase were included for all PCR reactions. Reactions were performed with an initial holding step at 95°C for 10 s, followed with 95°C for 15 s (melt) and 60°C for 30 s (anneal-extend) for 35 cycles, and a final holding step at 72°C for 7 min as recommended by Perkin Elmer. Under these conditions, all PCR reactions were on the linear portion of the time/product curve. PCR products, along with m.w. markers (100-bp DNA ladder, Life Technologies), were electrophoresed on 2% agarose gels and visualized by ethidium bromide.

Results and Discussion

Initially, we investigated the presence of PDE4B mRNA in purified T cells cultured in medium alone or after blast transformation with PHA plus IL-2, to detect possible differences in the expression of PDE4B after induction of cell cycling. We used RT-PCR with PDE4B isof orm-specific primers. As previously reported (31), these primers yield a 625-bp PCR product that is digested by Sall into two products of 284 bp and 341 bp. Fig. I shows that PDE4B isof orm-specific RNA was amplified by RT-PCR in purified resting and blast T cells. DNA contamination was excluded, because these products were not detected in the absence of the reverse transcriptase step. Furthermore, Sall digestion confirmed amplification of only the RNA coding for the correct PDE4B isof orm. As positive control, we used RNA from the U937 cell line, for which the expression of PDEs has been extensively characterized in previous reports (24, 30, 39). In the same experiment, we confirmed the expression of other PDE families in PBLs. As
shown in Fig. 1B, we detected PDE4A and PDE7 RNA but were unable to detect the expression of PDE4C transcripts. These findings are consistent with previous reports (11, 15, 20, 40). However, in contrast with previous reports in T cell clones (15) or CD4+ peripheral T cells (11, 20), we were not able to detect significant amounts of PDE4D RNA in blast T cells.

Next, we examined PDE4B protein expression in PBMC and blasts by Western blotting of cell lysates using a previously reported PDE4B-specific antiserum (31). As noted by Manning et al. (31) and as shown in Fig. 1C, PDE4B runs as a pair of doublets: a short form of ~65–67 kDa, which corresponds to PDE4B2, and a long form that runs as a broad band at 75–80 kDa, which corresponds to PDE4B1. These two forms of PDE4B correspond to alternatively spliced RNAs from the same gene (35). Both forms of PDE4B were present in very low amounts in PBMC. PDE4B protein levels did not change after short (10 min) or long (3 h) TCR ligation with an anti-CD3ε mAb (data not shown). However, upon cell entry into cycling, the levels of both forms of PDE4B increased significantly. The specificity of these findings was further strengthened by two additional experiments. First, we used the EBV-transformed B cell line as a negative control and the U937 monocytic cell line as a positive control for PDE4B expression. The U937 cell line has been extensively characterized at the gene and protein levels for expression and function of PDEs (24, 30, 39). Second, we repeated blotting for PDE4B after preabsorption of the antiserum with the peptide used as immunogen to raise the antiserum against PDE4B. In these experiments, disappearance of the PDE4B2-corresponding 65- to 67-kDa band occurred when blotting was performed in these conditions (Fig. 1C), whereas only a slight decrease in the 75- to 80-kDa band was observed. It is of interest to point out that we detected an additional immunoreactive protein of approximately 50 kDa in T cells and in U937 cells (data not shown). It is not known whether this protein represents another alternative spliced isoform of PDE4B (30) or merely represents a cross-reactive protein. However, we did not consistently observe a 100-kDa band compatible with a recently described form of PDE4B (PDE4B3) (41). Thus, the antiserum against PDE4B shows the expected reactivity in T cells (31).

Previous studies have suggested that PDE4 isozymes play a role in T cell activation, because selective PDE4 inhibitors can suppress cytokine production and T cell proliferation (11, 15, 20, 42–44). However, the role of individual CAMP-specific PDEs in T cell activation and the link between these enzymes and TCR/CD3-mediated signaling have not been established. We hypothesized that there may be a fraction of PDE4B that is associated to the TCR/CD3 complex as shown for other regulatory enzymes (45). We tested this hypothesis by blotting CD3ε immunoprecipitates from both PBMC and blast T cells for PDE4B. As shown in Fig. 2, only

**FIGURE 1.** Expression of PDE4B RNA and protein. **A**, RT-PCR analysis of PDE4B from PBMC and blast T cells. Two controls are shown: in the middle panel, the same PCR reactions were performed without previous reverse transcription; in the bottom panel, amplified products were digested with SalI. **B**, Expression of PDE4 isofoms in blast T cells and in the U937 promyelocytic cell line. **C**, Western blot analysis for PDE4B from cell lysates of PBMC, blast T cells, U937 cells, and the EBV-transformed B cell line (GM4672). Nonstimulated cells were lysed, and the lysates (6 × 10⁶ cell equivalents) were run in a 10% SDS-PAGE and immunoblotted with a specific antiserum against PDE4B or with the same antiserum after preabsorption with the peptide used for raising such antiserum.
a 65- to 67-kDa band, compatible with the short form of PDE4B, was detected in CD3ε immunoprecipitates from both PBMC and blasts when blotted with an antiserum against PDE4B. The level of this protein in CD3ε immunoprecipitates from blasts was higher than in the same immunoprecipitates from PBMC. TCR ligation did not induce further increases of this band. The possibility of the band being an artifact due to recognition of the immunoprecipitating Ab by the secondary Ab used in the immunoblots was ruled out by its absence when blotting the immunoprecipitating Ab alone without cell lysates and by its absence in immunoprecipitates with an irrelevant IgG2a isotype mAb (isotype-matched to mAb 64.1). Furthermore, a similar 65- to 67-kDa band was obtained when another anti-CD3ε Ab (UCHT-1) was used for immunoprecipitation (Fig. 2).

The previous result suggests that the short isoform of PDE4B (PDE4B2), but not PDE4B1, is associated with the TCR. To corroborate this finding, we investigated the presence of CD3ε chain in PDE4B immunoprecipitates. Given the lack of an adequate blotting Ab against CD3ε, we examined the association of CD3ε chain with PDE4B indirectly, by looking at tyrosine phosphorylated TCR subunits in PDE4B immunoprecipitates. Stimulation with anti-CD3 mAb of either PBMC or blasts resulted in tyrosine phosphorylation of both CD3ε and TCRζ as described previously (46, 47) (Fig. 3). In PBMC, a significant amount of phospho-CD3ε and phospho-TCRζ can be detected in nonstimulated T cells upon long exposure of the blotted membranes (46). In PDE4B immunoprecipitates from anti-CD3-stimulated PBMC and blasts (Fig. 3), we detected a 23- to 25-kDa tyrosine-phosphorylated protein that was compatible with phospho-CD3ε chain, further supporting our finding of PDE4B association with the CD3ε chain. This association was more intense in cycling T cells than in resting T cells. Similar results were obtained using ZAP-70 immunoprecipitates in addition to CD3ε immunoprecipitates (data not shown).

Our results indicate that T cells have a fraction of PDE4B that is constitutively associated, directly, or indirectly, with the TCR/CD3 complex. Differential cytosolic or membrane targeting of PDE isoforms has been reported for PDE4A and PDE4D, although the mechanism for this compartmentalization is unknown (48–50). One possibility to explain the association of PDE4B2 to the TCR is that this isoform is selectively targeted to the cell membrane. This was examined by sequence analysis of the N terminus of the PDE4B2 isoform and comparing it with the other two known PDE4B isoforms. We observed that the CD3ε chain-associated form of PDE4B (PDE4B2) had four potential myristylation sites at residues 6, 7, 16, and 17 in its N-terminal region. These sites were absent in PDE4B1 and PDE4B3. These myristylation sites likely correlate with predominant membrane targeting of PDE4B2. The sequence of events leading to its association with CD3ε remains to be established.

The functional significance of multiple localization patterns of cAMP-specific PDEs is not completely understood, but may be related to compartmentalization of cAMP signaling. Hence, spatial gradients of intracellular cAMP levels could be modulated by the specific site of PDE activity within the cell. It is plausible that the
short PDE4B form associated with the CD3ε chain could be more effective in decreasing intracellular cAMP levels than other forms located in the cytosol in response to TCR-mediated signaling. In this regard, a recent report has shown that membrane localization of the nonreceptor tyrosine kinase ZAP-70 in a particular configuration is required for its biological activity, suggesting that spatial orientation of membrane-associated signaling complexes could be an important feature for their function (51).

PDE4B2 is constitutively associated with the TCR complex in resting T cells, and the level of TCR-associated PDE4B2 does not change in response to TCR engagement. Therefore TCR-mediated regulation of PDE4B2 activity must require some mechanism other than TCR association. One potential mechanism may be tyrosine phosphorylation in response to TCR-mediated signaling. Three pieces of evidence support this idea. First, it is known that TCR-mediated signaling involves tyrosine phosphorylation of many signaling molecules that determines the formation of signaling complexes (reviewed in Ref. 52). Second, Stringfield and Moriomi have shown that tyrosine kinase inhibitors cause a decrease in phosphodiesterase activity in neural cells (53). Third, a comprehensive sequence analysis of PDE4B revealed the presence of a possible tyrosine phosphorylation site at residue 523 in the C-terminus region of this protein. This represents a unique feature of the PDE4B that is not present in the other PDE4 isoforms. Therefore, we examined if tyrosine phosphorylation of PDE4B could occur upon TCR/CD3 engagement.

PBMC or T cell blasts were stimulated with an anti-CD3 mAb, and phosphotyrosine immunoblotting was carried out in anti-PDE4B immunoprecipitates from cell lysates. As shown in Fig. 4A, there was no detectable tyrosine-phosphorylated PDE4B in either unstimulated PBMC or blasts. Stimulation with anti-CD3 mAb for 10 min induced tyrosine phosphorylation of PDE4B2. However, TCR ligation did not induce tyrosine phosphorylation of PDE4B1. It is important to note that we have detected interindividual variations in the level of tyrosine phosphorylation of PDE4B1 and PDE4B2. Pure T cells (10^6 per lane) isolated from PBMC (blasts) were stimulated with pervanadate (PV) (10 μl) for 10 min. Cells were lysed, and the lysates were immunoprecipitated with the PDE4B antiserum. Immunoprecipitates were blotted for phosphotyrosine. Ip, immunoprecipitating Ab; NRS, normal rabbit serum; H chains, heavy chains of the immunoprecipitating PDE4B antiserum and the anti-CD3-stimulating mAb.
The results indicate that the same PDE4B isoform that associates with PDE4B antiserum (post-PDE4B lane) or after two sequential immunoprecipitations was depleted after three sequential immunoprecipitations with the PDE4B-specific antiserum. Consistent with this result and previous studies (24, 26), the band corresponding to PDE4B2 in CD3 lysates was depleted after three sequential rounds of immunoprecipitations (lower blot of Fig. 4B). Overall, these results indicate that the same PDE4B isoform that associates with the CD3 chain (Fig. 2) can also be tyrosine-phosphorylated after TCR/CD3 ligation.

To confirm the association of tyrosine-phosphorylated PDE4B2 with the CD3ε chain, we carried out depletion experiments. As shown in the upper blot of Fig. 4B, the tyrosine-phosphorylated 65- to 67-kDa band present in PDE4B immunoprecipitates from stimulated blast T cells was depleted after three sequential rounds of immunoprecipitations (ips) with an anti-CD3 mAb (post-CD3 ips lane) or after two sequential rounds of immunoprecipitations with the PDE4B-specific antiserum. Consistent with this result and further supporting the association between PDE4B2 and CD3ε, the band corresponding to PDE4B2 in CD3ε immunoprecipitates was depleted after three sequential immunoprecipitations with the PDE4B antiserum (post-PDE4B ips lane) or after two sequential CD3ε immunoprecipitations (lower blot of Fig. 4B). Overall, these results indicate that the same PDE4B isoform that associates with the CD3ε chain (Fig. 2) can also be tyrosine-phosphorylated after TCR/CD3 ligation.

Although both PDE4B1 and PDE4B2 isoforms contain a potential tyrosine phosphorylation site in the C terminus, only PDE4B2 (the isoform associated with CD3ε) is tyrosine-phosphorylated after TCR ligation. However, nonspecific induction of protein phosphorylation by inhibition of phosphatase activity with pervanadate induced tyrosine phosphorylation of both PDE4B isoforms (Fig. 4C). This finding supports the concept that selective association of PDE4B2 to the TCR complex correlates with its selective tyrosine phosphorylation.

Next, we correlated tyrosine phosphorylation of PDE4B2 with changes in PDE4 activity and cAMP levels. We were not able to detect consistent changes in PDE4 activity using PBMC or T cell blasts. In addition, measurement of PDE4 activity in CD3 or PDE4B immunoprecipitates was not possible due to technical problems related to the binding/detachment of PDE4B in an active form, followed by ion exchange chromatography. Therefore, we focused on measurement of cAMP levels as an indicator of PDE4 activity. As shown in Fig. 5A, T cell blast TCR ligation with an anti-CD3 mAb (UCHT-1) increased cAMP levels at 2 min and reached a peak at 5 min. The cAMP levels declined thereafter by 10 min and 20 min. A similar cAMP kinetic profile was obtained in resting PBMC (data not shown). These results on cAMP levels correlated with a rapid appearance (1 min) of tyrosine-phosphorylated PDE4B2 after CD3 ligation that was sustained for 20 min and disappeared after 60 min of stimulation (Fig. 5B). This suggests that tyrosine phosphorylation may be involved in the regulation of PDE4B2 activity, because PDE4 activation correlates with decreased cAMP levels. This correlation may be oversimplified, because it does not take into account the role of other PDEs such as PDE3 and PDE7 in the regulation of cAMP levels following TCR-mediated activation (10, 11, 13, 14). In addition, T cell activation can be enhanced by coengagement of TCR and coreceptor (CD4/CD8) or costimulatory molecules (CD28), and this may further regulate cAMP levels through differential activation of PDEs. Previous studies have shown that cross-linking CD4 or CD8 induces accumulation of cAMP, whereas CD28 ligation does not affect cAMP levels (1, 2).

We have provided evidence that one of the PDE4B isoforms, PDE4B2, is selectively associated with the CD3ε chain of the TCR in basal conditions and is tyrosine-phosphorylated after TCR/CD3 ligation. The functional consequences of PDE4B2 tyrosine phosphorylation are not established. However, our data are compatible with tyrosine phosphorylation of PDE4B2 resulting in increased activity of this isoform, similar to the effect of PKA-mediated serine phosphorylation of the PDE4D3 isoform (24, 26). This mechanism may not be operational for other PDE types, because MAPK-mediated serine phosphorylation of PDE4B2 does not correlate with an increase in its activity (25). In support of this hypothesis, previous studies have shown that the use of tyrosine kinase inhibitors results in inhibition of PDE activity and increased cAMP levels, implying that a tyrosine kinase is regulating PDE activity and increasing cAMP degradation. Alternatively, tyrosine phosphorylation of PDE4B may not impact its activity but rather induce a conformational change in PDE4B that increases its stability. A similar possibility has been suggested by the finding that MAPK phosphorylation of a specific PDE4B renders it less susceptible to proteolysis than in the nonphosphorylated state (25). Finally, a third possibility involves the regulation of PDE4B interactions with other proteins containing SH2 domains, by phosphorylation of the phosphotyrosine motifs shown at the C terminus of PDE4B forms.

Understanding how specificity is achieved in signal transduction is a major issue in the field of cell activation. Cell compartmentalization has been proposed as a basic mechanism to explain it. Our data indicate that this may apply to the PDE4B family, and are consistent with the observation that TCR/CD3 ligation promotes the translocation of cAMP-dependent protein kinase type I to the TCR/CD3 complex (45). Taken together, these results suggest that ligation of the TCR triggers recruitment and/or activation of key elements of the cAMP/PKA pathway into a discrete microenvironment of the T cell. Such compartmentalization may contribute...
to the ability of cAMP to tightly regulate TCR signaling. Further studies will define the physiological role of PDE4B2 tyrosine phosphorylation, and the protein tyrosine kinases involved in this phosphorylation. The demonstration of PDE4B2 phosphorylation provided by our studies in primary T lymphocytes justifies a detailed structure-function analysis of this PDE isoform, using a transfection system to establish the functional link between PDE4B2 phosphorylation status and cAMP generation.

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