Overexpression of Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains induces cluster of differentiation 59-mediated apoptosis in Jurkat cells

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Abstract. Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains (CBP/PAG) is a membrane-bound adaptor protein that downregulates the activation of Src family kinases present in lipid rafts. To elucidate the role of CBP/PAG in human T cell activation, a cell line overexpressing CBP/PAG was constructed and the function of CBP/PAG in Jurkat cells was examined. The present study revealed that increased CBP/PAG expression in T cells significantly enhanced their apoptosis and reduced cellular activation and proliferation. Overexpression of CBP/PAG suppressed the growth of Jurkat cells by recruiting c-Src and its negative regulator, C-terminal Src kinase (CSK), to lipid rafts. The negative regulation of CBP/PAG was enhanced in the presence of anti-cluster of differentiation (CD)59 monoclonal antibodies. In addition, a significant association was revealed between the location of CBP/PAG and CD59, which were co-expressed in the same region of the cell membrane, implicating a potential overlap of the elicited signaling pathways. These results indicate that CBP/PAG functions as a negative regulator of cell signal transduction and suggest that CD59 may strengthen the role of negative feedback regulation.

Introduction

Csk-binding protein (CBP), also known as phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), is a transmembrane adaptor protein and located in glycosphingolipid-enriched membrane microdomains (GEMs), which are referred to as lipid rafts (1,2). In quiescent T cells, CBP/PAG is tyrosine phosphorylated and regulates the activity of Src family kinases (SFKs) by recruiting C-terminal Src kinase (CSK) (1,3). Ordinarily, SFKs contain an Src homology 2 (SH2) domain, combined with the phosphorylated carboxyl-terminal regulatory tyrosine of CSK, which inactivates SFKs (4). However, in response to the activation of human T cells, CBP/PAG is rapidly dephosphorylated and subsequently dissociates from CSK (5,6). Since CBP/PAG, CSK and SFKs are all ubiquitously expressed, this circuit is important in many cellular systems.

CBP/PAG has been described as a tumor suppressor; in human non-small cell lung cancer cell lines, the expression of CBP/PAG is significantly downregulated compared with normal human lung cells (7). CBP/PAG can recruit CSK into lipid rafts via phosphorylation and this directly contributes to regulating the oncogenicity of c-Src. The ability of CBP/PAG to suppress c-Src is dependent on CSK, so CSK-deficient cells are activated by overexpression of c-Src and drive the formation of tumors (8). CBP/PAG is a negative feedback regulator of T cells, but its absence triggers the negative feedback loop of cytotoxic T-lymphocyte protein 4 by activating Src family kinase activity (9). The results of these studies implicate that CBP/PAG regulates various cellular signaling pathways.

Similar to CBP/PAG, glycosylphosphatidylinositol (GPI)-anchored cluster of differentiation (CD)59 is also widely expressed on the majority of leukocytes, including T cells and attenuates cytolysis by inhibiting the insertion of additional C9 molecules into the C5b-9 complex during the assembly process of the complement membrane attack complex (MAC) (10,11). It also acts as a signaling molecule that activates T cells (12). Signaling pathways mediated by the T-cell receptor (TCR)/CD3 and CD59 differ due to the membrane localization of the TCR/CD3 complex and CD59 (13-15). It has been demonstrated that the antibody-mediated cross-linking of CD59 molecules promotes the activation of T cells. These include the phosphorylation of protein tyrosine kinases and increases

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in intracytoplasmic-free Ca^{2+}, T-cell proliferation and interleukin (IL)-2 production in response to phorbol 12-myristate 13-acetate stimulation (16-18). In addition, CD59 downregulates the antigen-specific activation of human T lymphocytes by binding with its ligand on antigen presenting cells (19). However, it has been suggested that CD59 may transmit intracellular signals by phosphorylating CBP/PAG, which inhibits the activity of Src kinase and maintains cell quiescence (20). Therefore, it is necessary to explore the function of CD59 on the signaling pathway of T cells.

The present study used a lentiviral vector to construct stable cell lines expressing high levels of CBP/PAG and aimed to elucidate the physiological relevance and mechanism of action of CBP/PAG in T cells. The results of the present study indicate that CBP/PAG negatively regulates T cell activation in Jurkat cells. In addition, it was demonstrated that the inhibitory effect of CBP/PAG on T cell activation was dependent on its ability to be tyrosine phosphorylated, recruit CSK and inactivate SFKs. Additionally, Jurkat cells were stimulated with anti-CD59 monoclonal antibodies (mAbs) to explore the role of CD59 molecules in T cell activation. It was demonstrated that CD59 and CBP/PAG co-localized in the same region of the cell membrane and that CD59 enhanced CBP-mediated apoptosis in Jurkat cells. Finally, it was determined that CD59 promotes the phosphorylation of CBP/PAG in T cells, indicating the important association between CBP/PAG and CD59 in T cell signaling pathways.

Materials and methods

Antibodies and reagents. The following antibodies were used: Anti-CD59 (cat. no. EPR6426; Abcam, Cambridge, UK), anti-CSK (cat. no. AP 13748a; Abgent, Inc., San Diego, CA, USA), anti-FYN (cat. no. AP 7709a; Abgent, Inc.), anti-LCK (cat. no. AP2831c; Abgent, Inc.), anti-phosphorylated (p)-LCK (Tyr505) (cat. no. D155167; Sangon Biotech Co., Ltd., Shanghai, China), anti-p-LCK (Tyr394) (cat. no. D155064; Sangon Biotech Co., Ltd.), anti-ZAP-70 (cat. no. D155208; Sangon Biotech Co., Ltd.) and β-actin (cat. no. D110001; Sangon Biotech Co., Ltd.). Guava Nexin Reagent for Flow Cytometry (cat. no. 4500-0450) was provided by EMD Millipore (Billerica, MA, USA). A horseradish peroxidase (HRP)-conjugated goat anti-rabbit Immunoglobulin G antibodies (cat. no. D110058) were obtained from Sangon Biotech Co., Ltd. Lysis buffer and phenylmethylsulfonyl fluoride (PMSF) were obtained from Beyotime Institute of Biotechnology (Shanghai, China). A Cell Counting kit (CCK)-8 kit was purchased from Beijing Fanbo Biochemicals Co., Ltd. (Beijing, China). The RevertAid First Strand cDNA synthesis kit was kindly provided by Roche Diagnostics (Basel, Switzerland).

Cell line and culture. Jurkat cells were purchased from the Chinese Academy of Sciences (Shanghai, China) and maintained in the laboratory. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin and streptomycin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37˚C with 5% CO_2. The cells culture medium was changed every 2-3 days to ensure that the cells were in the logarithmic growth phase.

Cell transfection. The CBP/PAG gene sequence was designed by Dr Wang in our laboratory. Lentiviruses containing CBP and enhanced green fluorescent protein (EGFP) genes, and lentivirus containing EGFP alone genes were constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China). Briefly, Jurkat cells were plated at 5,000 cells/well in 96-well plates 24 h prior to transfection. The lentiviruses [10^8 TU/ml; multiplicity of infection (MOI) is 50] and polybrene (Shanghai GeneChem Co., Ltd., Shanghai, China; 0.5 mg/ml; 10 μl/well) were added to the cell suspension, which was then cultured in at 37˚C in a 5% CO_2 incubator. At 8 h post-transfection, target cells were centrifuged at 200 x g for 10 min at 37˚C. Subsequently, cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin and streptomycin at 37˚C with 5% CO_2. At 48 h post-infection, cells were visualized using a fluorescent confocal microscope (FV-1000; Olympus Corporation, Tokyo, Japan) and single-cell sorted using a Guava EasyCyte mini flow cytometer (EMD Millipore). EGFP fluorescence (excitation at 488 nm) was detected using a 530/30-nm bandpass filter. Single cell sorting allowed the visualization of green fluorescence in microcultures.

Cell stimulation. Cells were divided into 2 groups: A negative control (transfected with lentivirus with EGFP only) and CBP-EGFP (transfected with lentivirus containing CBP-EGFP). When infected, the final concentration of lentivirus was $2.5x10^6$ TU/ml. CD59 antibody cross-linking can promote cell proliferation (16,17), therefore the 2 groups of cells were stimulated with anti-CD59 mAbs of 37˚C for 2 or 6 h.

Immunofluorescence. Cells were pre-incubated for 2 h with anti-CD59 mAbs at 37˚C in a 5% CO_2 incubator. Following washing, cells in the logarithmic growth phase were plated as monolayers on slides, allowed to dry naturally and fixed with 4% paraformaldehyde for 20 min at room temperature. Following blocking with 10% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature, cells were incubated with anti-CD59 mAbs (1:500) overnight at 4˚C. Cells were then incubated with rhodamine-labeled fluorescent secondary antibodies (cat. no. 111-295-144; 1:200; Jackson ImmunoResearch, Inc., West Grove, PA, USA) for 2 h in the dark at room temperature and coverslips were mounted on the glass slides. Images demonstrating the localization of CBP/PAG and CD59 were captured using a fluorescent confocal microscope (FV-1000; Olympus Corporation).

Flow cytometry. Cells incubated with the CBP-EGFP and negative control vector were stimulated on a 24-well-plate (each well contained 2 ml RPMI 1640 medium and the cell density was 10^6 cells/ml) coated with anti-CD59 mAbs for the indicated 2 or 6 h. Some cells from each group did not undergo stimulation with CD59. Cells were re-suspended in RPMI 1640 medium, subsequently, 100 μl Guava Nexin Reagent for Flow Cytometry containing AnnexinV-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) was added and cells were incubated at 20 min at room temperature in the dark. The apoptotic rate was detected using a Guava EasyCyte Mini flow cytometer and analyzed using Guava CytoSoft™ software version 4.2.1 (both EMD Millipore).

Cell proliferation assay. Cells incubated with the CBP-EGFP and negative control vector were seeded in a 96-well-plate (100 μl/well contained 10^4 cells). Stimulation of cells was performed for 24 h at 37˚C using wells pre-coated with anti-CD59 mAbs. Cells were pulsed with 10 μl CCK-8/well for 4 h at 37˚C to measure proliferation and experiments were repeated ≥5 times. Optical density was measured using an automatic enzyme mark instrument at a wavelength of 450 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from untreated cells and cells stimulated for 6 h with anti-CD59 mAbs in the CBP-EGFP and negative control groups was extracted using TRIzol (Thermo Fisher Scientific, Inc.). Reverse transcription was performed using the RevertAid First Strand cDNA synthesis kit and qPCR was performed using Fast SYBR™-Green Master Mix (Thermo Fisher Scientific, Inc.) Expression of the housekeeping gene GAPDH was used to normalize the amount of total RNA. The primers were as follows: CBP/PAG forward, 5'-TCAGCCTGA GAGGAGGAAAT-3' and reverse, 5'-GCTCCTGCTACTTGG GAGTC-3'; CD59 forward, 5'-CAAGGAGGGTCTGTCCT GTT-3' and reverse, 5'-GACCTGAATGGCAGAAGACA-3'; CSK forward, 5'-GCACTACCGCATCATGTACC-3' and reverse, 5'-ACAGAGTCCATCTGCGTCTG-3'; LCK forward, 5'-GCATGGCATTCATTGAAGAG-3' and reverse, 5'-CCT GGCTGTGTACTCGTTGT-3'; and GAPDH forward, 5'-GAT GACCTTGCCCACAGCCT-3' and reverse, 5'-ATCTCTGCC CCCTCTGCTGA-3'. PCR cycling was performed as follows: Denaturation at 95˚C for 4 min, and 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 70˚C for 30 sec. Each RNA sample test was repeated 3 times and the mean value of Cq was determined and relative mRNA expression was calculated using $2^{-\Delta\Delta Cq}$ as previously described (21).

Western blot analysis. Cells were washed with phosphate-buffered saline (PBS) three times. Subsequently, cells were dissolved in 500 μl lysis buffer and 5 μl PMSF prior to incubation for 20 min on ice. Samples were centrifuged at 12,000 x g for 5 min at 4˚C to separate the membrane fraction from the cytosolic fraction. The total protein concentration was measured using a BCA kit (cat. no. C503021; Sangon Biotech Co., Ltd.). Samples were boiled for 5 min to denature the protein. Equivalent amounts of protein (20 μg/lane) were resolved with 12% SDS-PAGE, electroblotted onto PVDF membranes. The membranes were blocked with Albumin Bovine V (Beijing Solarbio Science & Technology Co., Ltd.) for 2 h at room temperature and immunoreacted overnight with anti-CD59, ZAP70, FYN, CSK, LCK, p-LCK (Tyr505), p-LCK (Tyr394), β-actin antibodies (1:1,000), followed by a 2 h incubation with the HRP-conjugated goat anti-rabbit secondary antibodies (1:5,000). Chemiluminescent signals were generated by a SuperSignal West Pico PLUS chemiluminscent substrate (Thermo Fisher Scientific, Inc.). β-actin was used as a loading control. All blots were visualized and quantified using a UVP BioSpectrum 810 Imaging System (UVP LLC, Upland, CA, USA).

Statistical analysis. The Student's paired-samples t-test was used for statistical analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for analysis. Significant differences were determined from at least two independent experiments performed in triplicate and the data are presented as mean ± standard deviation. $P<0.05$ was considered statistically significant.

Results

CBP/PAG and CD59 co-locate in the cell membrane. To characterize the location and biochemical features of CBP/PAG, a lentivirus expressing EGFP was used. The following two recombinant lentiviral vectors were generated: The negative control, consisting of a viral vector containing EGFP alone and a vector comprised of CBP/PAG and EGFP (CBP-EGFP). Two stable cell lines were established by viral transfection. Flow cytometric analysis was conducted to determine the transfection rate of Jurkat cells. EGFP fluorescence (excitation at 488 nm) was detected using a 530/30-nm bandpass filter and a flow cytometer (Fig. 1A). E1 is a cell mass, which excludes the effects of cell debris on the results. Untreated Jurkat cells (the left side of M4) were defined as the blank control. At 48 h post-transfection, cells were able to express GFP and the transfection efficiency was deemed to be 99.97% (Fig. 1A, M5 right). The expression and localization of green fluorescence was observed using confocal microscopy. Green fluorescence can be observed at all angles using a microscope. CBP/PAG was uniformly expressed on the cell membrane and small aggregated clusters were occasionally observed (Fig. 1B). CD59, which is ubiquitously expressed, was present on the cell membrane as assessed by immunofluorescence.

To further study the association between CBP/PAG and CD59, CD59 mAb were used to stimulate the cells for 2 h. According to the analysis of the expression and distribution of red fluorescence in the control and the experimental groups, the expression of CD59 was increased following antibody cross-linking (Fig. 1C). As demonstrated in the negative control group of Fig. 1C, EGFP was expressed primarily homogenously in the cytoplasm prior to and following the cells being cross-linked with anti-CD59 mAbs. However, green fluorescence in the CBP-EGFP group was increased and its distribution was more concentrated following stimulation (Fig. 1C). Furthermore, the distribution of CBP/PAG and CD59 was more concentrated following antibody cross-linking and the two regions overlapped (as indicated by the arrows; Fig. 1C).

CD59 is a GPI-linked protein clustered in lipid rafts (22) and CBP/PAG is considered to be a constitutive tyrosine-phosphorylated molecule also located in lipid rafts (1,2). In the present study, CBP/PAG was expressed on the cell membrane and its location was unchanged following virus transfection. Meanwhile, these results demonstrated that CBP/PAG and CD59 are co-located at the cell membrane and that the expression of CBP/PAG was increased following stimulation with anti-CD59 mAbs.

CBP/PAG serves a negative feedback role in Jurkat cells. To examine whether CBP/PAG expression was altered following transfection, the expression of CBP/PAG mRNA in the CBP-EGFP and negative control groups was measured using RT-qPCR. The expression of CBP/PAG mRNA was significantly upregulated in the CBP-EGFP group compared with the negative control (Fig. 2A). It has been determined that

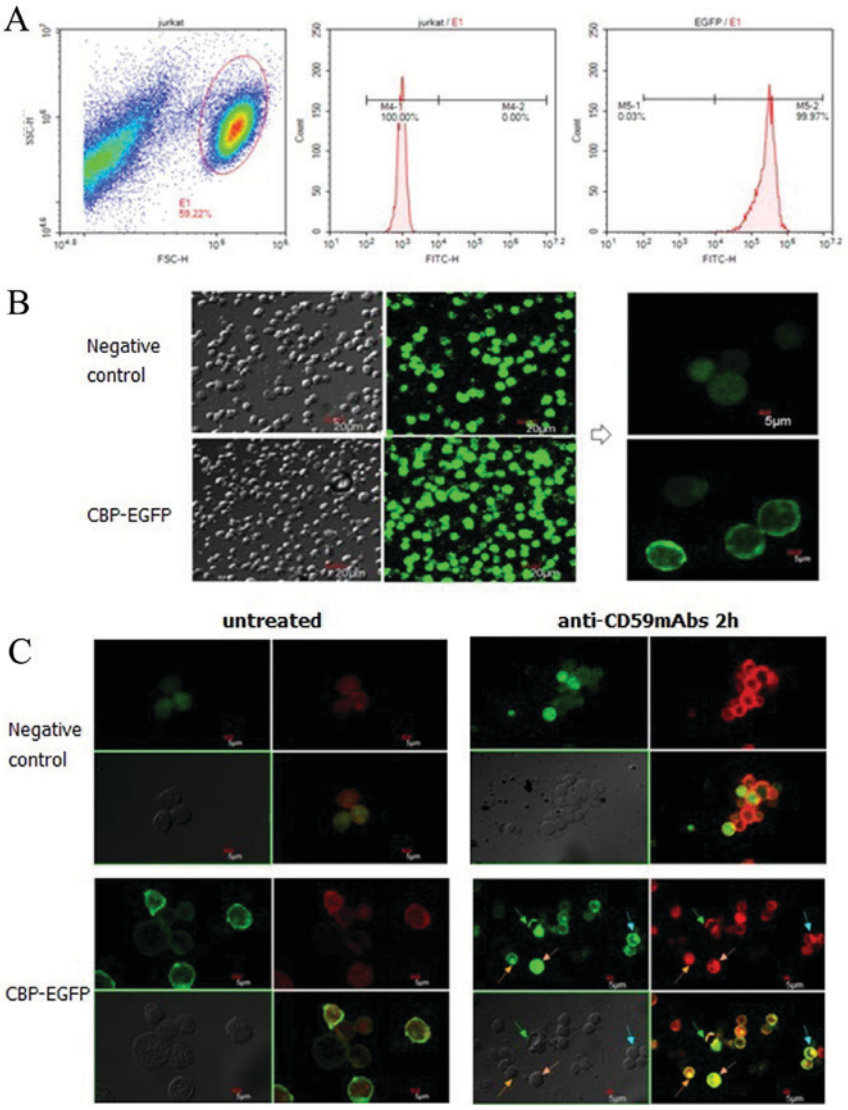

Figure 1. Association between CBP/PAG and CD59. (A) Virus transfection efficiency of negative control and CBP-EGFP groups: Physical parameter diagram, Jurkat only, Jurkat transfected by lentivirus. (B) Localization of green fluorescence at low (left) and high magnification (right). (C) Distribution of CBP/PAG and CD59 in the negative control and CBP-EGFP groups observed by confocal microscopy. One group of cells was treated with anti-CD59 mAbs 2 h (right) and one group was untreated (left). Following cross-linking of the antibody, the green fluorescence and red fluorescence were concentrated on the cell membrane and coincided, as indicated by the arrows. Top left, green fluorescence represents EGFP; bottom left, cells; top right, red fluorescence represents CD59; bottom right, synthetic map. CBP, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; EGFP, enhanced green fluorescent protein; CD, cluster of differentiation; mAbs, monoclonal antibodies.

CBP/PAG is expressed as a tyrosine-phosphorylated protein and serves a negative role in signal transduction in lymphoid cell lines (23). To identify whether this was the case in the transfected Jurkat cells, proliferation and apoptosis in the two cell lines was examined. It was demonstrated that the expression of CBP/PAG significantly suppressed the proliferation of Jurkat cells (Fig. 2B). The percentage of dead cells was measured using the mean fluorescence intensity of 7-amino actinomycin (7-AAD). The percentage of 7-AAD(+) Annexin V-PE(-) cells in the negative control and CBP-EGFP groups, handled without any additional processing, was 6.5 and 13.5%, respectively (Fig. 2C)-a significant difference (Fig. 2D). By contrast, the numbers of 7-AAD(-) Annexin V-PE(-) living cells in CBP-EGFP were significantly lower than those in the negative control (Fig. 2D) and this reduced trend was similar to the results of the proliferation assay. Collectively, these data indicate that CBP/PAG serve a negative role in Jurkat cells.

Increased CD59 expression on Jurkat cells promotes apoptosis when CBP/PAG is overexpressed. The antibody-mediated cross-linking of CD59 on human T cells induces protein tyrosine kinase (PTK) phosphorylation and increases intracytoplasmic free Ca^{2+} and cell proliferation (16-18). The present study investigated the response of negative control and CBP-EGFP cells to anti-CD59 mAbs. As an initial experiment, the expression of CD59 mRNA was compared with and without anti-CD59 stimulation using RT-qPCR. It was revealed that the expression of CD59 mRNA was significantly upregulated following stimulation with anti-CD59 mAbs in the negative control and CBP-EGFP groups (Fig. 3A). Indeed, these results demonstrated that anti-CD59 mAbs enhance the expression of intracellular CD59 molecules, while engagement with CD59 alone serves an important role in T lymphocyte signal transduction.

To examine whether CD59 upregulation alters biological processes, the activation and proliferation of Jurkat cells was

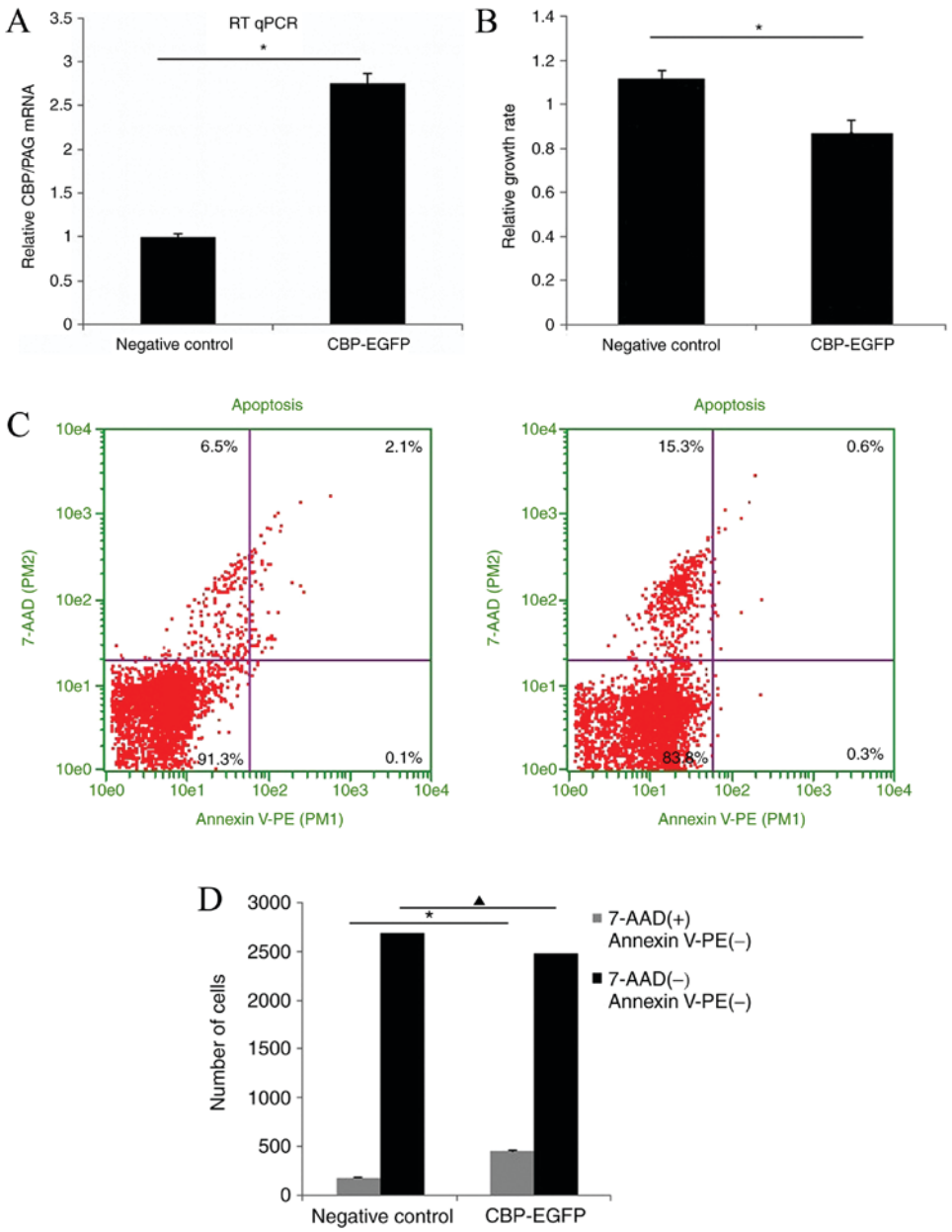

Figure 2. Apoptosis and proliferation of Jurkat cells following transfection. (A) RT-qPCR was performed using total RNA extracted from two cell lines. The relative expression of CBP/PAG mRNA, which was normalized to GAPDH mRNA expression, in the negative control and CBP-EGFP groups is presented. *P<0.05. (B) The proliferation of CBP-EGFP and negative control cells was assessed using a Cell Counting kit-8 assay. *P<0.05. (C) Cellular apoptosis was detected by flow cytometry. Annexin V-PE detected phosphatidyl serine that is exposed outside the cell membrane surface during apoptosis, while 7-AAD was used to measure the integrity of the cell membrane. Values presented in the left upper quadrants indicate the percentage of 7-AAD positive cells. (D) The number of double-negative cells and 7-AAD positive cells are presented. *P<0.05 and ▲P<0.05. CBP, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; EGFP, enhanced green fluorescent protein; CD, cluster of differentiation; mAbs, monoclonal antibodies; 7-AAD, 7-amino actinomycin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PE, phycoerythrin.

tested. Sitmulation with anti-CD59 mAbs significantly promoted the growth of cells in the negative control and CBP-EGFP groups, as assessed by a growth assay (Fig. 3B). In the negative control group, flow cytometry indicated that the number of apoptotic cells was significantly lower in Jurkat cells stimulated with anti-CD59 mAbs for 2 h than in unstimulated cells (Fig. 3C). The proportion of apoptotic cells in the CBP-EGFP group was higher than that in the negative control group, reaching about 15%. Furthermore, anti-CD59 mAb stimulation significantly increased the proportion of apoptotic cells to ~20% (Fig. 3D).

CD59 expression in Jurkat cells stimulated by anti-CD59 mAbs was therefore significantly increased. These results indicate that CD59 serves a positive role in signaling in the negative control group, further strengthening cell activation and proliferation. However, contrasting results were observed in the CBP-EGFP group, where CD59 coordinates with CBP/PAG to induce apoptosis in Jurkat cells.

CBP-CSK association regulates CD59-induced cell signal transduction. To determine the mechanisms for signal transduction in Jurkat cells induced by CBP/PAG expression, the interaction between CBP/PAG and CSK was examined in Jurkat cells (Fig. 4). CSK is a cytoplasmic protein tyrosine kinase consisting of an SH3, an SH2 and a kinase domain (24), and

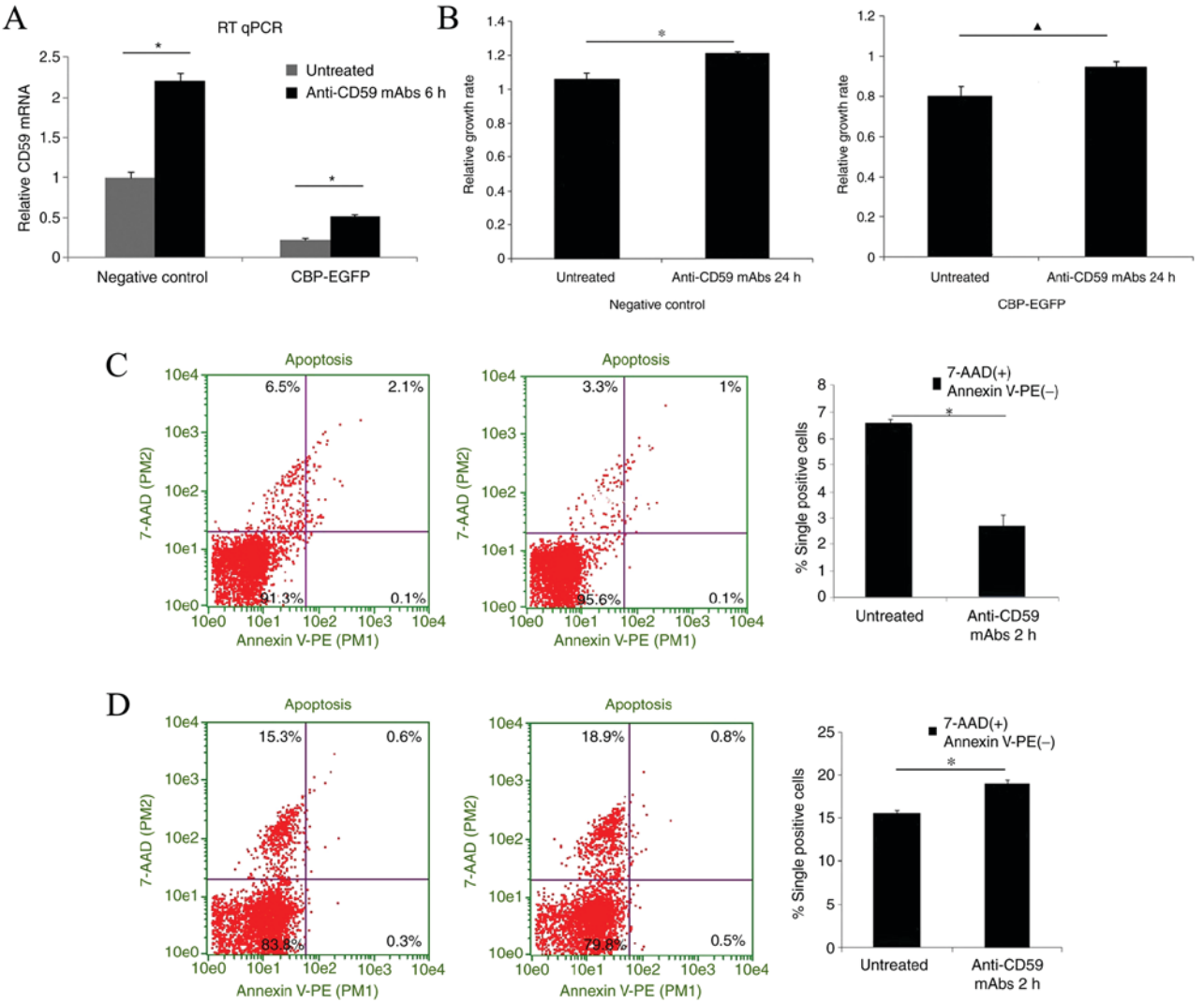

Figure 3. CBP/PAG and CD59 serve a synergistic role to promote apoptosis. (A) RT-qPCR was performed. The relative expression of CD59 mRNA, which was normalized to GAPDH mRNA expression, is presented. Left, negative control; right, CBP-EGFP. *P<0.05. (B) The proliferation of Jurkat cells was determined using a proliferation assay. Following anti-CD59 mAb crosslinking, proliferation was not observed in the CBP-EGFP group. *P<0.05 and ▲P<0.05. (C) Apoptosis was reduced following anti-CD59 mAb treatment in the negative control group. *P<0.05 (D) Apoptosis was increased in the CBP-EGFP group following anti-CD59 mAb treatment. *P<0.05. CBP, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; EGFP, enhanced green fluorescent protein; CD, cluster of differentiation; mAbs, monoclonal antibodies; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; 7-AAD, 7-amino actinomycin.

phosphorylated CBP/PAG binds tightly to the SH2 domain of CSK (1,2). Compared with the negative control, CSK mRNA and protein expression was upregulated in the CBP-EGFP group (Fig. 4A and B). Furthermore, the expression of CBP/PAG mRNA as well as CSK mRNA, were significantly increased in the CBP-EGFP group following stimulation with anti-CD59 mAbs (Fig. 4D). These results indicate that overexpression of CBP/PAG is associated with an increase in CSK expression. Furthermore, CSK may be transiently recruited to membrane ruffles following CBP/PAG phosphorylation, and the association between CBP and CSK induces a biological effect in Jurkat cells.

To examine whether the effects of CD59 on cell growth were mediated via an association with CBP/PAG, the effects of anti-CD59 mAbs on the growth of the negative control and CBP-EGFP groups were investigated. As depicted in Fig. 4C, the addition of anti-CD59 mAbs increased the expression of free CSK in CBP-EGFP groups. However, no changes were evident in the negative control group. It was also revealed that the expression of CSK mRNA was significantly decreased in the negative control group but was significantly increased in the CBP-EGFP group (Fig. 4D). These data indicate that CSK expression increases in Jurkat cells that overexpress CBP/PAG stimulated for 6 h by anti-CD59 mAbs and that this increase in CSK expression is accompanied by an increased in CBP/PAG expression (Fig. 4D). To verify the association between CBP/PAG and CD59, Jurkat cells were stimulated with anti-CD59 mAbs for 2 and 6 h. In the CBP-EGFP group, the ratio of living cells [7-AAD(-) Annexin V-PE(-)] was significantly decreased following 2 h treatment but did not decrease further following 6 h treatment (Fig. 4E).

Overexpression of CBP/PAG suppresses T cell receptor activation. Subsequently, the effects of CBP/PAG overexpression on c-Src activation were examined. CBP/PAG suppresses T cell receptor activation by recruiting CSK and it has been revealed that the phosphorylation of CBP/PAG Tyr314 creates

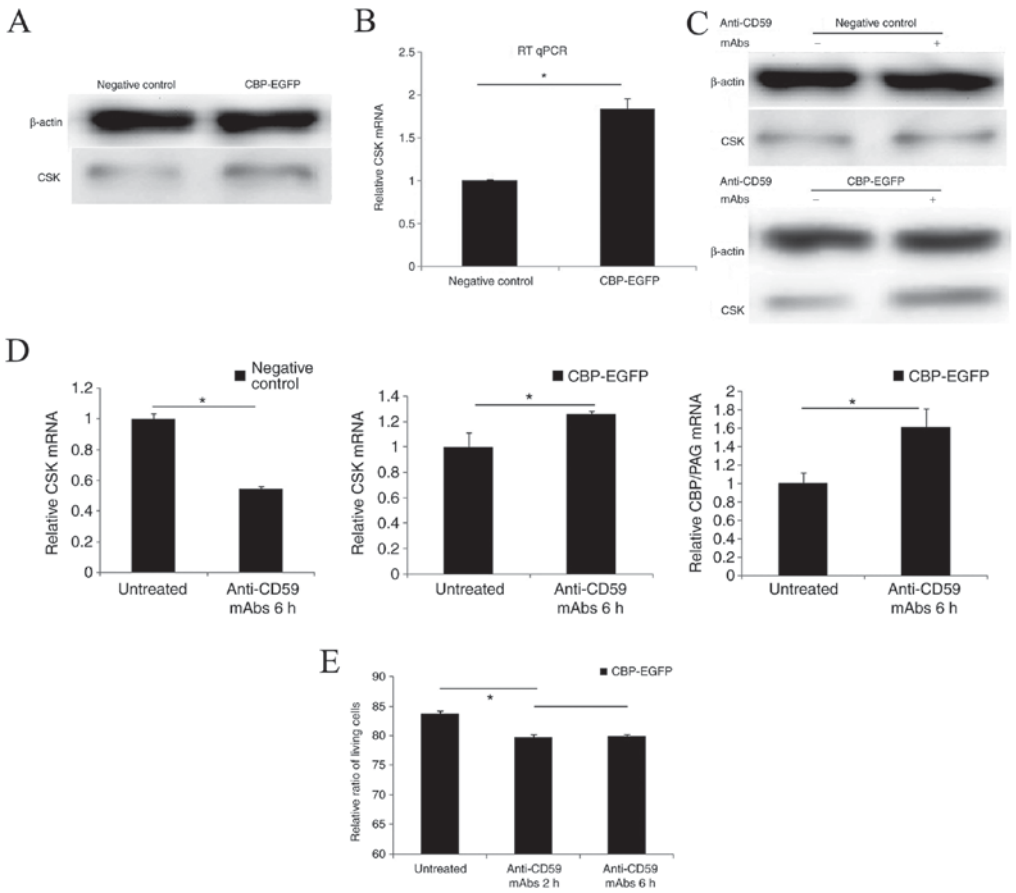

Figure 4. CBP-CSK association regulates CD59-induced cell signal transduction. (A) Total cell lysates from negative control and CBP-EGFP cells were analyzed using western blotting with anti-CSK antibodies. (B) Levels of CBP/PAG mRNA in the negative control group were decreased compared with the CBP-EGFP group. (C) Cells in the negative control and CBP-EGFP groups were stimulated with anti-CD59 antibodies for 6 h and total cell lysates were subjected to western blotting with anti-CSK antibodies. (D) The expression of CBP/PAG and CSK were analyzed by reverse transcription quantitative-polymerase chain reaction. Levels of CSK mRNA were decreased in the negative control and increased in the CBP-EGFP group following stimulation. (E) The ratio of living cells in CBP-EGFP group prior to and following stimulation with anti-CD59 mAbs was examined using flow cytometry. Data represent relative values. *P<0.05. CBP, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; EGFP, enhanced green fluorescent protein; CD, cluster of differentiation; mAbs, monoclonal antibodies.

a docking site for the CSK SH2 domain (24,25). This binding enhances CSK catalytic activity, allowing CSK to phosphorylate the inhibitory tyrosine on LCK (Tyr505) (26). Western blotting of the total cell lysates confirmed that LCK protein was adequately expressed in the transfected cells (Fig. 5A). In addition, the expression of LCK mRNA in the CBP-EGFP and negative control groups was measured using RT-qPCR (Fig. 5B). These results indicated that the expression of LCK protein and mRNA did not differ significantly between the two groups. The expression of p-LCK (Tyr505) was higher in the CBP-EGFP group than in the negative control group (Fig. 5A). By contrast, the expression of p-LCK (Tyr394) was decreased in the CBP-EGFP group. Total LCK content was slightly decreased in CBP-EGFP cells stimulated with anti-CD59 mAbs. However, the expression of the inhibitory tyrosine on LCK (Tyr505) was higher than the activation tyrosine on LCK (Tyr394) (Fig. 5C).

To further examine the nature of the Src family kinases responsible for CBP/PAG phosphorylation in Jurkat cells, the expression of the FYN and ZAP-70 proteins was detected by western blotting. It has been suggested that FYN may serve a critical role in the initiation and progression of cancer (27), as well as in T cell receptor signaling (28,29). The differentiation or activation of T cells depends on FYN activity and its knockout severely impairs the responses of T cells (30). The present study determined that FYN expression in the CBP-EGFP group was downregulated and this decrease was due to a change in CBP/PAG. Within the CBP-EGFP group, no significant difference in the expression of FYN protein was identified prior to and following anti-CD59 mAbs cross-linking (Fig. 5D). The expression of ZAP-70 in the signal transduction pathway of T cells was also measured. ZAP-70 was initially identified in TCR-stimulated Jurkat cells (31) and it was revealed that this is an essential kinase in T cell signaling (32). It has been demonstrated that following TCR stimulation, cells that do not express ZAP-70 fail to activate downstream signaling events (33). The results of the current study indicated that ZAP-70 expression was reduced in the CBP-EGFR group compared with the negative control group and decreased even further following cross-linking with anti-CD59 mAbs (Fig. 5D).

Discussion

A complex interaction may exist between the membrane-bound adaptor protein CBP/PAG and the GPI-anchored protein CD59 to regulate TCR activation pathways in T lymphocytes. The

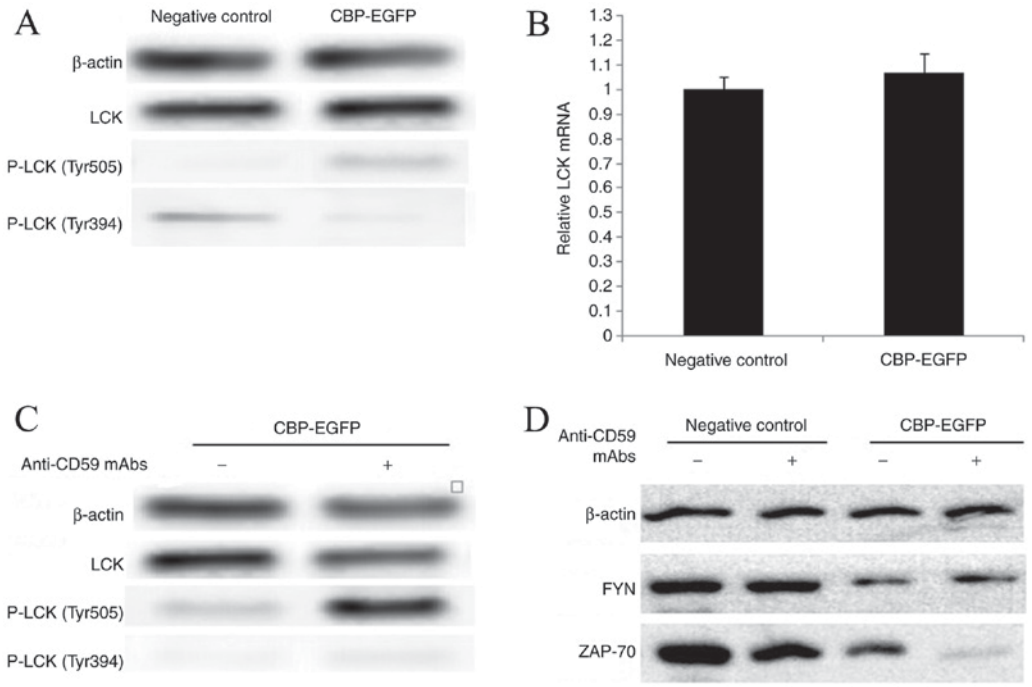

Figure 5. Overexpression of CBP/PAG suppresses T-cell receptor activation. (A) Total cell lysates were fractionated using high-speed centrifugation. The total protein expression of LCK was determined by western blotting and the extent of tyrosine (505,394) phosphorylation of LCK was also evaluated. (B) Levels of LCK mRNA in negative control cells were compared with those of CBP-EGFP cells. There were no significant differences between the groups. (C) Cells were stimulated for 6 h with anti-CD59 mAbs, and LCK, p-LCK (Tyr505) and p-LCK (Tyr394) proteins were assessed by western blotting analysis with the indicated antibodies. (D) Expression of FYN and ZAP-70. CD, cluster of differentiation; p-, phosphorylated; mAbs, monoclonal antibodies; CBP, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; EGFP, enhanced green fluorescent protein.

present study investigated whether CBP/PAG and CD59 regulate the signal transduction pathway in Jurkat cells. The results revealed that a negative feedback loop is formed by CBP/PAG, CSK and Src kinase in signaling pathways; that CBP/PAG and CD59 are co-expressed in the same region of the cell membrane and that the CD59-induced CBP-CSK complex promotes the apoptosis of Jurkat cells when CBP/PAG is overexpressed. These observations indicate that CBP/PAG and CD59 serve a critical role in cell signaling.

CBP/PAG is a protein consisting of 432 amino acids (aa) that contains a short extracellular domain (16-18 aa), a transmembrane domain (20 aa) and a large cytoplasmic domain (387-396 aa) (1,2). In the cytoplasmic domain, CBP/PAG contains 10 tyrosine residues, nine of which are potential sites for phosphorylation by Src kinases (34). It has been implied that CBP/PAG may function as a negative regulator by regulating c-Src signaling. The activity of Src kinases is mainly controlled by the equilibrium between phosphorylation and de-phosphorylation at a C-terminal inhibitory tyrosine and an activating tyrosine in the catalytic domain (35). CSK, a critical protein that can negatively regulate Src kinase activity, is recruited to lipid rafts by CBP/PAG (3,4). When Src kinase is phosphorylated by CSK, its C-terminal tyrosine binds intramolecularly to the SH2 domain, which together with the SH3 domain, interacts against the kinase domain (4).

The results of the present study revealed that the overexpression of CBP/PAG significantly inhibits cell signaling, primarily via the recruitment of CSK. The increase in CBP/PAG expression in the present study is accompanied by an increase in CSK. LCK, a member of the Src family non-receptor protein tyrosine kinases, is essential for normal T-cell development and activation (36,37). There are two tyrosine residues (Tyr394 and Tyr505) in the catalytic domain of LCK. Tyr394 is a self-phosphorylation site, which increases the activity of LCK by phosphorylation (26,38). However, LCK activity decreases following Tyr505 phosphorylation (39). The results of the present study revealed that the expression of phosphorylation of LCK (Tyr505) was higher in the CBP-EGFP group than in the negative control, whereas the opposite result was observed for LCK (Tyr394). Activated LCK (Tyr394) recruited and activated the Syk PTK ZAP-70 to stimulate the pathways that orchestrate T cell proliferation and differentiation. In addition, it was revealed that the expression of FYN, another member of the Src kinase family, was decreased when CBP expression increased.

One surprising result of the current study was that the expression of CD59 affected CBP/PAG during T cell signal transduction. CD59 is an inhibitor of cytolysis mediated by MAC; however, previous studies have implied that it participates in T cell activation and signaling. It has been demonstrated that anti-CD59 mAbs increase intracytoplasmic free Ca^{2+} and T cell proliferation in the presence of appropriate co-stimulators (16). Murray and Robbins (12) revealed that CD59 activation initiates a cascade of tyrosine kinase activation, including the phosphorylation of the cytoplasmic tyrosine kinase SYK and its close relative ZAP-70. CD59 also enhanced the effects of CD3-mediated T cell signal transduction (40). However, the glycosylphosphatidylinositol-linked CD59 molecule is expressed on the surface of Jurkat cells and the derived TCR/CD3-defective subline and when cross-linked by anti-CD59 mAbs, it can induce cellular apoptosis (41). The results of previous studies have demonstrated that CD59

negatively modulates the activity of $CD4^+$ T cells in response to polyclonal and antigen-specific stimulation (42,43). Since CD59 is not a transmembrane protein, the mechanism by which CD59 transmits a message to T cells when it is engaged with its ligand should be examined further.

The present study demonstrated that when CBP/PAG was overexpressed, Src activity was markedly suppressed in Jurkat cells. When induced by CD59 cross-linking, the negative regulation of CBP/PAG was significantly enhanced, which was observed early on following antibody cross-linking. Previous studies have demonstrated that CD59 is localized by its GPI anchor into the plasma membrane lipid microdomains in human T cells (44,45), whereas CBP/PAG is exclusively localized to lipid rafts (2). The results of the present study revealed that CBP/PAG and CD59 co-located at lipid rafts within the membrane and that this location was required to initiate specific stimulation. Phosphorylation at the inhibitory tyrosine (Y505) in LCK was increased by CD59 cross-linking. A possible explanation for this phenomenon is that the GPI-anchor protein CD59 and CBP/PAG contain an interaction site. The results of a previous study demonstrated that CD59 promotes the Linker for activation of T cells (LAT)-mediated activation of T cells and its mechanism may be associated with the transference of intermolecular palmitoylation (46).

The present study revealed that the cytoplasmic domain of PAG contains a potential site for palmitoylation located close to the transmembrane region. This modification may target CBP/PAG to the GEMs, as palmitoylation of LAT is essential for its recruitment into microdomains (47,48). Furthermore, CD59 and CBP/PAG may interact with each other via the palmitoylation sites. Following the antibody-mediated cross-linking of CD59 molecules on Jurkat cells, CD59 molecules combine with a large number of CBP/PAG molecules to inhibit cell proliferation.

In conclusion, the results of the current study indicate that CBP/PAG is implicated in the negative regulation of Src activity. Thus, CD59 may boost CBP/PAG phosphorylation in Jurkat cells when CBP/PAG is overexpressed and its interaction with SFKs may suppress the activation of signal transduction.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BBC conceived the work, acquired the data, played an important role in interpreting the results, and drafted and finished the manuscript. MHG was involved in drafting and reviewing the manuscript and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. BL and BZ provided the instruments of flow cytometry and laser confocal microscope, participated in the cell transfection experiment and analyzed the problems in the process of cell transfection. They also participated in writing and reviewing the manuscript. BW and SCZ discussed the role of CSK, LCK, p-LCK(Tyr394) and p-LCK(Tyr505) in this experiment and were responsible for analyzing apoptosis, RT-qPCR and protein expression data. LNW, HQL, ZW and SYH performed the immunofluorescence, western blotting, RT-qPCR and flow cytometry experiments.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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