Identification and Characterization of the CD226 Gene Promoter

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Running Title: CD226 gene promoter

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CD226 is one of the main activating receptors on natural killer cells, and it can induce cytotoxicity to target cells through interaction with its ligands CD155 or CD112. CD226 is also involved in T cell differentiation, activation and cytotoxicity. The expression of CD226 on natural killer cells and T cells can be regulated by cytokines and chemical stimuli, however the mechanism of the regulation of the CD226 gene is still unknown. In this study, we identified two promoters in the human CD226 gene, named P1 and P2, which locate to in –810 bp ~-287 bp and +33bp~+213 bp respectively, and a negative regulation element (NRE) between P1 and P2. Both P1 and P2 can be regulated by phorbol ester (TPA) and calcium ionophore (A23187). Bioinformatics analysis shows that within this CD226 gene region there are putative binding sites for transcription factors AP-1, Sp1, PEA3 and Ets-1. We found that transcription factor AP-1 can upregulate CD226 promoters P1 and P2 in HHCC cells, a hepatocarcinoma cell line with low expression of endogenous AP-1 and Ets-1. Interestingly, the transcription factor Ets-1 promotes AP-1-induced P2 activity but inhibits AP-1 induced P1 activity, for which a 10 bp AP-1/Ets-1 composite site CCTTCCTTCC in P1 may be responsible.

CD226, also named TLiSA1, PTA1 or DNAM-1, is a Mr 65,000 adhesion molecule containing two immunoglobulin V-like domains in its extracellular region (1,2). CD226 is broadly expressed in hematopoietic cells such as T cells, natural killer (NK) cells, NKT cells, a subset of B cells, monocytes/macrophages, dendritic cells, and megakaryocyte /platelet lineage as well as hematopoietic stem cells/progenitor cells (1-11). In Jurkat cells, CD226 mRNA and surface expression is greatly stimulated by treatment of the cells with phorbol ester (TPA) (2). IL-2 and TNF-α augment CD226 expression and cytotoxic function of effector cells generated from mixed lymphocyte culture (MLC), whereas transforming growth factor β (TGF-β) could inhibit both of these events. TGF-β also prevents the IL-2 induced upregulation of CD226 expression (12). In 2003, the poliovirus receptor (PVR)/CD155 and nectin-2/CD112 were identified as cell surface ligands for human CD226 (13). Soon after, the interaction of CD226 with its ligands was confirmed as one of the major mechanisms which trigger NK cell activation and cytotoxicity against some tumor cells and leukemic cells (14-16). Accumulating evidences indicate that CD226 is involved in a variety of immunological functions including T cell differentiation and cytotoxicity, NK cell cytotoxicity, NKT cell apoptosis, megakaryocyte polyploidization,

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platelet activation and aggregation, monocyte extravasation, dendritic cell maturation, and platelet and megakaryocytic cell adhesion to vascular endothelial cells (1-3, 5-15). It has been found that LFA-1 (CD11a/CD18), the αLβ2 integrin, physically associates with CD226 in NK cells and anti-CD3 mAb stimulated T cells, and that cross-linking of LFA-1 induced tyrosine phosphorylation of CD226, for which the Fyn protein tyrosine kinase is responsible, indicating that CD226 is involved in the LFA-1-mediated intracellular signals (5,16). CD226 on T cells forms a dynamic molecular complex with protein 4.1G and human discs large which may serve to cluster and transport LFA-1 and associated molecules (4). Protein kinase C (PKC) phosphorylates Ser329 of CD226 which plays a critical role in both CD226 adhesion and signaling as well as for lipid raft recruitment of CD226 and LFA-1-mediated signaling (17, 18). LFA-1, CD226, and Fyn are polarized at the immunological synapse upon the stimulation with anti-CD3 in T cells (5). Although progress in CD226 molecule distribution, function, and its relation to some diseases has been made in recent years, the mechanism of CD226 gene regulation is still unknown.

AP-1 (activating protein-1) is a collective term referring to dimeric transcription factors of Jun, Fos or ATF (activating transcription factor) subunits that bind to a common DNA site, the AP-1-binding site. The activity of AP-1 could be modulated by treatment of cells with TPA, known to stimulate PKC (19,20). AP-1 mediates gene regulation response to a variety of extracellular stimuli including growth factors, cytokines, tumor promoters, and chemical carcinogens (20,21). When regulating gene expression, AP-1 could cooperate with other transcription factors such as Ets-1. Ets is a family of transcription factors which share a highly conserved Ets domain, a winged helix-turn-helix DNA binding domain, which could bind different sites containing a core A/CGGAA/T motif existing in the promoter region of many genes including matrix metalloproteinase (MMPs), uPA, and TIMP-1 (22-24). Ets-1 is composed of Ets domain, transactivation domain, and pointed (PNT) domain and can cooperate with the c-Fos/c-Jun complex at AP-1 sites to regulate certain promoters (25).

In this study, we identified the promoters of human CD226 gene for the first time, and investigated the regulation of CD226 promoters by AP-1 and Ets-1 transcription factors.

**EXPERIMENTAL PROCEDURES**

5'-RACE and Nucleotide Sequencing - The 5' end of CD226 transcript was cloned by 5'-RACE using the 5'-RACE kit according to the manufacturer’s instructions (TaKaRa). After being reversed to single chain cDNA by using 5' phosphated gene specific primer (P5), RNA from TPA-treated Jurkat cells was degraded by using RNaseH. Nested PCR was carried out using two couple gene specific primers, P2 and P3 for the first round PCR, P1 and P4 for the second round PCR, in which the single chain cDNA was used as templates. The PCR products were cloned into pMD18-T vector for DNA sequencing. The sequences of primers were listed below:

P1: 5'-ATCCTGTATTACGGCCTCTAGCAC GT-3' position+75~+101
P2: 5'-AAGGCTGGTTCTTGAGATGTGAGT GC-3' position+155~+180
P3: 5'-CTTAACACAGGTGGAGTGGTTCAAGATC-3' position+329~+356
P4: 5'-CTACTCATGGCATGGTCATAAGGATGCACGT-3' position+391~+417
P5: 5'-AGAAAGAGTCATGTTATTGG-3' position+460~+480

Cell Culture and RNA Preparation - Jurkat cells were maintained in RPMI 1640 (HyClone) supplemented with 10% fetal calf serum (FCS); HyClone), and HHCC cells were maintained in Dulbecco’s modified Eagle’s medium
supplemented with 10% FCS at 37°C in 5% CO2. Total RNA was extracted from TPA-treated Jurkat cells using Trizol according to the manufacturer’s instructions (Invitrogen).

**Plasmid Construction** - A 2kb upstream regulation region of CD226 gene was amplified by PCR from genomic DNA from human peripheral blood mononuclear cells (PBMC) and cloned into pGL3-basic vector (Promega) by restriction endonucleases HindIII and KpnI. A series of different truncated fragments of the regulation region of CD226 gene were created by PCR amplification by using the primers listed in Table 1 and inserted into pGL3-basic vector by the same restriction endonucleases. Expression vectors of c-Jun, c-Fos and Ets-1 were kindly provided by Prof. D.K Watson (Medical University of South Carolina, USA).

**Transient Cell Transfection** - For promoter activity analysis, promoterless pGL3-basic vector was used as a negative control, and plasmid phRL-TK (Promega) was cotransfected as an internal control. Plasmid DNA was prepared using Maxi-prep Kit (Qiagen). Jurkat cells were seeded in 96-well plate with 1×10⁵ cells per well and triple wells were set for each group. 0.1 μg of pGL3 vector containing different truncated DNA fragments and 5.0 ng of phRL-TK vector were cotransfected for each well using the Lipofectamine 2000 (Life Technologies). For investigation of the regulation of CD226 promoter by AP-1 and Ets-1, 1 μg of total DNA was transfected into HHCC cells in 24-well plate using the Lipofectamine 2000. 48 h after transfection, cell lysates were prepared for measurement of luciferase activity. In some groups, Jurkat cells were treated with 30 ng/ml of TPA for 4 h, or 0.5 μg/ml of A23187 for 1 h, or 100 u/ml of IL-2 for 24 h, or 500u/ml of TNF-α for 24 h before luciferase assay.

**Luciferase Assays** - Cells were rinsed in phosphate buffered saline at 48 h after transfection and lysed in a Passive Lysis Buffer (Promega). Luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) with Turner luminometer and normalized for transfection efficiency to the Renilla Luciferase activity. Reported data were represented as the mean from three independent experiments.

**Western-blot** - Jurkat cells or HHCC cells were pelleted, and then lysed by 2× loading buffer. After SDS-PAGE resolution and membrane transfer, the target proteins were probed with rabbit antibody against human c-Jun, Ets-1 or β-actin (Santa Cruz). Then HRP-labeled goat anti-rabbit immunoglobulin was added and the proteins were detected by HRP substrate (Pierce).

**Electrophoretic Mobility Gel Shift Assay (EMSA)** - Nuclear extracts were prepared from TPA treated Jurkat cells according to the method of Dignam (26) with minor modification. Briefly, 3×10⁵ cells were washed twice with ice-cold phosphate buffered saline and resuspended in buffer A (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and left on ice for 15 min. After the lysates were passed several times through a 25-gauge needle, nuclei were recovered by centrifugation at 13 000 rpm for 10 min at 4 °C. Nuclei were resuspended in buffer B (20 mM HEPES, pH7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 20% glycerol, and 1 mM DTT) and incubated on ice for 30 min. The mixture was centrifuged at 13 000 rpm at 4 °C for 15 min, and the supernatant as nuclear extract was collected and stored at -70°C until use. Protein concentrations of nuclear extract were determined by Bradford assay. For preparation of probes, γ-32p was labeled to the end of AP-1 oligo consensus by using T4 Polynucleotide Kinase (Promega), while α-32p was labeled to P1 and P2 by PCR amplification. In some groups, 2.0 μg of nuclear extract was pretreated with AP-1 oligo consensus (5’-CGCTTGATGAGCCGGA A-3’), or Sp1 oligo consensus (5’-
ATTCGA TCGGGC GGGGCGAGC-3’) for 10 min in Binding Buffer provided in the EMSA kit (Promega). The 32p-labeled probes were added into non-pretreated or pretreated nuclear extract and reacted for 20 min. The reactions were electrophoresed in 6% non-denature polyacrylamide gel in 0.5×TBE buffer at 350 V for 15 min. The gel was dried and exposed to a Biomax-MS film (Eastman Kodak) in -70°C for about 10 h. After that, the films were developed, and the binding of transcription factors with promoters or oligo consensus was analyzed according to the density and area of each blot on the developed film.

RESULTS

Defining the Transcription Start Site (TSS) of Human CD226 Gene - The 5’-RACE was carried out to define the transcription start site of human CD226 gene. The mRNA from TPA-treated Jurkat cells was reverse transcribed using gene specific primers. One DNA band of ~500bp was obtained after nested PCR and then cloned into pMD18-T vector for DNA sequencing. The results from three randomly selected clones revealed that the transcription start site was located at –229bp upstream of ATG in mature mRNA of CD226, which was identical to the CD226 genomic sequence (Fig. 1).

Bioinformatics Analysis of CD226 Promoter Region - A 2 kb DNA sequence of CD226 upstream regulation region was obtained from GenBank, which was used for bioinformatics analysis. There are two TATA-box, located at -85bp--80bp and -196bp--191bp respectively, one GC-box, located at -249bp--243bp, and several putative binding sites of transcription factors, such as AP-1, Sp1, Ets-1, PEA3 and GATA-1 (Fig. 1).

Location and Regulation of CD226 Promoter - A series of truncated DNA fragments were inserted into pGL3 vectors and transfected into Jurkat cells, and promoter activity of each truncated fragment was measured by luciferase assay. The results show that truncated DNA fragment 1 (T1) has basic promoter activity, while T4 and T7, located at -810bp~287bp and +33bp+213bp respectively, have relative higher promoter activities; these regions are designated as P1 and P2 (Fig. 2). In Jurkat cells, TPA could increase P1 activity by 19.3% but slightly inhibit P2 activity, while A23187 could increase both P1 and P2 activities. When treated with TPA and A23187 together, the Jurkat cells showed inhibitory effects for all the 3 fragments (T1, T4/P1, and T7/P2) compared with the groups of TPA or A23187 treated T4/P1 or A23187 treated T7/P2 (Fig. 3A). This interesting phenomenon is consistent with previous described(2). The CD226 promoters could also be regulated by IL-2 and TNF-α. Thus after stimulation for 24h by IL-2 or TNF-α, the transfected Jurkat cells was lysed for measurement of luciferase activities. The results showed that IL-2 could increase the activities of T1, T4/P1 and T7/P2 by 40.8 %, 19.4% and 244%, respectively, and TNF-α could increase T1 and P2 promoter activities by 60.2% and 145%, couldn’t affect the P1 activity (Fig. 3B).

Tissue Specific Expression of CD226 Promoters - CD226 is mainly expressed in hematopoietic cells, including T cells, NK cells, monocytes, megakaryocytes, and some population of B cells (1-11). Therefore we examined the expression of CD226 promoters in different cell types to investigate whether the promoters contain tissue specific elements. The results show that P1 and P2 have relative high activities in Jurkat and Daudi cells which express CD226 and low activities in HL60 and K562 cells which do not express CD226. Moderate activities of P1 and P2 were found in Dami cells and NK92 cells. Interestingly, P1 and P2 showed relatively different activities among the cell lines above. P1 had higher activity in Jurkat cells, while P2 showed higher activity in Daudi cells, while the difference of activities for P1 and P2 in NK92
Identification of the NRE in CD226 Upstream Regulation Region - Based on the analysis of luciferase activities using T4~T7, we found a NRE, which was located between the two promoters P1 and P2. Both T5-pGL3 and T6-pGL3 containing a DNA fragment -286bp~+32bp had much lower promoter activities compared with T4-pGL3 and T7-pGL3 (Fig. 2), indicating the fragment -286bp~+32bp may be a NRE. To further test the inhibitory effect, the NRE was introduced to the downstream of cytomegalovirus (CMV) promoter and transfected into Jurkat cells. The luciferase assay showed that the NRE could inhibit CMV promoter activity with or without TPA treatment, and the inhibition rates reached 85.1% and 73.7% respectively (Fig. 5).

Regulation of CD226 Promoters by Transcription Factors AP-1 and Ets-1 - AP-1 is the main transcription factor activated by TPA, IL-2 or TNF-α. Ets-1 is a transcription factor involved in many lymphocyte functions and can also associate with AP-1 to regulate target genes. Bioinformatics analysis indicated that there were several putative binding sites of AP-1 and Ets-1. To investigate whether the two transcription factors could regulate CD226 promoters, HHCC cells were employed because of their low expression of endogenous AP-1 and Ets-1 and high efficiency for transient transfection (Fig. 6A). We co-transfected the expression vectors containing these transcription factors with P1-pGL3 or P2-pGL3 into HHCC cells and found that c-Jun, c-Fos or Ets-1 alone did not regulate P1 and P2. In contrast, the c-Jun/c-Fos heterodimer (AP-1) could increase P1 and P2 activities to 56.7-fold and 12.7-fold respectively. When co-transfected the AP-1 and Ets-1 expression vectors and P1-pGL3 or P2-pGL3 into the HHCC cells, we found that Ets-1 could inhibit AP-1 induced P1 activity but increase AP-1 induced P2 activity (Fig. 6B). Bioinformatics analysis showed that there was an AP-1/Ets-1 composite binding site in P1 (Fig.1), suggesting that Ets-1 may inhibit AP-1 induced P1 activity by competing for binding at the site. Deletion of the composite site of AP-1/ Ets-1 partially impaired AP-1 induced P1 activity and Ets-1 could no longer inhibit AP-1 induced P1 activity (Fig. 6C). These results indicate that the composite binding site was responsible for AP-1 induced P1 activity, substantiating the notion that Ets-1 inhibits AP-1 function on P1 by competing for the binding site.

Binding of Transcription Factors with CD226 Promoters - AP-1 could functionally regulate CD226 promoters. To investigate whether AP-1 could physically bind with CD226 promoter, we carried out EMSA analysis. In EMSA, the specificities of AP-1 bands were confirmed by adding excess amounts of unlabelled specific and non-specific AP-1 probes to the reaction mixtures. The results indicate that the nuclear extract from TPA-stimulated Jurkat cells could bind with CD226 promoters, P1 and P2. One main band could be found when P1 was incubated with the nuclear extract. This band could be partially inhibited by unlabelled AP-1 oligo consensus, but almost completely inhibited by the Sp1 oligo consensus. Two strong bands could be found when P2 interacted with the nuclear extract and they could be strongly inhibited by unlabelled AP-1 oligo consensus, but only partially by unlabelled Sp1 oligo consensus (Fig. 7). These results confirmed that AP-1 and Sp1 could bind CD226 promoters P1 and P2.

DISCUSSION

The phenomenon that a single gene owns alternative promoters is very common and is often related to tissue specific expression of that gene (27-29). In this study, we show that the human CD226 gene has at least two promoters, termed P1 and P2, and they have distinctive activity in some cell lines derived from different...
tissues. These results suggest that the two promoters may play a role in tissue specific expression of human CD226. Previous studies have shown that CD226 mRNA and surface expression in Jurkat cells was upregulated by treatment of the cells with TPA (2), and it is known that TPA can activate the transcription factors AP-1 and NF-xB through the PKC pathway (24). Here we also show that TPA can upregulate CD226 promoters in Jurkat cells, and that AP-1 could increase CD226 promoter activities. These results suggest that the mechanism of upregulation of CD226 expression by TPA may be through the activation of AP-1. We also found that ionophore/A23187 could upregulate CD226 expression and promoter activity in Jurkat cells. However, when Jurkat cells were treated with TPA, co-treatment with A23187 resulted in an inhibitory effect on CD226 promoter activities. This finding is consistent with the previous report on CD226 mRNA and protein expression (2), but the mechanism of this phenomenon is still unclear. It is well established that AP-1 and NFAT are the main downstream transcription factors of TPA and A23187 signal pathway. It is possible that the interaction of AP-1 and NFAT may be involved in the inhibitory effect of A23187 on TPA induced CD226 expression.

In the present study, we show that not only TPA could upregulate CD226 promoter activities, but also AP-1 upregulated CD226 promoters through an AP-1/Ets-1 binding site. Thus, it is most likely that TPA functions by activating AP-1, and AP-1 in turn promotes CD226 transcription through the binding site. Ets-1 can interact with AP-1 which displayed different regulatory on the two promoters of the CD226 gene. In our investigation of the binding of AP-1 to CD226 promoters in vitro, we found that both P1 and P2 could bind with the nuclear extract of TPA stimulated Jurkat cells. And the bands could be inhibited by unlabelled oligonucleotides of AP-1 and Sp1 to different extent. These results imply that the nuclear proteins which bind with CD226 promoters could be a protein complex, at least composed of AP-1 and Sp1, and the composition of the complex may influence the regulatory outcome.

It is very common for genes to contain negative regulatory elements within the promoter, and this is one of the major mechanisms of gene regulation. Regulations by NRE can occur by two mechanisms. The first is position-dependent or independent, and the second is gene specific or non-specific. Here we identified a NRE which is positioned between the two promoters of CD226 gene. Since the NRE could inhibit CD226 promoters, whether downstream or upstream, it is likely that this NRE works in position-independent manner. Moreover, the NRE of CD226 gene can also effectively inhibit the CMV promoter, suggesting that this NRE works in gene non-specific manner. The elucidation of the mechanism of the NRE inhibition will be helpful in further understanding CD226 gene regulation.

REFERENCES


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The abbreviations used are: CMV, cytomegalovirus; EMSA, electrophoretic mobility gel shift assay; HRP, horseradish perosidase; LFA-1, lymphocyte function associated antigen-1; NRE, negative regulation element; PKC, protein kinase C; TSS, transcription start site.

**TABLE LEGEND**

**Tab. 1.** Primers for PCR of truncated fragments in upstream regulation region of CD226 gene.

**FIGURE LEGENDS**

**Fig. 1.** Identification of the transcription start site (TSS) and bioinformatics analysis of upstream regulation region of CD226 gene. The TSS site is indicated by an arrow and putative binding sites of transcription factors are underlined.

**Fig. 2.** Location of promoters and the negative regulation element (NRE) of CD226 gene. Seven truncated DNA fragments in pGL3 vector (T1~T7-pGL3) and negative control vector pGL3 were transfected into Jurkat cells. Luciferase activities were measured at 48h after transfection. Two promoters, designated P1 and P2, at -810bp~+287bp, and +33bp~+213bp and one NRE at -286bp~+31bp were identified based on luciferase activity analysis.

**Fig. 3.** Regulation of CD226 promoters by stimuli (TPA1, A23187) and cytokines. A, T1-pGL3, T4/P1-pGL3 and T7/P2-pGL3 were transfected into Jurkat cells and cultured for 48 h before cell lysis. In some groups, transfected Jurkat cells were treated with TPA for 4 h, or A23187 for 1 h before luciferase assay. B, T1-pGL3, T4/P1-pGL3 and T7/P2-pGL3 were transfected into Jurkat cells and cultured for 24 h. Then IL-2 (100u/ml) or TNF-α (500u/ml) was added in the culture system for an additional 24 h before luciferase assay.

**Fig. 4.** Determination of CD226 promoters in different cell lines by luciferase assay. T4/P1-pGL3 and T7/P2-pGL3 were transfected into Jurkat, HL60, Dami, NK92, K562, and Daudi cells and cultured for
48h before luciferase assay.

Fig. 5. Inhibition of cytomegalovirus (CMV) promoter activity by the negative regulation element (NRE). pGL3, CMV-pGL3 and CMV-NRE-pGL3 were transfected into Jurkat cells respectively. The transfected cells were treated with TPA for 4 h or without TPA, and the promoter activities were determined by luciferase assay.

Fig. 6. Regulation of CD226 promoters by AP-1 and Ets-1. A, The expression of AP-1 and Ets-1 in Jurkat and HHCC cells. Jurkat cells or HHCC cells were pelleted, and lysed by 2×loading buffer. After SDS-PAGE and membrane transfer, the target proteins were probed with rabbit antibodies against human c-Jun, Ets-1 and β-actin. Then HRP-labelled goat anti-rabbit immunoglobulin was added and the proteins were detected by HRP substrate. B, Expression vectors of mock, c-Jun, c-Fos, Ets-1, AP-1 or AP-1 plus Ets-1 were transfected into HHCC cells respectively. Luciferase activities were measured at 48 h after transfection. C, Function of composite binding site of AP-1 and Ets-1 in P1. Wild type P1- pGL3 vector (WT-P1) or AP-1 site deleted P1-pGL3 vector (AP-1Δ-P1) were transfected into HHCC cells and luciferase activities were measured at 48 h after transfection.

Fig. 7. Binding of transcription factors to CD226 promoters. The 32p-labeled probes were added into TPA -treated or non-pretreated nuclear extract from TPA treated Jurkat cells and reacted for 20 min. The reactions were electrophoresed, and the gel was dried and exposed to a Biomax-MS film. After that, the films were developed, and the binding of transcription factors with promoters or oligo consensus were analyzed according to the density and area of each blot on the developed film. One predominant band could be observed when 32P-labeled AP-1 probe was incubated with the nuclear extract (lane 2). The unlabelled AP-1 oligo consensus but not the unlabelled Sp1 oligo consensus could compete the binding of 32P-labeled AP-1 probe with AP-1 in the nuclear extract (lane 3, 4). Both P1 and P2 could interact with nuclear extract from TPA-treated Jurkat cells (lane 6, 10) and the binding could be inhibited by unlabelled AP-1 and Sp1 oligo consensus at different levels (lane 7, 8, 11, and 12).
Table 1

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Seven pairs of primers were designed for PCR amplification of different truncated DNA fragments T1, T2, T3, T4, T5, T6 and T7 respectively. They are P1 and P8 for production of T1, P1 and P2 for T2, P3 and P8 for T3, P3 and P4 for T4, P5 and P8 for T5, P3 and P6 for T6, and P7 and P8 for T7.
Figure 3

A.

B.
Figure 6

A. Jurkat HHCC

B. [Bar graph showing relative light units (RLU) for mock, Jun, Fos, Ets-1, AP-1, AP-1+Ets-1]

C. [Bar graph showing RLU for mock, AP-1, AP-1+10μM, WT-P1, AP-1Δ-P1]
### Figure 7

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