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Increased SOCS6 stability with PMA requires its N-terminal region and the Erk pathway via Pkcδ activation

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Abstract

We investigated stability of the ectopically expressed the SOCS6 protein in HEK293T cells with PMA, which activates protein kinase C (PKC). The treatment of PMA could largely increase SOCS6 stability in HEK293T cells. But, we did not observe increased protein levels of SOCS3 or Erk1 with PMA. This result suggests that the increased stability of SOCS6 with PMA did not generally occur in other proteins. The stability of SOCS6 depended on the N-terminal region containing an unidentified domain. We then studied the role of signal pathways in SOCS6 stability with PMA. We found that both Erk and Pkcδ activation were required for the increased SOCS6 stability by PMA. The Erk activation by PMA appeared to be downstream from the Pkcδ activation. The increased SOCS6 stability and Erk activation by PMA were both conserved in another cell line, MCF7. In addition, we demonstrated that PMA, insulin, and PDGF increased both the stability of endogenous-expressed SOCS6 and Erk activation in MDA-MB231 cells. These observations suggest that Erk activation may be correlated in the cells with high expression of SOCS6.

Keywords: PMA; SOCS6; Pkcδ; Erk; Stability; PKC; SOCS box; N-terminal region; HEK293T

The suppressors of cytokine signaling (SOCS) members are negative feedback regulators of the Jak/Stat or receptor tyrosine kinases pathway, and they inhibit the signaling pathways that initially led to their production [1–4]. The SOCS includes eight family members, which are CIS and SOCS1–7. These proteins have been classified on the basis of their structural domains; a conserved C-terminus referred to as the SOCS box, a central SH2 (src homology-2) domain, and a unique N-terminal region of variable length [4–6]. The SOCS6 protein has a unique addition of 300 amino acids in the N-terminal region unlike other SOCS family members, and the role of this addition is unidentified so far [1–6]. Therefore, its function might be expected to be different from the other SOCS members. For examples, SOCS6 does not inhibit signaling from growth hormone, leukaemia inhibitory factor, or prolactin [7,8]. Unlike other SOCS members, SOCS6 does not interact with Jaks [7,9]. Studies of SOCS6 show that its protein levels are an important factor for cellular normality. Therefore, it is pivotal to understand which mediators are required for the increase or decrease of SOCS6 protein levels. Furthermore, it is important to identify the transcription factors or signal molecules that control SOCS6 protein levels.

There are reports showing that Protein kinase C (PKC) activation is able to regulate stability for some proteins [10,11]. The increased stability of these proteins was primarily due to the reduction of proteasome-medi-

Abbreviations: PMA, phorbol 12-myristate 13-acetate; SOCS, suppressors of cytokine signaling; STAT, signal transducer and activator of transcription; JAK, Janus kinase; SH2, Src homology 2; CIS, cytokine-inducible SH2-containing protein; PKC, protein kinase C.

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ated degradation. Although PKC activation was shown to be related to the Jak/Stat pathway, there was not much known about the relationship between PKC activation and protein stability of the SOCS members [12]. Phorbol 12-myristate 13-acetate (PMA) is a stimulatory modulator, which activates PKC. In our study, we found that HEK293T cells could largely increase SOCS6 stability by PMA, after cells were transfected with a SOCS6 plasmid. The stability of SOCS6 depended on the N-terminal region containing an unidentified domain. The Erk activation by PMA was required for the increase of SOCS6 stability, because the inhibition of the Erk pathway resulted in the prevention of increases in SOCS6. Using experiments with an inhibitor and overexpressing-plasmid for Pkcδ, we found that Pkcδ activation was required for the increase in SOCS6 stability with PMA. We also demonstrated that Erk activation by PMA was downstream from Pkcδ activation. In addition, we found that PMA, insulin, and PDGF increased both the stability of endogenous-expressed SOCS6 and Erk activation in MDA-MB231 cells. These observations suggest that Erk or Pkcδ activation could be observed in the cells with high expression of SOCS6 in vivo.

Materials and methods

Cell culture. The human kidney cell line HEK293T and breast cancer cell lines (MCF7 and MDA-MB231) were obtained from the Korean Cell Line Bank (Seoul, Korea). They were cultured using DMEM or RPMI1640 for MDA-MB231, containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA).

Plasmids and transfection. The plasmids used in this work are described in Supporting information on the BBRC web site. HEK293T cells were transiently transfected using the expression vectors, or empty vectors. This was achieved with the standard phosphate calcium transfection method described previously [13]. For a transfection of the MCF7 cell line, the transfection reagent (WelGENE, Seoul, Korea) was used in accordance with the manufacturer's instructions for the transfection of plasmids. We observed over a 80% transfection efficiency with the above cell lines, when we estimated them with a GFP-plasmid. Four hours after the transfection, the cells were supplied with fresh medium and treated with PMA or other specific inhibitors.

Western blot analysis. For the Western blot analysis, all the proteins were extracted using the TCA method described previously [14]. Briefly described, cells placed on 60 mm dishes were washed three times each with a 5 ml PBS buffer. The 500 μl of 20% TCA was added to each plate, and cells were dislodged by scraping and transferred to Eppendorf tubes. Using a centrifugation for 5 min at 3000 rpm, the proteins were pelletized. They were then resuspended with a Tris–HCl (pH 8.0) buffer, and protein concentrations were estimated using the dye-binding method. They were then dissolved in the SDS–PAGE loading buffer (Invitrogen, Carlsbad, CA), and subjected to Western blot analysis.

RT-PCR. Total RNA was extracted using RNAzol B (TELTEST, Friendswood, TX). First-stranded cDNA was synthesized from 2 μg of total RNA using a reverse transcription kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. To amplify double-stranded cDNA, PCR was performed in a 25 μl reaction mixture containing 5 μl of the reverse transcribed cDNA. The primer pairs used for PCR were as follows; for SOCS6 5′-AGATGAGGGGATGTATCCTT-3′ and 5′-AGTTCGGATCAAAATTCAAA-3′; for GAPDH, 5′-TGATG ACATCAAGAAGGTGGTGAAG-3′ and 5′-TCCTTGGAGGCCATG TGGGCCAT-3′. GAPDH was amplified to normalize the results. After PCR, 10 μl of the samples was electrophoresed in 1.5% agarose gels.

Results and discussion

Treatment with PMA increased SOCS6 stability in transfected HEK293T cells

We investigated how the SOCS6 protein is regulated, since SOCS6 levels have been shown to be an important cellular function determinant. We did not detect any SOCS6 protein band in the lysates from HEK293T cells by using commercial polyclonal anti-SOCS6 antibody. We could detect the SOCS6 protein after the transfected pCMV-SOCS6 plasmid encoded its ORF. We found that the SOCS6 protein reached a maximum level 20 h after the transfection (data not shown). PKC activation has been shown to be important in producing higher protein levels through protein stability [10,11]. To investigate the effect of PKC on the stability of the SOCS6 protein, we treated HEK293T cells with 1 μM PMA, 11 h after the transfection. Then, cell extracts were prepared at 9 h after the treatment, and we performed Western blot analysis. As shown in Fig. 1A, the SOCS6 protein was greatly increased with the PMA treatment, when compared to the control lane with an absence of PMA. PMA treatment did not induce transcriptional activation in chromosomal SOCS6 nor in the pCMV-SOCS6 plasmid (Fig. 1B). Therefore, we excluded the possibility of increased mRNA for the SOCS6 protein with PMA. We tested the protein stability with PMA in another member of the SOCS family, SOCS3. Both SOCS3 and SOCS6 have conserved SH2 and SOCS box domains [4–6]. We did not observe increased protein levels of SOCS3 with PMA when we transfected plasmids to overexpress SOCS3 (Fig. 1C). This result suggests that SOCS6 stability may be regulated by different mechanisms in other SOCS family members. In addition, we did not observe increased levels of the Erk1 protein with PMA, when we transfected the plasmid to overexpress Erk1. However, SOCS6 levels were increased by PMA treatment (Fig. 1D). This suggests that the increased stability observed in SOCS6 with PMA treatment did not generally occur in other proteins.

Increased SOCS6 stability with PMA required its N-terminal region

We sought to identify critical amino acid regions in the SOCS6 protein for the increased stability with PMA. SOCS members contain a SOCS box, which is targeted for ubiquitination and subsequently for proteasome-mediated degradation [4–6]. A proteasome-mediated degradation mechanism was shown to regulate the stability of the SOCS6 protein [7,9,15]. Although we did not observe increased stability of SOCS3 with PMA, possibility remains that PMA increased the SOCS6 stability through the inhibition of SOCS box-mediated degradation. SOCS6 has 300 additional amino acids in the N-terminal region in front of the SH2 domain, in comparison to SOCS3 (Fig. 2A). This may contribute to the SOCS6 stability with

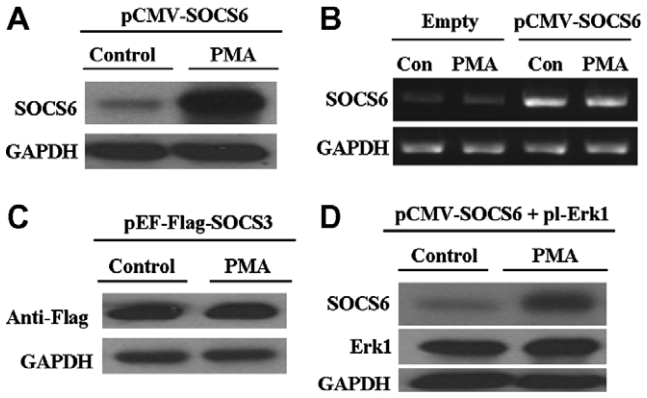

Fig. 1. Treatment with PMA increased SOCS6 stability in transfected HEK293T cells. (A) HEK293T cells were plated on 60 mm dishes with a 50% confluence and were grown for 24 h. Then, transfections of pCMV-SOCS6 were performed using a standard phosphate calcium transfection method, as described in Materials and methods. Cells were stimulated with non-treated (Control), or 1 μM of PMA, 11 h after the transfection. Then, cell extracts were prepared at 9 h after the treatment. Proteins were separated using the SDS–PAGE and then Western blot analysis was performed using antibodies for SOCS6 and GAPDH. Probing with GAPDH antibodies provided a loading control. (B) The pCMV-Tag2A (Empty), or pCMV-SOCS6 plasmid was transfected as described in (A). The transfected-cells were stimulated as described in (A). The total RNA was isolated and RT-PCR was performed for mRNA levels of SOCS6 and GAPDH, as described in Materials and methods. (C) The pEF-Flag-SOCS3 plasmids were transfected and stimulated as described in (A). Then, cell extracts were prepared as described in (A) and Western blot analysis was performed using antibodies for Flag and GAPDH. (D) The mixtures of pCMV-SOCS6 and pl-Erk1 plasmids were transfected using a standard phosphate calcium transfection method. The transfected-cells were stimulated as described in (A). Then, cell extracts were prepared at 9 h after the treatment. Western blot analysis was performed using antibodies for SOCS6, Erk1, and GAPDH.

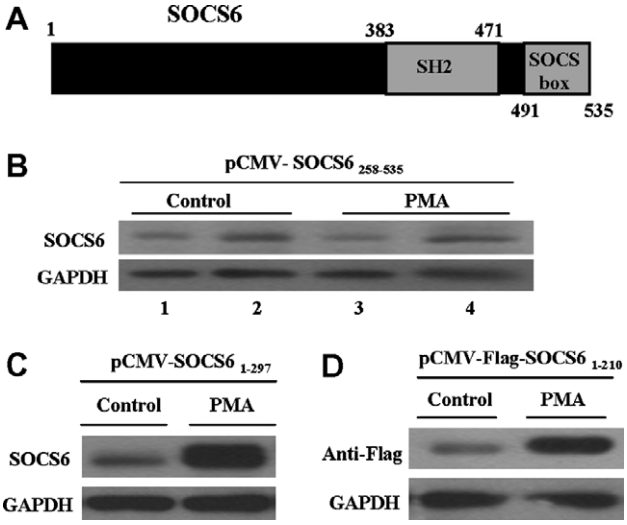

Fig. 2. Increased SOCS6 stability with PMA required its N-terminal region. (A) It represents the location of domains in the SOCS6 protein. The numbers indicate the amino acids order from the N-terminal of SOCS6. The conserved sequences in SOCS6 are following; SH2, SH2 domain (amino acid from 383 to 471) and SOCS box, SOCS box domain (amino acid from 491 to 535). (B) The pCMV-SOCS6$_{258–535}$ plasmid was transfected and stimulated as described in Fig. 1A. Then, cell extracts were prepared at 9 h after the treatment. Western blot analysis was performed using antibodies for SOCS6 and GAPDH. The samples were loaded with two different amounts of extracts in adjacent lanes; thus, the size of samples in lanes 1 and 3 were 50% of those loaded in lanes 2 and 4. (C,D) The pCMV-SOCS6$_{1–297}$ or pCMV-Flag-SOCS6$_{1–210}$ plasmid was transfected and stimulated as described in Fig. 1A. Then, cell extracts were prepared at 9 h after the treatment. Western blot analysis was performed using antibodies for SOCS6, Flag, and GAPDH.

PMA. We divided SOCS6 proteins into three parts in order to determine which region was important for the increased SOCS6 stability with PMA. We therefore generated PCR products containing amino acids from 258–535, 1–297, or 1–210 in SOCS6. Each fragment was cloned into pCMV vectors and generated the pCMV-SOCS6$_{258–535}$, pCMV-SOCS6$_{1–297}$, and pCMV-Flag-SOCS6$_{1–210}$ vectors. We then transfected them into HEK293T cells and observed that the protein stability of the fragments was increased by PMA. When we first examined the C-terminal region containing known domains, we did not observe any increased stability with PMA (Fig. 2B). Therefore, we concluded that the C-terminal region containing amino acids 258–535 did not contribute to increased SOCS6 stability with PMA. As we observed in SOCS3, this result was consistent with data that suggests that the conserved domains in SOCS family members do not contribute to increased stability with PMA. However, when we transfected either pCMV-SOCS6$_{1–297}$, or pCMV-SOCS6$_{1–210}$, we found that PMA largely increased the stability of the SOCS6 protein fragments containing N-terminal regions (Fig. 2C and D). This result suggests that the increased SOCS6 stability with PMA resulted from the N-terminal region containing an unidentified domain. We concluded that the SOCS6 protein was stabilized by a PMA-activated mechanism, and required an N-terminal region containing an unidentified and unconserved domain. It would be of interest to further investigate the mechanisms, the role of the N-terminal region, or the protein interactions required for the increased SOCS6 stability with PMA.

The presence of PMA largely increased the Erk activation, which was required for increases in the SOCS6 protein

In order to identify the role of signal pathways for the increased SOCS6 stability with PMA, we investigated the activation of Jnk, Erk, p38, and Akt signal pathways. In previous reports, these pathways have been shown to mediate Pkcδ activation [16,17]. We analyzed phosphorylation of Jnk1, Erk1/2, p38, and Akt and at the same time we measured increased SOCS6 protein levels. We found that Erk was highly activated, whereas the p38 and Akt activations were just slightly increased after the PMA-treatment (Supplementary Fig. 1A). We did not detect any Jnk1 activation by PMA (data not shown). The degree of Erk activation with PMA was similar to the increased SOCS6 level, shown in Fig. 1A. The Erk activation by PMA had already reached a saturated level at 1 h after the PMA treatment. This occurred earlier than the increases for SOCS6 proteins

(Fig. 3A). Since SOCS6 is known to be unstable, we assume that prolonged and activated signals from PMA were required for continuous increases in the SOCS6 level. Therefore, the Erk signal pathway was a good candidate for increased SOCS6 stability with PMA. However, it may be also possible that pathways other than Erk were activated at a very early time and then turned off, prior to the 9 h elapse time after the PMA-treatment. We examined whether Erk inhibitors would decrease the increased SOCS6 level with PMA. We treated cells with a MEK inhibitor, PD98059, which inhibits upstream of the Erk pathway. We found that 5 h of PD98059-pretreatment before PMA-treatment, abolished PMA-induced increases of SOCS6 protein. But, we did not detect any reduction of SOCS6 with a p38 inhibitor, SB203580, or an Akt inhibitor, LY294002 (Fig. 3B and C). We confirmed an Erk-dependence, using a pre-treatment from another Erk inhibitor, U0126 (Fig. 3C). It is possible that SOCS6 protein is increased even more, if more Erk proteins existed to be activated by PMA in the cells. We tested this with a PMA treatment after the co-transfection of SOCS6 and Erk2 plasmids. We found that SOCS6 protein levels were increased in the co-transfected cells with Erk plasmids, assuming that the SOCS6 level was determined by the amount of the Erk protein to be activated. As we expected, we did not observe increased SOCS6 protein levels when p38 was overexpressed (Fig. 3D). We conclude that Erk activation by PMA was the main signal pathway for increasing SOCS6 protein levels. Our conclusions also show that Erk activation was a prolonged and highly activated signal pathway, and was required for the increased SOCS6 stability with PMA. It would be of interest to further investigate the mechanisms underlying the increased SOCS6 stability in the N-terminal region by Erk activation. We assumed that the N-terminal region was involved in the targeting for ubiquitination and subsequently for proteasome-mediated degradation. Erk activation directly or indirectly contributed to the inhibition of this pathway. For example, Erk directly modified SOCS6 via phosphorylation of the N-terminal serine or threonine residues, and reduced the targeting for proteasome-mediated degradation. Erk may indirectly activate or repress some regulators, that may interact with the N-terminal region of SOCS6. This might prevent the ubiquitination-related degradation of SOCS6.

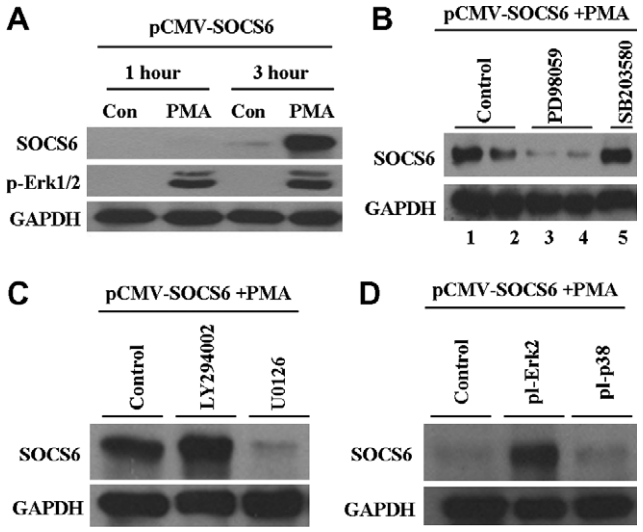

Fig. 3. The presence of PMA largely increased the Erk activation, and it was required to increase SOCS6 using PMA. (A) The pCMV-SOCS6 plasmids were transfected and stimulated as described in Fig. 1A. The protein extracts were collected at the time indicated after the treatment. Western blot analysis was performed with all the extracts, 12 (marked as 1 h) or 14 (marked as 3 h) hours after the transfection. (B,C) The pCMV-SOCS6 plasmids were transfected as described in Fig. 1A. The transfected-cells were stimulated with a non-treated (Control), 50 μM PD98059, 10 μM SB203580, 20 μM LY294002, or 10 μM U0126, 6 h after the transfection. Following a 5 h incubation, cells were treated with 1 μM of PMA. The protein extracts were collected 9 h after the PMA treatment. Western blot analysis was performed using antibodies against SOCS6 and GAPDH. (D) The pCMV-SOCS6 plasmids were co-transfected with an empty vector (Control), pl-Erk2, or pl-p38 plasmids as described in Fig. 1D. The co-transfected-cells were stimulated with 1 μM PMA, 11 h after the transfection, and protein extracts were collected 9 h after the PMA treatment. Then, Western blot analysis was performed using antibodies for SOCS6 and GAPDH.

Pkcδ activation was required for the increase in SOCS6 stability with PMA

Since PMA could activate several PKC subtypes, we tried to determine which subtype was activated or required for the increase in SOCS6. There are more than 10 PKC isoforms in the cell. Pkcδ is one of the most well-studied PKC isoforms in which activation with PMA resulted in an increase of some proteins [18,19]. In the absence and with the presence of PMA, there was no difference in Pkcδ protein levels. But, we found that a Pkcδ band was slightly shifted-up in the PMA-treated cells, suggesting that it was auto-phosphorylated. We also detected its active form, using the antibody for phosphorylation of Pkcδ (Supplementary Fig. 1B). Next, we tested whether there was a relationship between the increase in the SOCS6 protein and Pkcδ activation. If there were more Pkcδ proteins that could be activated by PMA, we assumed that the SOCS6 level would be increased. We treated cells with PMA, after the co-transfection of SOCS6 and Pkcδ plasmids. Cells co-transfected with the Pkcδ plasmid increased SOCS6 levels (Fig. 4A). This result showed that the increase in the SOCS6 protein by PMA was dependent on the amounts of the Pkcδ protein available in the cells. It also indicates that amount of the Pkcδ protein was a rate limiting factor for increased SOCS6 stability upon treatment with PMA. We used a specific inhibitor of Pkcδ, called rottlerin, in order to confirm Pkcδ-dependence [18]. We found that a rottlerin-pretreatment for 5 h prior to PMA-treatment, abolished PMA-induced increases in the SOCS6 protein (Fig. 4A). We did not detect any reduction in SOCS6 level with the rottlerin only treat-

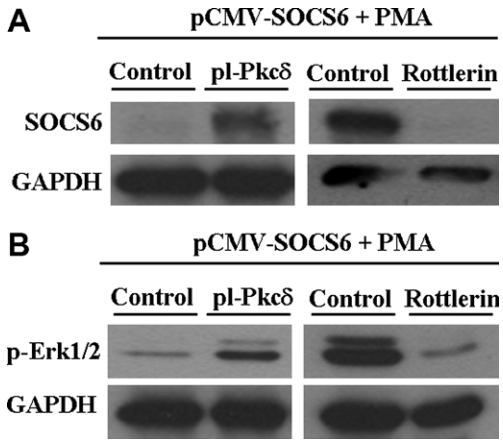

Fig. 4. Pkcδ activation was required for increased SOCS6 stability by PMA. (A) Left blot: the pCMV-SOCS6 plasmids were co-transfected with the empty vector (Control) or pl-Pkcδ plasmid expressing Pkcδ. The co-transfected-cells were stimulated with 1 μM PMA, 11 h after the transfection, and protein extracts were collected at 9 h after the PMA-treatment. Then, Western blot analysis was performed using antibodies for SOCS6 and GAPDH. Right blot: the pCMV-SOCS6 plasmids were transfected as described in Fig. 1A. The transfected-cells were stimulated with a non-treated (Control), or 10 μM rottlerin, 6 h after the transfection. Following a 5 h incubation, cells were treated with 1 μM PMA. The protein extracts were collected, 9 h after the PMA treatment. Western blot analysis was performed using antibodies against SOCS6 and GAPDH (B) Pkcδ activation by PMA was upstream, and required for Erk activation. The same protein extracts used in (A) were separated using the SDS–PAGE and then Western blot analysis was performed using antibodies for phospho-Erk1/2 and GAPDH.

ment, suggesting that Pkcδ did not play a role in the SOCS6 protein levels (Supplementary Fig. 2A). It also suggests that HEK293T cells do not have activated-Pkcδ in the absence of PMA. With this study, we determined that Pkcδ activation would be the major pathway for the increase in SOCS6 stability with PMA. Since our results show that Pkcδ activation could increase the SOCS6 protein, it would be interesting to investigate whether a correlation exists between the activated Pkcδ level and the SOCS6 protein in vivo.

Pkcδ activation by PMA was upstream and was required for the Erk activation

We found that both the activation of Erk and Pkcδ are involved in increased SOCS6 stability with PMA. Although we assume that Pkcδ activation occurred earlier than Erk activation, we wanted to determine whether Erk activation required Pkcδ activation. In addition, we could hypothesize that Erk activation by PMA was independent of Pkcδ activation. In order to show dependence between the activation of these proteins, we tested whether Erk activation depended on the quantity of Pkcδ activation. As shown in Fig. 4A, Pkcδ activation was required for increased SOCS6 protein, when either co-transfected with the Pkcδ overexpression-plasmid or pre-treated with rottlerin before PMA-treatment. When we observed the Erk activation using these conditions, we detected that Erk activation was dependent on the quantity of Pkcδ activation (Fig. 4B). Therefore, we have demonstrated that Erk activation by PMA was downstream from Pkcδ activation.

Both increased SOCS6 stability and Erk activation by PMA were found in another cell line

We determined whether another cell line could have both increased levels of SOCS6 protein and Erk activation with a PMA treatment. We used a breast cancer cell line, MCF7, which is an estrogen receptor positive cell line. It is widely used in transfection experiments for in vitro cancer investigations, because of its high transfection efficiency. As shown in Supplementary Fig. 2B, we detected both increased levels of SOCS6 protein and Erk activation by PMA in the MCF7 cell. This suggests that increased SOCS6 stability by PMA is conserved in cell lines derived from different organs.

PMA, insulin, and PDGF increased both the stability of endogenous-expressed SOCS6 and Erk activation

We sought to determine whether PMA treatment could increase the levels of both the endogenous SOCS6 protein and Erk activation. The cell line used in our study was a breast cancer cell line, MDA-MB231. We detected increased levels of both the endogenous SOCS6 protein and Erk activation by PMA, as shown in Supplementary Fig. 3A. We found that the cells predominantly exhibited Erk2 activation over Erk1 activation. We also confirmed the Erk-dependence using a pre-treatment from an Erk inhibitor, U0126. The results suggest that endogenous-expressed SOCS6 follows the same pathway and mechanism for the increased stability of overexpressed-SOCS6 shown in HEK293T cells. We then sought to determine whether the Erk activation by physiological stimulants could increase the stability of the endogenous-SOCS6 protein. We used two cytokines that can activate Erk, insulin, and PDGF. We found that both stimulants were able to activate Erk and increased endogenous-SOCS6 (Supplementary Fig. 3B). Therefore, we demonstrated that both Erk activation and increased SOCS6 stability occurred simultaneously by PMA or other physiological stimulants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.175.

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