

Differential inhibition of IL-6-type cytokine-induced STAT activation by PMA

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Abstract Prior activation of mitogen-activated protein kinases by phorbol 13-myristate 12-acetate (PMA) results in an inhibition of interleukin (IL)-6-induced activation of the Janus kinase/signal transducer and activator of transcription (STAT) signaling pathway which is most likely mediated by the induction of suppressor of cytokine signaling-3 and requires the specific SHP2 binding site Y759 of the IL-6 signal transducer gp130. In this study, we demonstrate that PMA inhibits STAT activation by IL-6 and the related cytokine leukemia inhibitory factor (LIF) but not by oncostatin M (OSM). Since the LIF receptor also contains an SHP2 recruitment site whereas the OSM receptor lacks such a module, we propose that two SHP2 binding modules within a homo- or heterodimeric receptor are necessary to mediate the PMA inhibitory effect. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cytokine signaling; Cross-talk; Mitogen-activated protein kinase; Signal transducer and activator of transcription; Suppressor of cytokine signaling

1. Introduction

The Janus kinase (Jak)/signal transducer and activator of transcription (STAT) pathway transduces the signals of numerous cytokines and growth factors from their respective surface receptors to target genes in the nucleus. In the case of the interleukin (IL)-6-type cytokines IL-6, oncostatin M (OSM), leukemia inhibitory factor (LIF), IL-11, ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1) binding to their specific receptors induces the homodimerization (IL-6, IL-11) or heterodimerization (OSM, LIF, CNTF, CT-1) of the signal transducing receptor chains [1]. Associated Janus family tyrosine kinases Jak1, Jak2 and Tyk2 become activated and in turn phosphorylate specific tyrosine residues within the receptor chains, thereby creating recruitment sites for Src homology 2 (SH2) domain-containing signaling components

such as STAT1, STAT3 and SHP2 [1–3]. Activated STATs homo- or heterodimerize, translocate to the nucleus and bind to regulatory elements of target genes. SHP2 negatively regulates the IL-6-induced gene transcription and links the Jak/STAT pathway to the mitogen-activated protein (MAP) kinase pathway [4,5].

Phorbol 13-myristate 12-acetate (PMA) is a known activator of protein kinase C and the Raf-MEK-Erk pathway [6,7]. Recently, it was shown that preincubation of cells with PMA inhibits the IL-6-induced STAT3 and STAT1 activation [8,9]. This negative effect was found to be dependent on the activation of MAP kinases and de novo transcription and protein synthesis. Since PMA leads to a rapid increase of suppressor of cytokine signaling (SOCS)-3 mRNA, a negative regulator of the Jak/STAT pathway, we suggested that the inhibitory effect of PMA on the Jak/STAT pathway is mediated by SOCS-3. This hypothesis was supported by the finding that the inhibitory effect of both PMA [9] and over-expressed SOCS-3 [10] is dependent on tyrosine-759 within the cytoplasmic part of gp130. This phosphorylated tyrosine module was initially found to bind the tyrosine phosphatase SHP2 [11,12]. However, recent findings suggest that also SOCS-3 can selectively bind to this phosphotyrosine residue thereby exerting its inhibitory function [10,13].

In this study, we addressed the question whether pre-activated MAP kinases also inhibit the activation of STAT factors induced by other IL-6-type cytokines. We find that PMA also inhibits the LIF-induced STAT activation but surprisingly not the one by OSM. Using mutant chimeric receptors, we present evidence that two potential binding sites for SHP2/SOCS-3 are necessary for the negative effect of PMA.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and DMEM/F-12 mix were from Life Technologies, Inc. (Eggstein, Germany), fetal calf serum from Seromed (Berlin, Germany). PMA was purchased from Sigma (Deisenhofen, Germany). Recombinant human IL-6 was prepared as described [14,15]. Recombinant human IL-5 was prepared as described previously [16].

2.2. Cultivation and stimulation of cells

HepG2 cells were grown in DMEM/F-12 mix, and COS-7 cells in DMEM at 5% CO₂ in a water-saturated atmosphere. Culture medium was supplemented with 10% (v/v) fetal calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l). Cells grown in a 100 mm dish to 80% confluency were preincubated with PMA (10⁻⁷ M) for 45 min. Cells were then stimulated with IL-6 (10 ng/ml), OSM (25 ng/ml) or LIF (50 ng/ml) for an additional 15 min.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; IL, interleukin; Jak, Janus kinase; LIF, leukemia inhibitory factor; MAP, mitogen-activated protein; OSM, oncostatin M; PMA, phorbol 13-myristate 12-acetate; SH2, Src homology 2; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling

2.3. DNA constructs and transfection procedures

Standard cloning procedures were performed. The construction of pSVL-based expression vectors (Pharmacia) encoding the receptor chimeras IL-5R α /gp130 and IL-5R β /gp130 has been described in [17], for the IL-5R β /OSM-R and IL-5R β /LIF-R in [18], for the IL-5R β /LIF-RY974A in [19]. The integrity of all constructs was verified by DNA sequence analyses using an ABI PRISM[®] 310 Genetic Analyzer (Perkin Elmer). COS-7 cells were transfected with 20 μ g total DNA by using standard calcium phosphate precipitates. Cells were incubated with the precipitate for 18 h, washed three times with phosphate-buffered saline (PBS) and cultured in fresh medium for at least 8 h. Cells were then split and incubated for another 24 h.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described in [20]. EMSAs were performed using a double-stranded ³²P-labeled mutated m67SIE oligonucleotide from the c-fos promoter (m67SIE: 5'-GAT CCG GGA GGG ATT TAC GGG AAA TGC TG-3') [21,22]. The protein–DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (20 mM Tris, 20 mM boric acid, 0.5 mM EDTA) at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid and 80% water for 1 h, dried and autoradiographed.

2.5. Immunoprecipitation

Cells were washed twice with PBS and solubilized in 1 ml lysis buffer (0.5% Nonidet P40, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM Na₃VO₄, 0.25 mM PMSF, 5 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin and 15% glycerol) for 30 min at 4°C. Insoluble material was removed by centrifugation, and the cell lysate was incubated with specific antibodies at 4°C for a minimum of 2 h. The immune complexes were bound to protein A-Sepharose (5 mg/ml in lysis buffer) for 1 h at 4°C. After centrifugation, the beads were washed three times with 0.05% Nonidet P40, 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM Na₃VO₄ and 15% glycerol, boiled in gel electrophoresis sample buffer and the eluted proteins were separated on sodium dodecyl sulfate (SDS)–polyacrylamide (7.5 or 10%) gels. The following antibody was used: anti-SHP2 rabbit polyclonal antibody (Santa Cruz, CA, USA).

2.6. Immunoblotting and immunodetection

Electrophoretically separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by the semidry Western blotting method. Non-specific binding was blocked with 10% bovine serum albumin in TBS-N (20 mM Tris–HCl, pH 7.4, 137 mM NaCl and 0.1% Nonidet P40) for 15 min. The blots were incubated with primary antibodies in TBS-N for 1 h. After extensive rinsing with TBS-N, blots were incubated with secondary antibodies, goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase for 1 h, and developed with the enhanced chemiluminescence system (ECL, Amersham, Braunschweig, Germany). The following primary antibodies were used: phosphotyrosine-specific STAT1 (Tyr-701) and STAT3 (Tyr-705) rabbit polyclonal antibodies (New England Biolabs, MA, USA), anti-phosphotyrosine mouse monoclonal antibody (4G10; Upstate Biotechnology, NY, USA) and anti-SHP2 rabbit polyclonal antibody (Santa Cruz, CA, USA).

3. Results

Recent findings of several groups indicate that the IL-6-induced STAT activation is negatively modulated by pre-activation of the MAP kinase pathway [8,9,23]. Therefore, we investigated whether STAT activation induced by other IL-6-type cytokines (OSM, LIF) is also inhibited by pre-activated MAP kinases. Human hepatoma HepG2 cells were used because they are equipped with the specific receptor molecules for IL-6, OSM and LIF binding.

HepG2 cells were pretreated with 0.1 μ M PMA or vehicle for 45 min before IL-6 (10 ng/ml), OSM (25 ng/ml) or LIF (50 ng/ml) were added for 15 min. At these concentrations, maximal STAT activation was achieved (data not shown). In EMSA, we observed a strong binding of STAT3 to the spe-

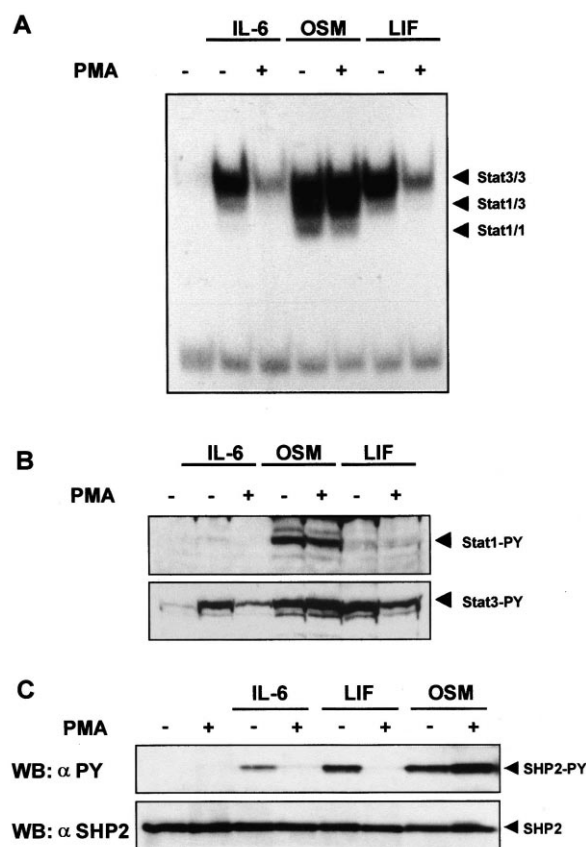


Fig. 1. Inhibition of IL-6-type cytokine-induced STAT1 and STAT3 activation by PMA. HepG2 cells were pretreated with PMA (10^{-7} M) for 45 min before stimulation with IL-6 (10 ng/ml), OSM (25 ng/ml) or LIF (50 ng/ml) for 15 min. (A) Cells were harvested, nuclear extracts were prepared and EMSAs performed as described under Section 2. (B) 50 μ g of nuclear proteins were separated by 10% SDS–PAGE and blotted onto PVDF membrane. Membranes were incubated with (upper panel) phosphospecific STAT1 (Tyr-701) or (lower panel) phosphospecific STAT3 (Tyr-705) antibodies. Immunogenic proteins were visualized with the ECL system. (C) Cell lysates were prepared and immunoprecipitation with anti-SHP2 antibodies was performed as described under Section 2. Precipitated proteins were separated by 10% SDS–PAGE, blotted onto PVDF membrane and analyzed by immunodetection with a specific anti-phosphotyrosine antibody (4G10) (upper panel). Blots were stripped and reprobed with anti-SHP2 antibodies (lower panel) for verification of equal loading.

cific SIE probe after stimulation of cells with IL-6, OSM or LIF (Fig. 1A). Upon OSM treatment also STAT1 was activated. In case of IL-6 and LIF, preincubation of HepG2 cells with PMA for 45 min resulted in a strongly diminished DNA binding of STAT3 which in the case of IL-6 is in agreement with previous observations [8]. In contrast, PMA had no effect on the OSM-induced STAT activation in HepG2 cells (Fig. 1A).

Since activation of STAT1 and STAT3 requires phosphorylation of specific tyrosine residues, i.e. Tyr-701 in STAT1 and Tyr-705 in STAT3, we performed Western blot analyses of nuclear extracts using specific antibodies against the tyrosine-phosphorylated forms of these two transcription factors. Tyrosine phosphorylation of STAT3 was induced by IL-6, OSM and LIF (Fig. 1B). Incubation with PMA prior to a stimulation with IL-6 or LIF leads to a diminished tyrosine phosphorylation, whereas the OSM-induced tyrosine phos-

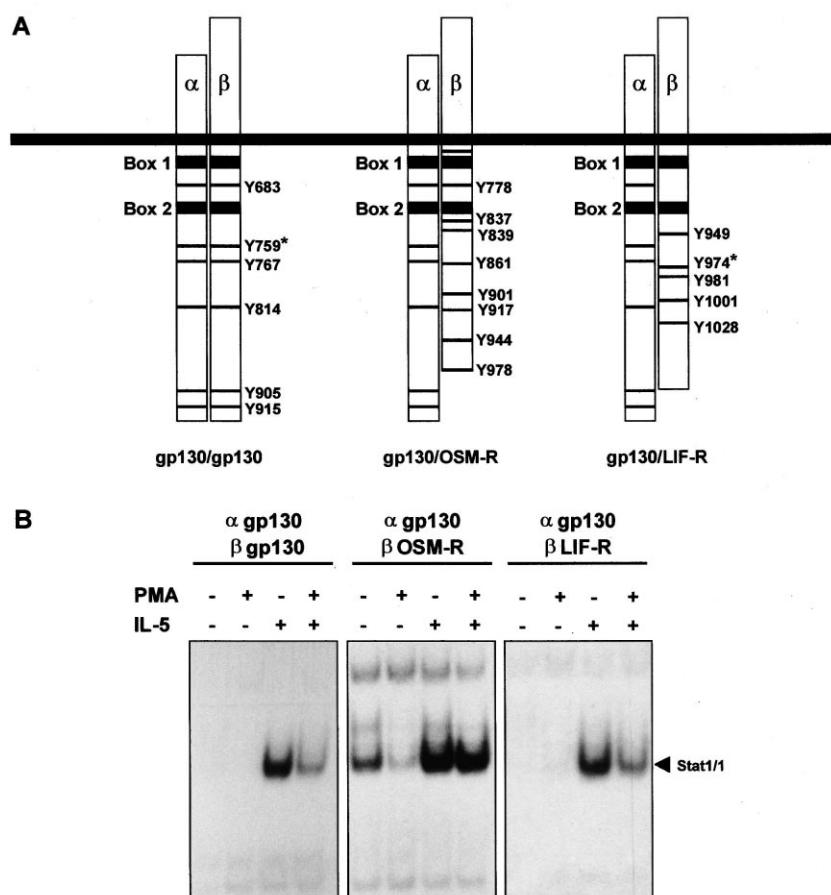


Fig. 2. (A) Schematic representation of IL-5 chimeric receptors used in this study. Tyrosine residues in the intracellular regions are indicated as black lines. Mutated tyrosine residues are indicated by asterisks. (B) COS-7 cells were transiently transfected as described under Section 2 with expression vectors for the chimeric receptors depicted. Cells were preincubated with PMA (10^{-7} M) for 45 min followed by stimulation with IL-5 (80 ng/ml) for 15 min. Nuclear extracts were prepared and EMSAs performed as described in the legend to Fig. 1.

phorylation of STAT3 is not affected. Tyrosine phosphorylation of STAT1 was only observed after OSM treatment and was resistant to the inhibitory effect of PMA. These results are consistent with the data of the EMSA (Fig. 1A). Blots were stripped and reprobed with antibodies against STAT1 and STAT3 for verification of equal loading (data not shown).

Very similar findings were obtained when the tyrosine phosphorylation of the phosphatase SHP2, another down-stream component of the IL-6-induced JAK/STAT pathway [11,24], was analyzed. After stimulation of cells with IL-6, LIF or OSM, a prominent tyrosine phosphorylation of SHP2 was observed (Fig. 1C). Interestingly, OSM and LIF turned out to be stronger inducers of SHP2 phosphorylation than IL-6. PMA inhibited the tyrosine phosphorylation in the cases of IL-6 and LIF but not after OSM (Fig. 1C). Binding of IL-6 to its specific α -receptor chain (gp80) leads to a subsequent homodimerization of gp130 receptor subunits, whereas OSM and LIF trigger the formation of heterodimers. The LIF-R complex is composed of one gp130 molecule and the specific LIF-R, whereas OSM can transduce its signal either via a gp130/LIF-R heterodimer or a gp130/OSM-R dimer. Our data indicate that PMA negatively modulates signaling via a gp130 homodimer or a gp130/LIF-R heterodimer but not via a gp130/OSM-R heterodimer.

To investigate this differential effect of PMA on IL-6-type

cytokine signaling in more detail, we made use of a chimeric receptor system based on the extracellular parts of the IL-5 receptor α - and β -chains and the transmembrane and intracellular parts of gp130, OSM-R or LIF-R, respectively [17,18]. This system allows the directed formation of homo- and heterodimers thereby mimicking the proposed natural receptor complexes (gp130/gp130, gp130/OSM-R or gp130/LIF-R) (Fig. 2A). Transient expression of the respective receptor chimera in COS-7 cells and stimulation with IL-5 resulted in a prominent activation of STAT1 in all three cases (Fig. 2B). STAT3 is not activated efficiently in these cells unless over-expressed [12]. Preincubation with PMA led to similar results as obtained with HepG2 cells: PMA reduced the STAT signal triggered by the formation of the gp130/gp130 homodimer and the gp130/LIF-R heterodimer but not signaling via the gp130/OSM-R heterodimer (Fig. 2B). These data suggest that the down-regulation of the Jak/STAT pathway by PMA is dependent on the intracellular part of the participating receptor subunits.

We have recently shown that the inhibitory effect of PMA on IL-6-induced STAT activation is dependent on tyrosine-759 of gp130 [9]. The tyrosine phosphatase SHP2 is recruited to gp130 via its SH2 domains that interact with phosphotyrosine-759 of gp130. Furthermore, the negative regulator of the Jak/STAT pathway SOCS-3 was recently found to mediate its

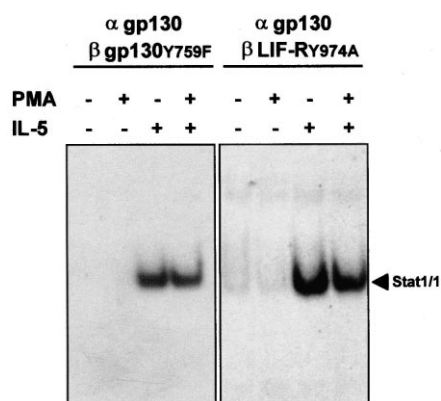


Fig. 3. COS-7 cells were transiently transfected as described under Section 2 with expression vectors for the indicated IL-5 β chimeric receptor mutants. Cells were preincubated with PMA (10^{-7} M) for 45 min followed by stimulation with IL-5 (80 ng/ml) for 15 min. Nuclear extracts were prepared and EMSAs performed as described in the legend to Fig. 1.

inhibitory effect via Y759 [10,13]. The motif Y759STV agrees well with the consensus sequence YXXL/V/I deduced from SHP2 binding to other receptors [11,12]. Inspection of the amino acid sequence of the LIF-R cytoplasmic domain revealed a potential recruitment site, Y974IDV (Fig. 2A, asterisks), for SHP2, whereas none could be found in the OSM-R. In order to examine the role of the YXXL/V/I motifs in the cytoplasmic tails of gp130 and LIF-R in the PMA-dependent inhibition of STAT activation, we made use of chimeric IL-5-R β mutants in which the respective tyrosine residues (Tyr-759 in gp130 and Tyr-974 in LIF-R) were replaced by phenylalanine or alanine. We transiently transfected COS-7 cells with IL-5-R α /gp130 and IL-5-R β /gp130Y759F or IL-5-R α /gp130 and IL-5-R β /LIF-RY974A, respectively, thereby creating receptor dimers with only one YXXL/V/I motif available in the homo- or heterodimer after IL-5 stimulation. In both cases, prestimulation with PMA did not inhibit the IL-5-induced STAT activation (Fig. 3). These findings led us to the suggestion that two YXXL/V/I motifs within a receptor dimer are necessary for the inhibitory effect of PMA.

4. Discussion

Two new findings are presented in this study. First, in contrast to IL-6 and LIF, the OSM-induced STAT activation is not inhibited by preincubation of cells with PMA. Second, within the gp130/gp130 homodimer and the LIF-R/gp130 heterodimer, respectively, a YXXL/V/I motif in each receptor subunit is necessary for the inhibitory effect of PMA on the STAT activation by IL-6-type cytokines.

There is now clear evidence that in respect to the IL-6 receptor complex, both negative regulators of the Jak/STAT pathway, SHP2 and SOCS-3, elicit their function via the phosphorylated Y759STV module within the cytoplasmic tail of gp130 [4,5,10,13,25]. In both cases, an SH2 domain of the respective protein directly binds to the phosphorylated module. We have recently found that the inhibition of the IL-6-induced STAT activation by PMA is most likely mediated by induction of SOCS-3 and requires interaction of SOCS-3 to phosphorylated Tyr-759 of gp130 [9]. Our finding that signaling via a gp130/gp130 homodimer with only one intact

SHP2/SOCS-3 binding site is not inhibited by PMA (Fig. 3) suggests that two SOCS-3 molecules have to bind to a gp130/gp130 homodimer in order to inhibit Jak/STAT signaling.

For the crucial LIF-R module Y974IDV, it has very recently been demonstrated that it interacts with SHP2 [26]. Whether also SOCS-3 binds to the LIF-R SHP2 recruitment site still has to be established. However, obviously the Y974IDV module of the LIF-R is analogous to the gp130 Y759STV module in mediating the inhibitory effect of PMA. In contrast, the OSM-R does not contain an obvious SHP2/SOCS-3 binding site. Thus, the failure of PMA to inhibit OSM signaling could be explained by the fact that within the gp130/OSM-R heterodimer only one negative regulatory site is available.

It is currently difficult to discriminate which negative modulatory effects on Jak/STAT signaling are mediated by SHP2 and which by SOCS-3 since knock-out mice for both are embryonically lethal [27,28]. However, the fact that in our scenario the IL-6-type cytokine-induced SHP2 tyrosine phosphorylation is impaired by PMA in exactly the same way as the STAT tyrosine phosphorylation (Fig. 1C) makes a direct inhibitory role of SHP2 rather unlikely. Also the fact that the PMA effect is dependent on de novo protein synthesis [9] excludes a significant role of SHP2 since this enzyme is extremely long-lived [29]. Of course, we currently cannot exclude that additional proteins are involved in the negative regulation of Jak/STAT signaling by PMA.

It is now well established that the Jak/STAT and the MAP kinase pathways are interdependent signaling cascades. Both positive and negative effects of MAP kinases on the activity of STAT factors have been described. Ser-727 which has been reported to be the MAP kinase phosphorylation site of STAT1 as well as STAT3 is required for transcriptional activity [30]. Recently, we could show that the hyperosmolarity-induced STAT1 activation [31] is dependent on the activation of the p38 MAP kinase [32]. On the other hand, pre-activation of MAP kinases by different stimuli was found to inhibit the subsequent IL-6-induced activation of STAT3 [8,9,33,34]. The data of this study demonstrate the complexity of these cross-talk mechanisms in which the inhibitory action of certain MAP kinases on Jak/STAT signaling does crucially depend on the stimulus and the involved receptor system.

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