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Involvement of Tyrosine Phosphatase PTP1D in the Inhibition of Interleukin-6-Induced Stat3 Signaling by α-Thrombin

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We previously demonstrated that exposure of CCL39 lung fibroblasts to α-thrombin inhibits interleukin-6 (IL-6)-induced tyrosine phosphorylation of Stat3 (signal transducers and activators of transcription 3) via activation of mitogen-activated protein (MAP) kinase kinase 1 [Bhat *et al.* (1998) *Arch. Biochem. Biophys.* 350, 307–314]. In this study, using CCL39/MRC-5 cells, we investigated if additional signaling intermediates are involved in α-thrombin's inhibitory effects on IL-6-induced Stat3 signaling. We also determined if α-thrombin inhibits oncostatin M (OSM)-induced Stat3/Stat1, and interferon-γ (IFN-γ)-induced Stat1 tyrosine phosphorylation. We demonstrate that, although both IL-6 and OSM belong to the same cytokine family, α-thrombin inhibited only the IL-6-induced Stat3 tyrosine phosphorylation. The tyrosine phosphatase PTP1D coprecipitated with Stat3 from α-thrombin + IL-6, but not from α-thrombin + OSM-treated cells. Pretreatment of cells with a phosphatase inhibitor reversed the inhibitory actions of α-thrombin, suggesting a role for PTP1D in α-thrombin-mediated inhibition of IL-6-induced Stat3 signaling. Interestingly, α-thrombin failed to inhibit OSM- and IFN-γ-induced Stat1 tyrosine phosphorylation. Cytokine-specific inhibition of the Stat3 signaling involving MAP kinase kinase 1 and PTP1D by α-thrombin may play an important role in regulation of gene expression. © 2001 Academic Press

Key Words: **interleukin-6; oncostatin M; tyrsosine phosphatases; signal transduction; gene expression.**

The interleukin-6 (IL-6) cytokine family members activate the JAK-STAT (Janus kinase-signal transducers and activators of transcription) pathway to exert diverse biological responses in many cell types (1, 2). These include growth stimulation, growth inhibition, cell differentiation and acute phase protein synthesis (3). Some of the genes activated by IL-6 family members are: c-*fos*, α_2-macroglobulin, intercellular adhesion molecule-1, tissue inhibitor of metalloproteinases-1, gp130 and FcγR1 (4–6). The six IL-6 family members identified to date are IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), interleukin-11, and cardiotrophin-1. They share significant similarity in amino acid sequence and secondary structure (1, 2). These cytokines utilize the transmembrane protein gp130 as a common subunit in combination with cytokine specific receptor subunits for signal transduction (2). Cytokine binding to these receptors induces either homodimerization of gp130, or heterodimerization of gp130 with cytokine-specific receptors (1, 2). Receptor/gp130 dimerization activates JAK kinases, which in turn causes tyrosine phosphorylation (activation) of Stat3. However, in some cases, depending upon cell types, activation of Stat3 and Stat1 has been reported (1). Activated STATs dimerize and translocate to the nucleus to induce gene transcription (7).

In a previous study, using CCL39 (hamster lung fibroblast cell line) cells, we showed that α-thrombin inhibits Stat3 signaling induced by IL-6, LIF and CNTF, via activation of MAP kinase kinase 1 (MAPKK1) (8). This suggested that the MAP kinase pathway was involved in α-thrombin's inhibitory effect. Inhibition of the IL-6 signaling by α-thrombin was also observed in the human lung fibroblast cell line, MRC-5 (8). In the present study, we addressed the question of whether additional signaling intermediates are involved in α-thrombin mediated inhibition. Here, we demonstrate that the protein tyrosine phosphatase, PTP1D coprecipitates with Stat3 from α-thrombin + IL-6-treated cell extracts, suggesting that this signaling intermediate may be involved in α-thrombin's inhibitory effect. Interestingly, in contrast to the potent inhibition of the IL-6-induced response, α-thrombin failed to inhibit OSM-induced Stat3/Stat1, and IFN-γ-induced Stat1 tyrosine phosphorylation. We discuss

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the differential effects of α-thrombin on IL-6, OSM and IFN-γ-induced signaling response in CCL39/MRC-5 cells.

MATERIALS AND METHODS

Reagents. Tissue culture flasks, fetal bovine serum, cell culture media, antibiotics and human recombinant IL-6 were purchased from Life Technologies (Gaithersburg, MD). Human OSM and IFN-γ were purchased from R & D systems (Minneapolis, MN). Phospho-specific anti-Stat3 antibody (specific for the detection of tyrosine phosphorylated Stat3), phosphospecific anti-Stat1 antibody (specific for the detection of tyrosine phosphorylated Stat1) and anti-PTP1D antibody were purchased from Upstate Biotechnology Inc (Lake Placid, NY). Polyclonal anti-Stat3 antibody (detects both phosphorylated and non-phosphorylated Stat3) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals such as α-thrombin and sodium orthovanadate were purchased from Sigma (St. Louis, MO).

Cell culture. CCL39 (hamster lung fibroblast) cells and MRC-5 (human lung fibroblast) cells were obtained from American Type Culture Collection. Cells were grown as previously described (8) in RPMI medium containing 10% heat inactivated fetal bovine serum at 37°C for 24–48 h. Cells were serum starved for 12 h before the addition of various agents.

Total cell extraction, immunoprecipitation and Western blots. Cells were treated with various agents for the indicated times and washed in phosphate buffered saline. Cell lysis, immunoprecipitation and Western blots were performed as previously described (8).

Electrophoretic mobility shift assays. This was performed as previously described, using ^{32}P-labeled SIE (8). The sequence of the SIE DNA strand was 5′CAGTTCCCGTCAATC-3′.

RESULTS

α-Thrombin Inhibits IL-6, but Not OSM-Induced Stat3 Tyrosine Phosphorylation

Treatment of CCL39 cells with α-thrombin potently inhibits IL-6-induced Stat3 tyrosine phosphorylation (8). Since IL-6 and OSM belong to the same cytokine family (1), we determined if α-thrombin would inhibit the OSM-induced Stat3 tyrosine phosphorylation. For this, cells were left untreated, or treated with OSM alone, or first with α-thrombin and then with OSM. As a control for α-thrombin's inhibitory effect, we also treated cells with IL-6 alone, or first with α-thrombin and then with IL-6. Cell lysates were prepared and immunoblotted with an antibody specific for the detection of tyrosine phosphorylated Stat3 protein (anti-phospho-Stat3 antibody). Figure 1A demonstrates that both IL-6 and OSM induce Stat3 tyrosine phosphorylation (lanes 3 and 4), whereas pretreatment of cells with α-thrombin inhibits only IL-6 (lane 5), but not OSM-induced (lane 6) tyrosine phosphorylation. When the samples were immunoblotted with anti-Stat3 antibody (detects both phosphorylated and non-phosphorylated Stat3), we observed similar amounts of Stat3 protein in all lanes (Fig. 1B) (loading control).

In serum starved cells, Stat3 is present in the cytoplasm, but not in the nucleus (1). Upon cytokine stimulation, tyrosine phosphorylated Stat3 forms a dimer in the cytoplasm, translocates to the nucleus, and binds to specific promoter DNA sequences to induce gene transcription. Tyrosine phosphorylated Stat3 also has the ability to bind *sis*-inducing element (SIE) in the promoter of the proto-oncogene, *c-fos* to form a DNA-protein complex referred to as SIF (*sis*-inducing factor) (9)

To confirm the results of Fig. 1, we performed electrophoretic mobility shift assays for nuclear extracts treated with cytokines alone, or first treated with α-thrombin and then with cytokines. Figure 1C demonstrates that α-thrombin inhibits only IL-6, but not OSM-induced SIF activity. Consistent with this, Stat3 was not detected in cells treated with α-thrombin + IL-6 (Fig. 1D, lane 5); however it was present in the nuclear extracts from cells treated with α-thrombin and OSM (lane 6). These results further confirm that α-thrombin selectively inhibits IL-6, but not OSM-induced Stat3 signaling.

Stat1 Coprecipitates with Stat3 in OSM-Treated Cells

We next sought to determine the reason for the differential effects of α-thrombin on IL-6 and OSM-induced Stat3 tyrosine phosphorylation. We observed in CCL39 cells that, in contrast to IL-6 which activated only Stat3, OSM induced tyrosine phosphorylation of Stat3 and Stat1 in equal amounts (data not shown). These differences in STAT member activation would cause the formation of only Stat3:Stat3 homodimers in IL-6-treated cells; whereas, in OSM-treated cells, three types of dimers would be formed: (a) Stat3:Stat3 homodimer; (b) Stat1:Stat1 homodimer; (c) Stat3:Stat1 heterodimer (1). We hypothesized that: (a) exposure of CCL39 cells to α-thrombin activates a tyrosine phosphatase, and that this phosphatase may selectively dephosphorylate the Stat3 in Stat3:Stat3 homodimer in α-thrombin + IL-6-treated cells; (b) Stat3 in Stat3:Stat1 heterodimer may be resistant to the action of this tyrosine phosphatase in α-thrombin + OSM-treated cells. To address this, we first determined if Stat1 coprecipitates with Stat3 in extracts from IL-6 or OSM-treated cells. Cells were left untreated, or treated with α-thrombin alone, or IL-6 alone, or OSM alone, or first with α-thrombin and then with IL-6 or OSM. Cell lysates were immunoprecipitated with anti-Stat3 antibody, immunocomplexes immunoblotted with phospho-specific Stat1 antibody (detects only tyrosine phosphorylated Stat1 protein). Figure 2A demonstrates that tyrosine phosphorylated Stat1 coprecipitates with Stat3 in OSM (lane 4), and α-thrombin + OSM (lane 6)-treated cells. Coprecipitation of Stat1 with Stat3 was not detected with IL-6 alone (lane 3), or α-thrombin + IL-6 (lane 5)-treated cells. This suggests that Stat3 has the potential to form a heterodimer with Stat1 in OSM-treated CCL39 cells. Reprobing the blot

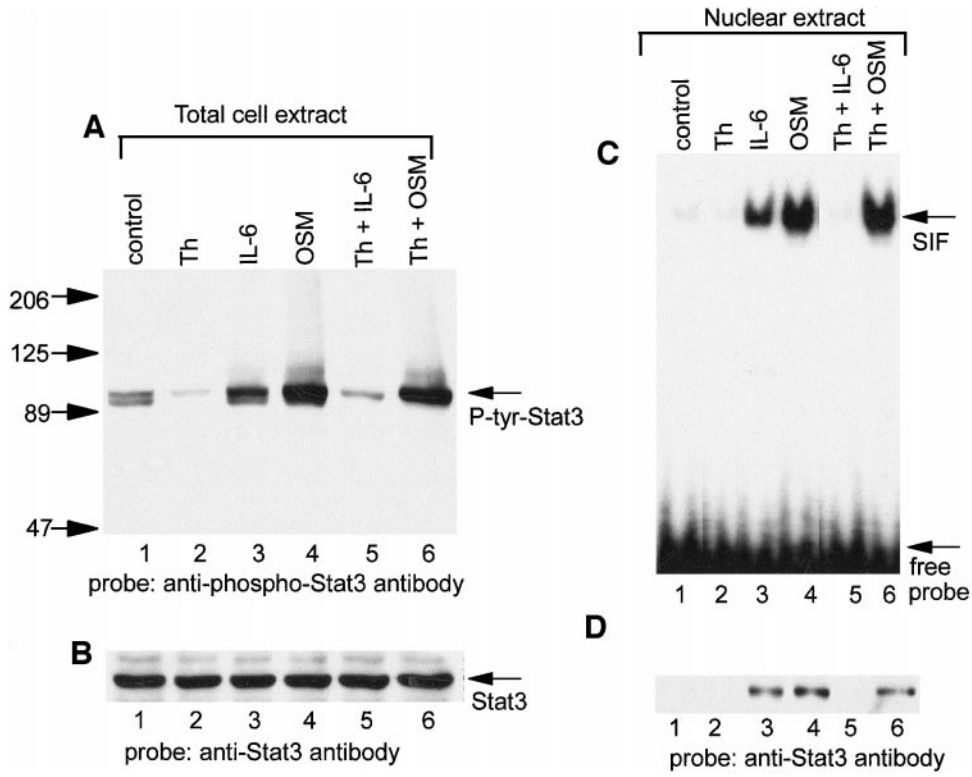

FIG. 1. α-Thrombin inhibits IL-6, but not OSM-induced Stat3 tyrosine phosphorylation. (A) Western blot probed with phospho-specific anti-Stat3 antibody. Serum-starved cells were left untreated (lane 1) or treated with α-thrombin (Th) alone (0. 4 units/ml) for 40 min (lane 2), or IL-6 alone (20 ng/ml) for 15 min (lane 3), or OSM alone (10 ng/ml) for 15 min (lane 4), or first with α-thrombin for 25 min and then with IL-6 for 15 min (lane 5), or first with α-thrombin for 25 min and then with OSM for 15 min (lane 6). Cell lysates were prepared, immunocomplexes run on an 8% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody specific for the detection of tyrosine phosphorylated Stat3 protein (anti-phospho-Stat3 antibody). (B) The blot in A was stripped and reprobed with anti-Stat3 antibody (detects both phosphorylated and non phosphorylated Stat3 protein). (C) α-Thrombin inhibits IL-6, but not OSM-induced SIF activity. Nuclear extracts were prepared from untreated, or cells treated with α-thrombin alone, or cytokine alone or first with α-thrombin followed by cytokines. Treatment conditions for C were identical to those in A. Nuclear extracts were incubated with ^{32}P-labeled SIE and analyzed in an electrophoretic mobility shift assay. (D) The samples of 1C were immunoblotted with anti-Stat3 antibody. P-tyr-Stat3: tyrosine phosphorylated Stat3; SIF, *sis*-inducing factor.

showed similar amount of Stat3 protein in all the lanes (Fig. 2B).

The Protein Tyrosine Phosphatase 1D (PTP1D) Coprecipitates with Stat3 in α-Thrombin + IL-6-Treated Cells

Previous reports by other investigators have shown that exposure of PS200 cells (a derivative of CCL39 cells) to α-thrombin induces tyrosine phosphorylation of PTP1D (10). To establish a role for this phosphatase in α-thrombin mediated inhibition, we determined if PTP1D preferentially coprecipitates with Stat3 in extracts from cells treated with α-thrombin + IL-6 and compared this to PTP1D precipitation in α-thrombin + OSM-treated cells. For this, the samples of Fig. 2A were immunoprecipitated with anti-Stat3 antibody and immunoblotted with anti-PTP1D antibody. Figure 2C demonstrates that PTP1D coprecipitates with Stat3 in cells treated with α-thrombin alone (lane 2), or α-thrombin + IL-6 (lane 5). Coprecipitation of PTP1D was minimal in cells treated with α-thrombin + OSM (lane 6). This suggests that PTP1D may associate with Stat3:Stat3 homodimer to cause dephosphorylation in cells treated with α-thrombin + IL-6.

Pretreatment of Cells with Sodium Orthovanadate Prevents α-Thrombin's Inhibitory Effect on IL-6-Induced Stat3 Tyrosine Phosphorylation

To further establish a role for PTP1D in α-thrombin's inhibition of the IL-6-induced Stat3 response, we determined if pretreatment of the cells with sodium orthovanadate, a inhibitor of tyrosine phosphatases (11), would prevent the inhibitory action of α-thrombin on IL-6-induced Stat3 tyrosine phosphorylation. For this, cells were left untreated, or treated with sodium orthovanadate alone, or IL-6 alone, or first with α-thrombin and then with IL-6, or first with sodium orthovanadate followed by α-thrombin and IL-6. Cell

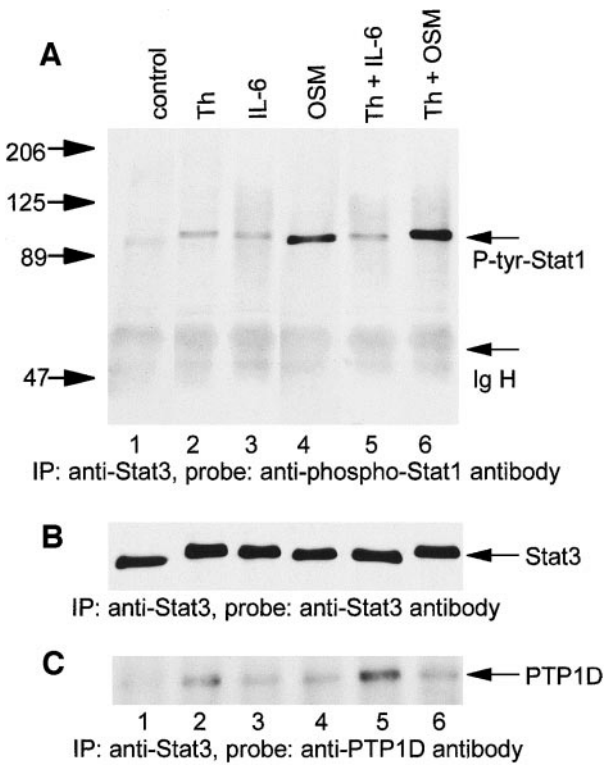

FIG. 2. Stat1 coprecipitates with Stat3 in extracts from α-thrombin + IL-6-treated cells (A); and PTP1D coprecipitates with Stat3 in extracts from α-thrombin + OSM-treated cells (C). Treatment conditions were identical to those in Fig. 1A. For A, cell lysates were immunoprecipitated with anti-Stat3 antibody (this antibody will immunoprecipitate both phosphorylated and nonphosphorylated Stat3), immunocomplexes run on an 8% SDS–polyacrylamide gel, and immunoblotted with phospho-specific anti-Stat1 antibody (this antibody is specific for tyrosine phosphorylated Stat1 protein). (B) The blot in A was stripped and reprobed with anti-Stat3 antibody (same antibody that was used for immunoprecipitation). (C) The blot in B was stripped and reprobed with anti-PTP1D antibody. Ig H, immunoglobulin heavy chain. P-tyr-Stat1: tyrosine phosphorylated Stat1.

lysates were prepared and immunoblotted with phosphospecific anti-Stat3 antibody. Figure 3A demonstrates that sodium orthovanadate treatment alone slightly enhanced tyrosine phosphorylation of Stat3 (lane 2) compared to the control (lane 1). However, pretreatment of cells with the phosphatase inhibitor significantly reversed the inhibition of IL-6-induced Stat3 tyrosine phosphorylation by α-thrombin (compare lanes 1, 3, 4 and 6). Reprobing the blot with anti-Stat3 antibody showed a similar amount of Stat3 in all lanes (Fig. 3B).

Pretreatment of MRC-5 Cells with IFN-γ Reverses the Inhibitory Action of α-Thrombin on IL-6-Induced Stat3 Tyrosine Phosphorylation

We next determined if Stat3 in Stat3:Stat1 heterodimer would be resistant to the inhibitory effect of α-thrombin. To address this, we determined if treatment of cells with IFN-γ would reverse the inhibitory action of α-thrombin on IL-6-induced Stat3 tyrosine phosphorylation. IFN-γ has been shown to induce tyrosine phosphorylation of Stat1 (1, 2). We hypothesized that simultaneous treatment of cells with IFN-γ and IL-6 would induce tyrosine phosphorylation of both Stat1 and Stat3. We argued that because IL-6-induced Stat3 would form a heterodimer with IFN-γ-induced Stat1 in these cells, α-thrombin should fail to inhibit IL-6-induced Stat3 tyrosine phosphorylation. Because IFN-γ is highly species specific and hamster IFN-γ is commercially not available, we used the human lung fibroblast cell line, MRC-5, instead of CCL39 in this experiment. MRC-5 cells express abundant receptors for α-thrombin, IL-6 and IFN-γ (8). Cells were left untreated, or treated with IFN-γ alone, or IL-6 alone, or first with α-thrombin and then with IL-6, or first with α-thrombin and then with IFN-γ, or initially with α-thrombin followed by IFN-γ and IL-6. Cell lysates were prepared and immunoblotted with phospho-specific anti-Stat3 antibody. Figure 4A demonstrates

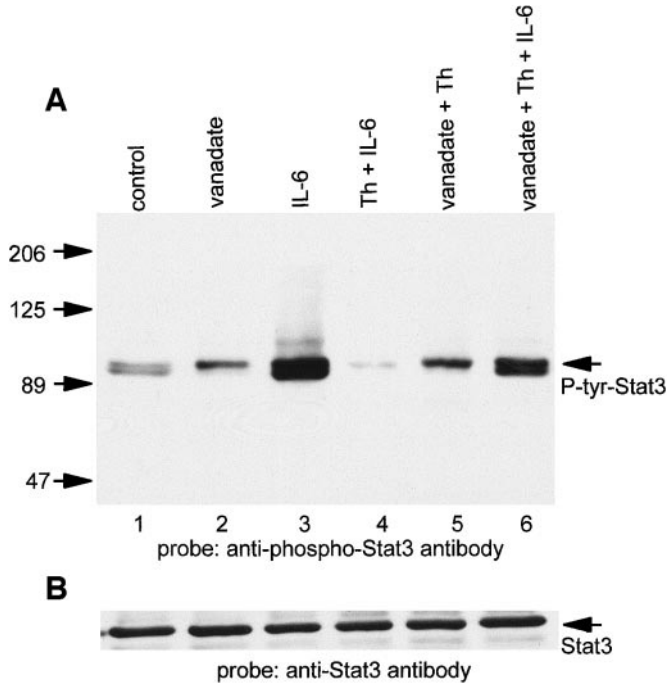

FIG. 3. Inhibition of the tyrosine phosphatase by treatment with sodium orthovanadate reverses the inhibitory action of α-thrombin. (A) Serum-starved cells were left untreated (lane 1), or treated with sodium orthovanadate (1 mM) alone for 2 h (lane 2), or IL-6 alone (20 ng/ml) for 15 min (lane 3), or first with α-thrombin for 25 min and then with IL-6 for 15 min (lane 4), or first with sodium orthovanadate for 1 h 20 min and then with α-thrombin for 40 min (lane 5), or first pretreated with sodium orthovanadate for 1 h 20 min and then with α-thrombin for 25 min followed by IL-6 for 15 min (lane 6). Cell lysates were prepared and immunoblotted with anti-phospho-Stat3 antibody (specific for tyrosine phosphorylated Stat3). (B) The blot in A was stripped and reprobed with anti-stat3 antibody.

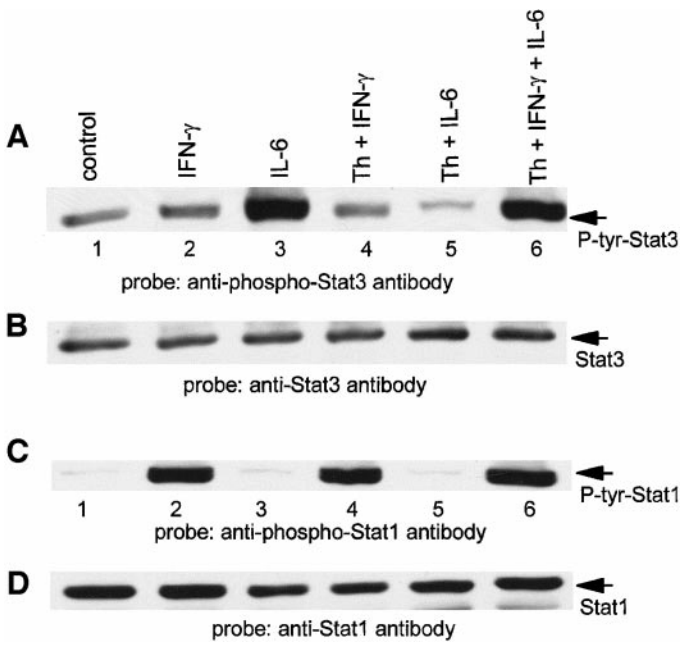

FIG. 4. Pretreatment with IFN-γ reverses the α-thrombin's inhibition of IL-6-induced Stat3 tyrosine phosphorylation in MRC-5 cells. (A) Serum-starved cells were left untreated (lane 1), or treated with IFN-γ (5 ng/ml) alone (lane 2), or IL-6 (20 ng/ml) (lane 3), or first with α-thrombin (0.4 units/ml) for 25 min and then with IFN-γ for 15 min (lane 4), or first with α-thrombin for 25 min and then with IL-6 for 15 min (lane 5), or first pretreated with α-thrombin for 25 min followed by IFN-γ and IL-6 for 15 min (lane 6). Cell lysates were prepared and run on an 8% SDS–polyacrylamide gel and immunoblotted with anti-phospho-Stat3 antibody (specific for tyrosine phosphorylated Stat3). (B) The blot in *A* was stripped and reprobed with anti-Stat3 antibody. (detects both phosphorylated and nonphosphorylated Stat13protein). (C) The sample of A was run on a SDS–polyacrylamide gel and immunoblotted with anti-phospho-Stat1 antibody (specific for tyrosine phosphorylated Stat1). (D) The blot in C was stripped and reprobed with anti-stat1 antibody (detects both phosphorylated and nonphosphorylated Stat1 protein). P-tyr-Stat3, tyrosine phosphorylated Stat3; P-tyr-Stat1, tyrosine phosphorylated Stat1. Th, α-thrombin.

that, only IL-6 (lane 3), but not IFN-γ (lane 2) induced tyrosine phosphorylation of Stat3 in MRC-5 cells. More importantly, pretreatment of cells with IFN-γ significantly reversed α-thrombin's inhibition of IL-6-induced Stat3 tyrosine phosphorylation (lane 6). Reprobing the blot with anti-Stat3 antibody (detects both phosphorylated and non-phosphorylated Stat3) showed that all lanes contain similar amount of Stat3 protein (Fig. 4B).

To demonstrate that MRC-5 cells are responsive to IFN-γ, the samples of Fig. 4A were immunoblotted with phospho-specific anti-Stat1 antibody. Figure 4C demonstrates that treatment of cells with IFN-γ potently induced Stat1 tyrosine phosphorylation (lane 2). As expected, IL-6 did not induce Stat1 tyrosine phosphorylation (lane 3). α-Thrombin failed to inhibit IFN-γ-induced Stat1 tyrosine phosphorylation (lane 4). Reprobing the blot with anti-Stat1 antibody (detects both phosphorylated and nonphosphorylated Stat1) showed similar amount of Stat1 in all lanes (Fig. 4D). This further supports the view that Stat1:Stat1 homodimers, and Stat3:Stat1 heterodimer are resistant to the inhibitory action of α-thrombin.

DISCUSSION

In this paper, we demonstrate that pretreatment of CCL39 cells with α-thrombin selectively inhibits IL-6, but not OSM-induced Stat3 tyrosine phosphorylation. In these cells, IL-6 induced activation of only the Stat3, whereas OSM induced activation of both Stat3 and Stat1. Consistent with this, Stat1 coprecipitated with Stat3 in OSM, but not in IL-6-treated cells. Stat3 immunoprecipitates from α-thrombin + IL-6-treated cells contained PTP1D, whereas no PTP1D was detected in α-thrombin + OSM-treated cells. Inhibition of the tyrosine phosphatases by pretreatment with sodium orthovanadate reversed the inhibitory effects of α-thrombin on IL-6-induced Stat3 activation. This demonstrates a role for PTP1D in α-thrombin mediated inhibition of IL-6-induced Stat3 signaling.

The three major steps in signaling by IL-6 family cytokines are: (a) binding of cytokine to their respective receptor and dimerization; (b) activation by tyrosine phosphorylation of the JAK kinases; (c) tyrosine phosphorylation of Stat3. Although IL-6 and OSM bind to distinct receptors (1, 12), both require gp130 and members of JAK family to induce tyrosine phosphorylation of STAT proteins. In CCL39 cells, IL-6 activated Stat3, and OSM activated both Stat3 and Stat1. Yet, inhibition of the Stat3 signaling by α-thrombin is observed only with respect to IL-6. α-Thrombin failed to inhibit OSM-1 and IFN-γ induced Stat1 tyrosine phosphorylation. This suggests that one site of interference by α-thrombin is likely to be at the level of Stat3 during IL-6-induced signaling. Since PTP1D coprecipitates with Stat3 from α-thrombin + IL-6-treated cells, it is likely that this tyrosine phosphatase is involved in α-thrombin mediated interference. One possibility is that the Y-705 phosphotyrosine group of Stat3 in the Stat3:Stat3 homodimer is accessible for PTP1D for interaction and dephosphorylation in α-thrombin + IL-6-treated cells. In contrast, the Y705-phosphotyrosine may not be accessible in the Stat3:Stat1 heterodimer for PTP1D actions in α-thrombin + OSM-treated cells. In a previous report, we demonstrated that α-thrombin's inhibitory effect could be partially reversed by pretreatment of cells with the MAPKK1 inhibitor, PD98059, suggesting that MAP kinase pathway is at least partially involved in α-thrombin's inhibitory action on IL-6-induced Stat3 signaling (8). The data presented in this paper suggests that PTP1D is also involved in α-thrombin's inhibitory effects. Whether these signaling intermediates (MAPKK1 or PTP1D) act independently of the other to cause inhibition of the IL-6-induced response requires further investigation.

The cross-talk between α-thrombin and IL-6 may have significance in cells/tissues which express receptors for both agents. High concentrations of α-thrombin and IL-6 have been detected in bronchoalveolar lavage fluid obtained from patients with pulmonary fibrosis, and patients suffering from adult respiratory distress syndrome (13, 14). Under these circumstances, cells in the injured lung (e.g., fibroblasts, endothelial and epithelial cells) are exposed to both α-thrombin and IL-6, and in these potentially responsive cell types, α-thrombin may regulate IL-6-induced response via inhibition of the JAK-STAT pathway.

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