Non-redundant Signal Transduction of Interleukin-6-type Cytokines

THE ADAPTER PROTEIN Shc IS SPECIFICALLY RECRUITED TO THE ONCOSTATIN M RECEPTOR*

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The common use of the cytokine receptor gp130 has served as an explanation for the extremely redundant biological activities exerted by interleukin (IL)-6-type cytokines. Indeed, hardly any differences in signal transduction initiated by these cytokines are known. In the present study, we demonstrate that oncostatin M (OSM), but not IL-6 or leukemia inhibitory factor, induces tyrosine phosphorylation of the Shc isoforms p52 and p66 and their association with Grb2. Concomitantly, OSM turns out to be a stronger activator of ERK1/2 MAPKs. Shc is recruited to the OSM receptor (OSMR), but not to gp130. Binding involves Tyr861 of the OSMR, located within a consensus binding sequence for the Shc PTB domain. Moreover, Tyr861 is essential for activation of ERK1/2 and for full activation of the α₂-macroglobulin promoter, but not for an exclusively STAT-responsive promoter. This study therefore provides evidence for differential signaling mechanisms exerted by IL-6-type cytokines.

The 28-kDa protein oncostatin M (OSM) belongs to the family of interleukin (IL)-6-type cytokines, which additionally comprises IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, cardiotoxin-1, and the recently described novel neurotrophin-1/B-cell stimulatory factor-3 (1, 2). These cytokines play an important role in hematopoiesis, inflammation, the acute phase response, bone, and heart development as well as neurogenesis. Their redundant effects can be attributed to the shared use of the common signal transducing receptor chain glycoprotein (gp) 130. gp130 is homodimerized by IL-6 and IL-11 upon binding to their ligand-specific α receptors. The other cytokines of this family trigger the heterodimerization of gp130 with the LIF receptor (LIFR) or the OSM-specific receptor (OSMR). Whereas human OSM has the capability to signal both via gp130-LIFR and gp130-OSMR heterodimers, murine OSM solely utilizes the gp130-OSMR heterodimer for signal transduction (1, 3).

OSM is involved in various biological responses. It supports the growth of AIDS-associated Kaposi's sarcoma cells (4, 5), whereas it leads to growth inhibition of various solid tumors (6). Due to its ability to induce TIMP-1 and TIMP-3, profibrotic properties have been attributed to this cytokine (7–9). Indeed, transgenic mice expressing OSM in islet β-cells develop severe fibrosis (10). In addition, OSM is suggested to play a role in the wound healing process and in attenuation of the inflammatory response (11). Compared with other IL-6-type cytokines, OSM often induces stronger effects e.g. with regard to STAT and MAPK activation, induction of protease inhibitors, or growth inhibition (11–15). The molecular basis for this phenomenon is not known.

Concerning the functional properties of the three signal transducing receptor subunits, much progress has been achieved in elucidating the signaling events initiated by the gp130 receptor chain (reviewed in Ref. 1). gp130 associates with tyrosine kinases of the Janus family (Jak1, Jak2, and Tyk2). Upon ligand binding and receptor dimerization, Jaks are activated and in turn phosphorylate gp130 on several cytoplasmic tyrosine residues, which then provide docking sites for SH2 domain-containing molecules, such as transcription factors of the STAT family (STAT3 and STAT1). Upon phosphorylation, the STATs translocate as dimers into the nucleus, where they bind to promoter regions of their specific response genes. Additionally, the tyrosine phosphatase SHP-2 becomes recruited to gp130 via tyrosine residue 759 (16). SHP-2 has been implicated in the down-regulation of gp130-mediated signals (17–19). In addition, SHP-2 serves as an adapter molecule linking cytokine receptors like gp130 to the Ras/Raf/MAPK pathway (20).

In the present study, we addressed the question whether the OSMR contributes distinguishably to signal transduction. We demonstrate that in contrast to gp130, the human OSMR does not recruit SHP-2 but utilizes another protein implicated in activation of the Ras/Raf/MAPK pathway, Shc. This protein exists in three different isoforms of 46 kDa (p46), 52 kDa (p52), and 66 kDa (p66); p46 and p52 are produced by using alternative translation initiation sites of the same transcript, whereas p66 results from a differentially spliced message (21–24). The 46- and 52-kDa isoforms are expressed ubiquitously, whereas the 66-kDa isoform is predominantly found in cells of epithelial origin. Shc contains an SH2 and a PTB domain, separated by a proline/glycine-rich collagen homology domain (CH1). The p66 isoform contains a further CH domain (CH2) at the N terminus (23, 24). The SH2 and the PTB domain of Shc are involved in phosphorylation-dependent association with membrane receptors upon stimulation of cells with a number of growth factors and cytokines (21). Moreover, the PTB domain can interact with acidic phospholipids, thereby possibly mediating membrane association (25). Shc can be phosphorylated at three different tyrosine residues, Tyr861, Tyr866, and Tyr860 (26–28). Grb2/Sos binds to Shc phosphorylated at Tyr861, which then
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leads to the activation of the Ras/Raf/MAPK pathway (26). However, She is also involved in mediation of Ras-independent signals (29, 30). Other signaling molecules able to associate with She include phosphatidylinositol 4,5-bisphosphate (31, 32) and the inositol 5-phosphatases SHIP-1 and SHIP-2 (33, 34). She proteins have been implicated in processes like endocytosis (35), cell migration (36–38), cell survival (27, 39), or mitogenesis (40–43). Moreover, p66 She is part of a signal transduction pathway that regulates stress apoptotic responses and life span in mammals (44).

In the present study, we show a clear difference in signal transduction between gp130 and the OSMR for which so far mainly shared functions have been reported (12, 45, 46). We provide comparative data showing that upon receptor activation She binds to the OSMR (and not to gp130), whereas SHP-2 only binds to gp130 (and not to the OSMR). We locate the site of Shc/OSMR interaction to tyrosine residue 861 of the OSMR.

Point mutation of this residue abrogates ERK activation and reduces the induction of an αM-promoter-driven reporter gene. This finding promises to contribute to the understanding of specific characteristics that have been attributed to OSM.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Growth Factors, and Transient Transfections—**Simian monkey kidney cells (COS-7) were maintained in Dulbecco’s modified Eagle's medium; human hepatoma cells (HepG2) were maintained in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 60 μg/ml penicillin. The human melanoma cell line A375 was maintained in RPMI medium supplemented with 5% fetal calf serum, 100 μg/ml streptomycin, and 60 mg/ml penicillin. Recombinant human IL-5 and human OSM were obtained from Cell Concepts (Umkirch, Germany), recombinant human LIF was a kind gift from Dr. N. Nicola (Walter and Elisa Hall Institute, Melbourne, Australia), and recombinant IL-6 was prepared as described (47). The specific activity was 2 × 10^7 B units/mg of protein. Soluble human IL-6 receptor (sIL-6R) was prepared in insect cells as described previously (48). Approximately 1.5 × 10^7 COS-7 cells were transiently transfected with 10–20 μg of plasmid DNA using a modified DEAE-dextran method as described (12). HepG2 cells were transfected with 14 μg of plasmid DNA using the calcium phosphate method as described (19).

**Expression Constructs—**The construction of the IL-5R chimera β/γgp130, β/γgp130-B1/2, β/OSMR, β/OSMRA, and β/OSMRA1 has been described previously (12, 49). a/gp130YFFFFF was constructed by exchanging the cDNA encoding the extracellular part of gp130YFFFFF (50) with the cDNA encoding the extracellular part of the IL-5 receptor chain (49). Two further C-terminal deletion mutants, β/OSMRA4 and β/OSMRA1, were generated by polymerase chain reaction using an antisense oligonucleotide incorporating an in-frame termination codon followed by the recognition site for BamHI. They retain 105 and 75 amino acids of the OSMR cytoplasmic tail, respectively. The point mutated constructs containing the amino acid substitution Y861F were generated by polymerase chain reaction using the respective mutated oligonucleotides with either the cDNA for β/OSMR or β/OSMRA1 as a template. The resulting products were cloned into the EcoRV/BamHI digestion expression plasmid pSVL-IL-5Rβ/OSMR, thereby generating the constructs encoding β/OSMRY861F and β/OSMRA1Y861F. The integrity of all constructs was verified by DNA sequence analyses using an ABI PRISM 310 Genetic Analyzer (PerkinElmer Life Sciences). For transfection of HepG2 cells, XhoI/BamHI fragments comprising the cDNA encoding the various receptor constructs were inserted into XhoI/BamHI-digested pCAGGS expression vector (51). Expression plasmids for Jak1 and Jak1K907E were kindly provided by Dr. I. M. Kerr (Imperial Cancer Research Fund, London).

**Cell Lysis, Immunoprecipitations, and Western Blotting—**A375 and HepG2 cells were stimulated for different periods of time with either LIF, OSM, or IL-6 in the presence of sIL-6R, which is known to act agonistically (53, 54). Interestingly, a phosphorylation of the p66 and p52 isoforms of She occurs only after stimulation with OSM but not after treatment with IL-6 or LIF as demonstrated upon immunoprecipitation of the Shc proteins from lysates. The phosphorylation was transient with a maximum reached after 5 min, which continued for 30 min. Then the proteins become dephosphorylated again (Fig. 1A, upper right panel). The p46 isoform of She could not be detected, although equally well expressed (data not shown), due to comigration with the Ig heavy chain. As shown in Fig. 1A (upper right panel), several additional tyrosine-phosphorylated proteins of high molecular mass were coimmunoprecipitated with She. One protein might represent the wild-type OSMR. Since there are no antibodies available for Western blot analysis, we are presently unable to verify this prediction. The phosphoproteins could not be identified as Sos, ErbB2, or members of the Janus kinase family (data not shown), which have either been implicated in signal transduction downstream of She or coprecipitated with She (21, 55, 56). Future studies will aim at the identification of the coimmunoprecipitated proteins.

Several reports have demonstrated that She is able to bind Grb2 after tyrosine phosphorylation of Tyr317, thereby linking cytokine or growth factor receptors to the activation of the Ras/Raf/MAPK pathway. Indeed, we could demonstrate a transient coimmunoprecipitation of Grb2 with She that parallels
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Shc phosphorylation (Fig. 1A, lower right panel). The lack of Shc phosphorylation in response to IL-6 and LIF was not due to limited amounts of cytokines applied, since no Shc phosphorylation was detectable after increasing the concentration of IL-6 up to 200 ng/ml and of LIF up to 500 ng/ml (data not shown). In contrast, an OSM concentration of 10 ng/ml was sufficient to induce Shc phosphorylation (not shown). However, all three cytokines induced tyrosine phosphorylation of STAT3, indicating that the Jak/STAT pathway is activated by IL-6, LIF, and OSM (Fig. 1B, top panel). Analyzing the activation of the Ras/Raf/ERK1/2 pathway as an additional signaling route activated by IL-6-type cytokines showed that OSM has a much higher potential to stimulate ERK1/2 phosphorylation compared with IL-6 or LIF (Fig. 1B, third panel). This suggests that Shc may play a key role in bridging the gp130-OSMR complex to the Ras/Raf/ERK1/2 pathway.

**OSM Mediates Shc Activation via the OSMR-gp130 Heterodimer**—Human OSM is able to transduce signals via gp130-LIFR and gp130-OSMR complexes. The experiments performed in HepG2 cells showed that stimulation with OSM but not with LIF mediates Shc activation, indicating the involvement of the OSM-specific receptor in phosphorylation of Shc. To verify this assumption, we took advantage of A375 melanoma cells expressing only gp130 and the OSMR, but not the LIFR (45, 57). Therefore, all signals occurring after OSM stimulation must be transduced via the gp130-OSMR heterodimer. Indeed, Shc p66 and p52 isoforms were also phosphorylated in A375 cells in response to OSM but not to IL-6 (Fig. 2, upper right panel). We conclude that the phosphorylation of Shc can be attributed to the OSM-specific receptor.

**Shc Binds to the Cytoplasmic Part of the OSMR Receptor between Akm236 and Se256**—Since Shc phosphorylation turned out to be an OSMR-dependent event, we investigated whether the OSMR is able to recruit Shc. The studies were performed in COS-7 cells, since these cells can be transfected efficiently and yield high levels of heterologously expressed proteins. To analyze mutated receptors independently of endogenous receptors, we took advantage of receptor chimeras that have been described previously (12, 49): the transmembrane and intracellular parts of the OSMR or gp130, respectively, were fused to the extracellular region of the IL-5 receptor β-chain (Fig. 3). Moreover, this approach enabled us to use the same precipitating antibody (α-IL5Rβ) for a comparative side-by-side analysis of OSMR and gp130. We overexpressed a Janus kinase (Jak1) along with the IL-5Rβ chimeras in COS-7 cells. As demonstrated by others (58), thereby a stimulation-independent receptor phosphorylation can be achieved (not shown). After lysis of the cells and immunoprecipitation of the receptor chimeras, we observed binding of Shc p66 and p52 to chimeras containing the intracellular part of the OSMR but not to chimeras containing gp130 cytoplasmic sequences (Fig. 4, upper left panel) or LIFR sequences (not shown). In contrast, SHP-2 bound exclusively to β/gp130 but neither to truncated β/gp130-B1/2, which lacks the SHP-2 recruitment site Tyr759 (Fig. 4, second left panel, lanes 1 and 2), nor to β/OSMR (Fig. 4, second left panel, lane 3). When a kinase-negative mutant of Jak1 (JAK1K907E) was coexpressed with the receptors, no Shc could be coprecipitated with β/OSMR, indicating that it becomes recruited to the receptor in a phosphotyrosine-dependent manner (data not shown). Thus, we demonstrate a major difference between gp130 and OSMR in engaging downstream signaling molecules; SHP-2 binds to activated gp130 (but not to OSMR), and Shc binds to activated OSMR (but not to gp130).

To delineate the Shc binding region, we generated C-terminal deletions of the OSMR. OSMRΔ3, OSMRΔ4, and OSMRΔ5 retain 153, 105, and 75 cytoplasmic amino acids, respectively (Fig. 3). The truncated IL-5Rβ chimeras were coexpressed with Jak1 in COS-7 cells. As demonstrated previously (12, 46), truncation of the OSMR yielded higher expression levels compared with the full-length OSMR (Fig. 4, lower panels, lanes 4–6 versus lane 2). While β/OSMRΔ3 and β/OSMRΔ4 were still able to bind Shc, β/OSMRΔ5 lost the ability to precipitate Shc from
cell lysates (Fig. 4, upper left panel, lanes 4–6), although association with Jak1 was comparable for all OSMR constructs (Fig. 4, third left panel). These findings indicate that the region between Asn836 and Ser866 of the OSM-specific receptor contains the sequence responsible for recruitment of Shc.

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Exchange of OSMR Tyr861 with phenylalanine led to a significant decrease in luciferase activity (Fig. 6B). The Y861F mutation did not affect receptor surface expression (not shown). These data indicate that one pathway activated after recruitment of Shc to the OSMR is the Ras/Raf/MAPK pathway. As we have previously demonstrated, STAT activation can be mediated by the OSMR even in the absence of gp130 STAT recruitment sites (12). Dimerization of OSMR with the OSR receptor chimera Y861F with β/OSMRΔ1 constructs in COS-7 cells, the MAPKs ERK1 and ERK2 were activated upon coexpression of α/gp130YFFFFF and the β/OSMRΔ1 constructs. These data indicate that the Jak/STAT pathway remains unaffected by this mutation.

**Activation of ERK1/2 through the OSMR Is Dependent on Tyr861**—As shown in Fig. 1B, OSM has a higher potential to activate the MAPKs ERK1/2 than IL-6 or LIF. After having shown that the adapter protein Shc is recruited to Tyr861 of the OSMR (Fig. 5), we were curious to see whether ERK activation is dependent on this tyrosine residue. To mimic signal transduction of a gp130-OSMR heterodimer, we used receptor chimeras based on the heterodimeric IL-5R, which enabled us to force the dimerization of two different cytoplasmic tails (12, 49). To avoid gp130-mediated ERK activation (via Tyr705), we took advantage of the mutant α/gp130YFFFFF, which is a chimera of the IL-5Ra extracellular region and the gp130 cytoplasmic part with all tyrosine residues downstream of box 2 replaced by phenylalanine (see scheme in Fig. 3). To yield higher surface expression of the chimeras (see Ref. 12), we introduced the point mutation Y861F into the receptor chimera β/OSMRΔ1, which lacks the C-terminal 27 amino acids. After coexpression of α/gp130YFFFFF and the β/OSMRΔ1 constructs in COS-7 cells, the MAPKs ERK1 and ERK2 were activated when α/gp130YFFFFF was dimerized with β/OSMRΔ1, but not after IL-5 stimulation of the α/gp130YFFFFF and β/OSMRΔ1Y861F heterodimer (Fig. 6A, upper left panel). The Y861F mutation did not affect receptor surface expression (not shown). These data indicate that one pathway activated after recruitment of Shc to the OSMR is the Ras/Raf/MAPK pathway. As we have previously demonstrated, STAT activation can be mediated by the OSMR even in the absence of gp130 STAT recruitment sites (Fig. 6A, upper right panel (12)). Dimerization of α/gp130YFFFFF with β/OSMRΔ1Y861F still led to tyrosine phosphorylation of STAT3, indicating that the Jak/STAT pathway remains unaffected by this mutation.

**DISCUSSION**

gp130, the common receptor chain of IL-6-type cytokine receptor complexes, has been the subject of extensive research...
during the past years. Extension of the studies to the LIFR and, more recently, to the OSMR mainly revealed shared properties of the three signal-transducing receptor chains, i.e., their ability to activate Jaks and to recruit transcription factors of the STAT family (12, 46, 61). Comparative studies have been hampered by the fact that the receptor components are expressed at variable levels in different cells. Thus, it is difficult to distinguish signaling differences of the receptor complexes from mere quantitative effects caused by differential expression patterns of the proteins. Moreover, human OSM has the capability to signal both via the gp130-LIFR complex and via the gp130-OSMR complex (15, 45), which complicates the assignment of specific signaling capacities to either receptor even more. Minor differences that have been noted include stronger activation of STAT5 by LIFR-gp130 and OSMR-gp130 heterodimers in comparison with the gp130 homodimer (45, 46) and often stronger signals of OSM compared with the ones induced by IL-6 or LIF (11–14). Our finding that the OSMR, unlike gp130 and the LIFR, recruits Shc as a downstream signaling molecule therefore reveals a qualitative signaling difference of the OSMR and gp130 and thereby significantly contributes to the knowledge about specific properties of the OSMR.

**Shc Associates with the OSM Receptor via Tyrosine Residue 861, Which Is Located within a Consensus Binding Site for the Shc PTB Domain**—One major finding of this study is that Shc binds to the tyrosine-phosphorylated cytoplasmic part of the human OSM receptor. Shc proteins have been described to associate with growth factor receptors by virtue of their PTB and/or SH2 domains in a phosphorylation-dependent manner. Using receptor truncations, we were able to localize the region responsible for Shc binding between amino acids 836 and 866 of the OSMR. The amino acids preceding the tyrosine residue Tyr 861 (FENLTY) match the consensus binding sequence of the Shc PTB domain \( (D/E)NXXpY \) (59). Replacing Tyr 861 with phenylalanine revealed that it is a major binding site (Fig. 5), since its mutation completely abrogated the recruitment of Shc. Similarly, Shc binding to other receptors like epidermal growth factor receptor (62–64), GM-CSFR \( \beta_{c} \) (41), or TrkA (65) occurs primarily via the PTB domain.

**Mutation of Tyr861 Attenuates OSMR-mediated Phosphorylation of ERKs and Activation of an \( \alpha_{M} \) Promoter**—She has been proposed to function upstream of the Ras/Raf/MAPK pathway. After recruitment to receptor chains, phosphorylation of She leads to its association with Grb2. Thereby, the guanine nucleotide exchange factor Sos is recruited to the membrane and can activate Ras (21, 66). The tyrosine phosphatase SHP-2 is another adapter protein that binds to receptor and, once phosphorylated, provides a docking site for Grb2/Sos (67). The link to the Ras/Raf/MAPK pathway via SHP-2 has been documented for gp130 (20, 68) and the LIFR (69). In accordance with this, we also found an association of SHP-2 with the tyrosine-phosphorylated cytoplasmic part of gp130 but
not, under the same conditions, with the β/OSMR chimera. Instead, Shc proteins were observed to interact with β/OSMR but not with β/gp130 (Fig. 4).

After having shown that mutation of Tyr861 abrogates the potential of OSMR to associate with Shc, we investigated the effect this mutation has on ERK activation. We coexpressed the β/OSMR constructs with the IL-5R YFFFFF chimera in which all tyrosine residues downstream of box 2 have been replaced by phenylalanine. Thereby, we prevented ERK activation by SHP-2, which associates with Tyr759 of gp130 (16, 19, 20). Mutation of Tyr861 in the OSMR leads to a strong reduction of OSMR-mediated ERK1/2 activation. This indicates that recruitment of Shc to the OSMR contributes to activation of the Ras/Raf/MAPK pathway within an OSMR-gp130 heterodimeric receptor complex.

Both the p66 and the p52 isoforms of Shc were found to associate with the OSMR in COS-7 cells. In HepG2 and A375 cells, these two forms of Shc become phosphorylated upon OSM stimulation. Unfortunately, p46 phosphorylation could not be demonstrated due to its comigration with the Ig heavy chain. It is likely that ERK activation is mediated by the p52/p46 isoforms, since p66 has no stimulatory or even an inhibitory effect on the activation of the Ras/Raf/MAPK pathway (24, 44).

Interestingly, Shc binding to the OSMR is not involved in the tyrosine phosphorylation of STAT proteins. As demonstrated in Fig. 6, both β/OSMR constructs were able to induce STAT3 tyrosine phosphorylation as well as induction of the STAT-responsive SIE-tk promoter to a similar extent, irrespective of their ability to associate with Shc. This further indicates that the MAPKs ERK1/2 are not involved in STAT3 activation.

However, cytokine-mediated activation of the α2M promoter-luciferase construct was reduced when the OSMR mutant with the point mutation at position 861 was used. Thus, the Shc recruitment site in the OSMR contributes to the induction of this acute phase protein gene, which contains binding sites for both STAT3 and the transcription factor complex AP-1, which is known to be a target of the Ras/Raf/MAPK pathway (60).

Shc Is Specifically Phosphorylated upon Stimulation with OSM, but Not with IL-6 or LIF—OSM, IL-6, and LIF belong to the same family of cytokines. Shared as well as distinct biological properties have been attributed to these three cytokines. In this study, we provide evidence for a qualitative difference in signal transduction: OSM has a much stronger potential to activate Shc than IL-6 or LIF. Interestingly, IL-6-dependent activation of Shc could be demonstrated in certain cells of B cell origin (56, 70–73). In rat hepatoma cells, however, Shc was not found to be involved in IL-6-dependent signal transduction. Instead, ERK1/2 activation by gp130 could be attributed to SHP-2 (68). Although we cannot rule out cell type-specific differences in Shc recruitment by gp130, our side-by-side study indicates that the potential of gp130 to bind Shc is negligible in comparison with that of the OSMR.

Since the Shc proteins are involved in several signaling pathways, they might represent a key control point at which signal transduction can be modulated. Shc is implicated in various cellular functions such as endocytosis, migration, mitogenesis, and cell survival. Moreover, p66 Shc as a sensor of oxidative stress has recently been found to be involved in the determination of the life span of mice (44). These examples of the wide ranging functions of Shc point to important roles Shc may also play in OSM-induced signal transduction, thereby...
allowing new views of existing specific responses elicited by this cytokine.

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Note Added in Proof—Involvement of Shc in OSM-mediated signal transduction (in rat hepatoma cells) has also been described in a recent publication by Wang, Y., Robledo, O., Kinzie, E., Blanchard, F., Richards, C., Miyajima, A., and Bauman, H. (2000) J. Biol. Chem. 275, 25273–25285.

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