BOX 2 REGION OF THE ONCOSTATIN M RECEPTOR DETERMINES SPECIFICITY FOR RECRUITMENT OF JANUS KINASES AND STAT5 ACTIVATION

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Running title: Evolutionary distinct mechanisms to activate STAT5 by OSM

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Human and murine Oncostatin M (OSM) induce their bioactivities through a heterodimeric receptor complex consisting of gp130 and the OSM receptor (OSMR), which initiates a signaling pathway involving Janus kinases (JAKs) and transcription factors of the signal transducers and activators of transcription (STAT) family. In contrast to the signal transducing receptor subunit gp130, the OSMR allows strong activation of STAT5B. The underlying molecular mechanism, however, remained unclear. Here we demonstrate that the human and murine OSM receptors use distinct mechanisms for STAT5B activation. The human receptor contains a STAT5B recruiting tyrosine motif (Y837/Y839) C-terminal to the box1/2 region which is absent in the mouse receptor. In contrast, the murine receptor initiates STAT5 activation directly via the receptor bound gp130, the OSMR allows strong activation of STAT5B. The underlying molecular mechanism, however, remained unclear. Here we demonstrate that the human and murine OSM receptors use distinct mechanisms for STAT5B activation. The human receptor contains a STAT5B recruiting tyrosine motif (Y837/Y839) C-terminal to the box1/2 region which is absent in the mouse receptor. In contrast, the murine receptor initiates STAT5 activation directly via the receptor bound Janus kinases. Intriguingly, the murine receptor preferentially recruits JAK2 while the human receptor seems to have a higher affinity for JAK1. We identify a single amino acid (F820) in the human receptor which is responsible for this preference. Exchange by the murine counterpart (C815) allows recruitment of JAK2 by the human receptor and consequently activation of STAT5B independently of receptor tyrosine motifs. STAT5B interacts directly with JAK2 only in response to activation of the murine OSMR or the mutated human OSMR. Additionally, we show that OSM-induced STAT1 phosphorylation occurs independently of receptor tyrosine motifs and is mediated directly by Janus kinases while the two C-terminally located tyrosine residues Y917/Y945 of the OSMR are crucial for STAT3 activation.

Oncostatin M (OSM) belongs to the family of interleukin (IL)-6-type cytokines, comprising so far nine members: IL-6, IL-11, IL-27, OSM, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC) and neuropoietin (NP) (1-3). These cytokines are renowned for their pleiotropic functions and have been shown to play important roles in hematopoiesis, inflammation, the acute phase response, bone and heart development as well as neurogenesis (1,4). The pleiotropy can be attributed to the shared use of the signal transducing receptor glycoprotein (gp) 130 (5), which homo- or heterodimerizes after stimulation with the LIF receptor (LIFR) (6), the OSM receptor (OSMR) (7) or WSX-1, the receptor of IL-27 (3). Human OSM has the exceptional capability to recruit two different types of receptor complexes: the type I receptor complex composed of gp130/LIFR or the type II receptor complex of gp130/OSMR. In contrast, murine OSM solely utilizes the gp130/OSMR heterodimer for signal transduction (7,8). Recently, the OSMR has been shown to assemble the IL-31 receptor complex together with the IL-31R (9).

Whereas IL-6 represents one of the best studied cytokines to date, the relevance and physiological activities of OSM are less well known. OSM is predominantly secreted by activated T lymphocytes, macrophages and neutrophils (10,11) and seems to be involved in the regulation of the inflammatory response (12,13). Corresponding to its name, OSM supports growth inhibition of various solid tumors (14). However, it also induces the growth of AIDS-associated Kaposi’s sarcoma cells (15,16). Due to its ability to induce TIMP-1 and TIMP-3, pro-fibrotic properties have also been
attributed to OSM (17-19). Indeed, transgenic mice expressing OSM in islet β-cells develop severe fibrosis (20). Increased OSM-levels have also been found in the synovial fluid of rheumatoid arthritis patients (21) and in dermal lesions of psoriasis patients (22). OSMR knockout mice display defects in hematopoiesis and liver regeneration (23,24).

It is commonly accepted that OSM, besides activating the MAPK- and phosphatidylinositol 3-kinase pathway, is one of the strongest inducers of the JAK/STAT pathway (4,25,26). After ligand binding and receptor dimerization, the Janus kinase family members JAK1, JAK2 and TYK2 are activated (27,28), consequently mediating the phosphorylation of tyrosine residues within the cytoplasmic regions of either gp130 or the OSMR. The so far prevailing perception of the further downstream STAT activation involves recruitment of the STAT factors, particularly STAT1 and STAT3, to phosphorylated tyrosine motifs via their SH2 domains. Only for STAT5 a number of studies describe a receptor tyrosine-independent activation mechanism, which involves direct binding of STAT5 to the receptor recruited Janus kinases (29-31). However, it remains unclear to date which determinants render receptor-bound kinases susceptible for direct recruitment of STAT5.

Here we can show that the human and murine OSM receptor use distinct mechanisms to activate STAT5B. While the human receptor relies on two tyrosine motifs located proximal to the box1/box2-region, the murine receptor can activate STAT5B receptor-tyrosine independently. This tyrosine-independent activation requires a direct interaction of STAT5B with JAK2, which is only achieved by ligation of the murine OSMR. Indeed, JAK2 can only be precipitated with the murine OSMR, but not with the human OSMR. Interestingly, a single amino acid exchange within the human box 2 region to the equivalent amino acid in the murine receptor changes the preference of the human receptor for binding JAK1 to JAK2 and allows STAT5B activation independently of the tyrosine motifs. In contrast, the activation of STAT1 and STAT3 is mediated by conserved molecular mechanisms, i.e. for STAT3 through YXXQ-motifs in the cytoplasmic part of the receptors and for STAT1 directly via the Janus kinases.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection and Cytokines**

Primary human dermal fibroblasts (HDF), human embryonal kidney cells transformed with SV40 (HEK293T), murine embryonic fibroblasts (MEF) and human fibrosarcoma cells 2C4, U4C, γ2A, 2TGH and U1A (kindly provided by Dr. I. M. Kerr, Cancer Research UK, London) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen). All media were supplemented with 10% fetal calf serum, 100 µg/ml streptomycin and 60 µg/ml penicillin. Stably transfected HEK293T cells were selected in medium containing 100 µg/ml hygromycin, 500 µg/ml G418 and 10 µg/ml blasticidin. Cells were grown at 37°C in a water-saturated 5% CO₂ atmosphere. HDFs were generated as described previously (32). Transient transfections of HEK293T cells were carried out using FuGENE6 (Roche Applied Science) according to the manufacturer’s recommendations. HEK293T cells stably expressing the IL-5Ra/gp130YFFFFF as well as one of the following IL-5Rβ chimeras β/OSMRΔ1, β/OSMRΔ1Y917F/Y945F and β/OSMRΔ1-YFFFFF were generated by two rounds of transfections: first the IL-5Ra/gp130YFFFFF was integrated by transfection using FuGENE6 and selection using G418. Thereafter, clones were again transfected using the Flp-In™ T-Rex™ system from Invitrogen according to the manufacturer’s recommendations. Recombinant human IL-5 and human OSM were obtained from Cell Concepts (Umkirch, Germany), human LIF from Sigma (Taufkirchen, Germany) and murine OSM from R&D Systems (Minneapolis, MN).

**Expression Vectors**

The construction of the pSVL based expression plasmids encoding IL-5R chimeras α/gp130YFFFFF, β/OSMRΔ1, β/OSMRΔ1Y861F, β/OSMRbox1/2 has been described previously (25,28). As it was demonstrated in earlier studies that chimeras containing the full-length cytoplasmic region of OSMRβ are weakly expressed (28,33). Hence, we used truncated chimeric constructs lacking the 28 C-terminal amino acids (β/OSMRΔ1) which is expressed better, without losing any of the activation sites of STATs or MAPKs (28). The additional point mutated constructs containing the amino acid substitutions Y837F/Y839F, P813A and F820C were generated by polymerase chain reaction using...
the respectively mutated oligonucleotides with the cDNA for β/hOSMRΔ1 or β/hOSMRbox1/2 as a template, respectively. The C-terminal deletion mutants, β/mOSMRΔ1 and β/mOSMRbox1/2 were generated by polymerase chain reaction using the cDNA for murine OSMR as a template (kindly provided by M. Tanaka, University of Tokyo, Japan). The sense oligonucleotide incorporates an EcoRI site and the antisense oligonucleotides incorporate an in-frame termination codon followed by the recognition site for BamHI. They retain 191 and 65 amino acids of the murine OSMR cytoplasmic tail, respectively, and were subcloned into the EcoRI/BamHI-digested expression plasmid pSVL-IL-5Rβ/hOSMRΔ1 to generate the constructs encoding pSVL-β/mOSMRΔ1 and pSVL-β/mOSMRbox1/2. The human/murine OSMR chimeras were generated by standard PCR techniques and sequences are depicted in figure 3C. The pcDNA5/FRT/TO plasmids for β/hOSMRΔ1, β/hOSMRΔ1-Y917F/Y945F and β/hOSMRΔ1YFFFFFFF were generated by transferring a NotI/EcoRV fragment from the pSVL-constructs to the modified vector pcDNA5/FRT/TO (Invitrogen) containing an inverted multiple cloning site. The integrity of all constructs was verified by DNA sequence analysis using an ABI PRISM 310 Genetic Analyzer (PerkinElmer Life Science).

Innsbruck, Austria). The expression plasmid for STAT5B was kindly provided by W. Doppler (University of Innsbruck, Austria).

Cell Lysis, Immunoprecipitation and Western Blotting-HDFs, MEFs, fibrosarcoma cells and HEK293T cells were stimulated for the indicated periods of time with 1-100 ng/ml LIF, 1-100 ng/ml human OSM, 20 ng/ml murine OSM and 10 ng/ml IL-5. A 30 min preincubation with the pharmacological inhibitor AG490 (Calbiochem, Darmstadt, Germany) was used as indicated to inhibit activation of JAK2. HDFs were preincubated with LIF-05 (kindly provided by Prof. Dr. J. Heath, University Birmingham, UK) to inhibit signal transduction via the LIFR (34). Immediately after stimulation, cells were lysed in Triton lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml pepstatin, 5 μg/ml aprotinin and 5 μg/ml leupeptin) as described previously (25). All steps of cell lysis were performed at 4°C using ice-cold buffers. Proteins were separated by SDS-PAGE in 10 % gels, followed by electroblotting onto a polyvinylidene difluoride membrane (PALL, Dreieich, Germany). Western blot analysis was conducted using the indicated antibodies and the enhanced chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s instructions. Before reprobing, blots were stripped in 2 % SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.7) for 20 min at 70°C.

Antibodies-The phosphospecific polyclonal antibodies against STAT1(Tyr701), STAT3(Tyr705), STAT5(Tyr694), JAK1 (Tyr1022/1023) and JAK2 (Tyr1007/1008) as well as the monoclonal JAK2 antibody (24B11) were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal antibodies against STAT3 and Calnexin were purchased from Transduction Laboratories (Lexington, KY). Monoclonal IL-5Rα (S-16), polyclonal IL-5Rβ (N-20), polyclonal α-STAT1 (E-23), polyclonal STAT5B (C-17), polyclonal SOCS3 (C-20), polyclonal IRF-1 (C-20) and monoclonal Lamin-A/C antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) were used for detection. The horsedradish peroxidase-conjugated secondary antibodies were purchased from Dako (Hamburg, Germany).

Peptide Precipitation Assay and Immunoblot Analysis-The amino acid sequences of the used peptides were published previously (26). 0.003μmol of the biotinylated peptides were immobilized by incubation with 10 μl of NeutrAvidin-coupled agarose (Pierce). For STAT5B precipitation, HEK293T cells overexpressing STAT5B were lysed in 500 μl of lysis buffer (150 mM NaCl, 50 mM Tris/HCl, 0.1 mM EDTA, 10 % glycerol, 0.5 % Nonidet P-40, pH 8.0 supplemented with Na3VO4 (1 mM), pepstatin (3 μg/ml), leupeptin (5 μg/ml), aprotinin (5 μg/ml) and phenylmethylsulfonyl fluoride (1 mM). Endogenous STAT1 and STAT3 or overexpressed STAT5B were precipitated by incubation of total cell lysates with the immobilized peptides at 4°C overnight. Precipitates were then washed three times with 1 ml of lysis buffer. The precipitated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. STAT proteins were detected with antisera recognizing the proteins irrespectively of their activation.
Cell Fractionation—All fractionation and centrifugation steps were performed at 4°C using ice-cold buffers. Cytoplasmic, membrane and nuclear fractions were prepared using protocols described in detail before (35).

RT-PCR—Total RNA was isolated from HEK293T cells stimulated for the indicated times with 20ng/ml IL-5 using the RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RT and PCR were performed with 1ug of total cell RNA using a OneStep RT-PCR kit (Qiagen, Hilden, Germany). Detection of specific mRNA for IRF1, SOCS3 and CIS was achieved by using primers designed to amplify at least one exon (across one intron/exon border to exclude contamination of cDNA with genomic DNA). GAPDH was used as an internal standard (36). Amplification was carried out with 35 cycles of 40sec denaturation at 94°C, 30sec annealing at 58°C and 30sec extension at 72°C. The amplification was terminated with an extension step of 10min duration at 72°C after the last cycle. PCR products were separated on 1% agarose gels and stained with ethidium bromide.

RESULTS

OSM induces nuclear translocation of STAT1, STAT3 and STAT5

Primary human dermal fibroblasts (HDF) were stimulated for different periods of time with 20 ng/ml OSM and subsequently plasma membrane, cytosolic and nuclear fractions were collected to analyze the activation pattern of STATs. As expected and previously described (25,26,37,38), OSM leads to the phosphorylation of STAT1, STAT3 and STAT5 which could be detected in the cytosolic as well as in the nuclear fraction (Fig. 1A, left and right panel). Interestingly, the STAT activation profile in the nucleus indicates that STAT5 is the most transiently activated STAT protein since no tyrosine phosphorylated STAT5 could be detected in the nucleus at 60 min stimulation with OSM (Fig. 1A, upper panel). In contrast, phosphorylated STAT3 can be found in the nucleus at least up to 3 hours (lower panel). Analyses of whole cellular extracts confirmed the prolonged activation of STAT3 for at least up to 16 hours (suppl. Fig. 1).

Dose- and receptor-dependent STAT5 phosphorylation

HDF express gp130, the LIFR as well as the OSMR (not shown) and therefore formation of the type I as well as the type II receptor complex in response to OSM stimulation is possible. In order to compare the potential of the LIFR and the OSMR to activate STAT5 we stimulated HDF with increasing concentrations of OSM and LIF. While 1 ng OSM/ml is sufficient to induce a clearly detectable STAT5 activation (Fig. 1B, lane 7), a concentration of >50 ng/ml LIF was necessary to activate STAT5 (lane 1). To examine whether OSM-induced STAT5 activation is preferentially mediated by LIFR or can also be initiated through the OSMR, we pretreated the cells with LIF-05, a mutated form of LIF which is still able to bind the LIFR, but cannot recruit gp130 (34). Therefore, the LIFR cannot be used and signaling by OSM can only occur through the type II receptor complex (gp130/OSMR). Interestingly, the OSM-induced STAT5 phosphorylation shows no reduction (Fig. 1C, lane 4) and therefore has to be transduced via the OSMR, whereas the LIF-mediated STAT5 activation is completely absent (Fig. 1C, lane 8).

The human OSMR uses the double tyrosine motif Y837/Y839 to activate STAT5

STAT factors can be recruited to phosphorylated tyrosine motifs within receptor chains by virtue of their SH2-domains, and specific recruitment motifs for the different STAT isoforms have been established. To analyze the capability of the tyrosine motifs within the OSMR to recruit different STATs, we took advantage of a well-established chimeric receptor system (25,28): the transmembrane and intracellular parts of the gp130 or OSMR are fused to the extracellular region of the IL-5 receptor α or β chains, respectively. By stimulating transfected cells with IL-5 we induced receptor complex formation and initiated gp130- and OSMR-mediated signal transduction. To focus on the OSMR-initiated signaling we prevented gp130 tyrosine-based signal transduction by point mutating all five tyrosines C-terminal of box1/2 (α/gp130YFFFFF). Using this chimeric receptor system, we can show that, as expected, STAT3 tyrosine phosphorylation occurs through Y917/Y945, both of which are located within typical STAT3 consensus motifs (p)YXXQ. Mutation of Y917/Y945 within the OSMR completely abrogates STAT3 phosphorylation (Fig. 2A, upper panel, lane 4) and consequently the induction of the STAT3 target gene SOCS3.

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on RNA and protein level (Fig. 2A, middle and lower panel). In striking contrast, these two tyrosines are dispensable for STAT1-activation (Fig. 2B, upper panel, lane 4) and the induction of the classical STAT1 target gene IRF1 as demonstrated both on RNA and protein level (Fig. 2B, middle and lower panel). Indeed, even point mutation of all tyrosines located C-terminally of box1/2 of the OSMR did not lead to the abrogation of STAT1 activation (Fig. 2B, upper panel, lane 6). Thus, in contrast to STAT3, activation of STAT1 occurs independently of receptor tyrosine recruitment sites. In case of STAT5B, activation cannot occur tyrosine independently (Fig. 2C, lane 8). The receptors of well-known STAT5 activators such as Epo, IL-2 or GM-CSF contain characteristic YΦXΦ–STAT5 binding motifs (Φ, hydrophobic residue). Interestingly, the tyrosines Y837/Y839 of the OSMR are located within such a motif and we therefore tested if these tyrosines might serve as STAT5 recruitment sites. Indeed, mutation of the double tyrosine motif Y837/Y839 completely abrogated OSMR-mediated STAT5B tyrosine phosphorylation (Fig. 2C, compare lanes 2 and 4). Cells expressing an OSMR Y861F variant served as a negative control, since the Y861 residue is known as the specific recruitment site for the adapter protein Shc and therefore important for MAPK but not for STAT activation (25). Indeed, IL-5-induced STAT5 phosphorylation occurs normally (Fig. 2C, lane 6). To investigate the importance of the Y837/Y839-mediated STAT5B phosphorylation on target gene expression, we analyzed the RNA levels of the STAT5 responsive gene cis (cytokine-inducible protein with SH2 domain). Indeed stimulation through the OSMR allowed transcription of the cis gene (Fig. 2D, lane 2), whereas no RNA is detectable in the cells expressing the Y837/839F-mutated OSMR (Fig. 2D, lane 4). With no reliable CIS-antibodies available, the protein levels could not be analyzed.

To confirm our observations by an independent approach, we performed peptide precipitations with peptides encompassing OSMR tyrosine motifs to precipitate STAT1, STAT3 or STAT5, either endogenously or overexpressed in HEK293T cells. We used peptides containing all tyrosine motifs C-terminal to the box1/2 region in the intracellular part of the OSMR except Y978, which is the second last amino acid in the OSMR and therefore unable to support a receptor/SH2 domain-containing protein inter-

action. Fig. 2E strongly supports our findings using mutated receptors: STAT5B can be precipitated with phosphorylated Y837 or Y839. The single tyrosines within the double motifs seem to be redundant in this process. None of the tested phosphotyrosine-containing peptides deduced from the OSMR sequence is able to interact with STAT1 and STAT3 does interact with the tyrosines Y917 and Y945. Biacore analyses using a purified STAT3-SH2 domain and peptides encompassing each of the two phosphorylated tyrosine motifs (pY917 or pY945) showed that both tyrosines bind STAT3 with similar affinities (data not shown).

The murine OSMR relies on receptor-bound Janus kinases to activate STAT5

When comparing the sequences of the OSMR from various species, one notices that in contrast to the conserved recruitment sites for STAT3 in all receptors, the murine and rat receptors lack the double tyrosine motif responsible for the STAT5 phosphorylation in its human counterpart (Fig. 3). Nonetheless, human fibroblasts and murine fibroblasts elicit equal STAT5 tyrosine phosphorylation kinetics in response to the respective OSM (Fig. 4A, upper panel) and consequently an indistinguishable expression of the STAT5 target gene cis (Fig. 4B). Since the murine OSMR does not contain any additional tyrosine motifs compared with the human receptor we hypothesized that in contrast to the human receptor, the murine OSMR might be capable of STAT5 activation in a tyrosine-independent fashion. To test this assumption, we transfected HEK293T cells with either the human or the murine receptor (hOSMRΔ1, mOSMRΔ1) or shortened variants containing only the box1/2 region and therefore lacking all tyrosine motifs. As expected from the previous experiment, both long versions of the human and the murine wild type receptors initiate STAT5B activation after stimulation with IL-5 (Fig. 4C, upper panel, lane 2, 6). Whereas the human OSMRbox1/2 is not able to mediate STAT5B tyrosine phosphorylation anymore, as it lacks the crucial double tyrosine motif (Fig. 4C, lane 4), the murine OSMRbox1/2, still supports phosphorylation of STAT5B (Fig. 4C, lane 8). Transient transfection of the same constructs into murine fibroblasts resulted in the same activation profile (suppl. Fig. 2). In contrast, activation of STAT1 and STAT3 occurs via similar molecular mechanisms for the murine and the human OSMR: STAT1 tyrosine-
independently, STAT3 depending on the tyrosine motifs (Fig. 4C, lane 4, 8).

The box 2 region of the murine receptor determines the STAT5 activation mode

Analysis of the box1/2 region of human and murine OSMR indicated that the box 1 is highly conserved between both species (Fig. 4D). However, the interbox and particularly the box 2 regions are less well conserved. Therefore, we generated new receptor chimeras in which either the interbox region or the box 2 region were exchanged between human and murine receptor (Fig. 4D, constructs 3-6). These novel chimeras were transfected into HEK293T cells along with the gp130 variant used before, which is devoid of any intracellular tyrosine motifs. Exchange of the interbox region did not alter the mode of STAT5B activation – the human (mu interbox) variant still failed to activate STAT5B (Fig. 4E, lane 6). Consequently, the murine OSMR retained its ability to activate STAT5B in the presence of the human interbox region (Fig. 4E, lane 12). However, exchanging the box 2 region generated a human OSMR with mouse capacities, i.e. activation of STAT5B can be achieved without any receptor tyrosines (Fig. 4E, lane 4). Vice versa, if the box 2 region in the murine receptor was replaced by the human box 2, the murine OSMR lost its ability to activate STAT5B (Fig. 4E, lane 10).

Closer inspection of the 18 amino acids present in the human and murine box 2 region revealed two prominent differences between both species: P813A and F820C. These residues were point mutated in the human receptor to their murine counterparts and again expressed transiently in HEK293T cells along with the gp130 variant used before, which is devoid of any intracellular tyrosine motifs. Exchange of the interbox region did not alter the mode of STAT5B activation – the human (mu interbox) variant still failed to activate STAT5B (Fig. 4E, lane 6). Consequently, the murine OSMR retained its ability to activate STAT5B in the presence of the human interbox region (Fig. 4E, lane 12). However, exchanging the box 2 region generated a human OSMR with mouse capacities, i.e. activation of STAT5B can be achieved without any receptor tyrosines (Fig. 4E, lane 4). Vice versa, if the box 2 region in the murine receptor was replaced by the human box 2, the murine OSMR lost its ability to activate STAT5B (Fig. 4E, lane 10).

F820C mutation of the human OSMR box 2 region changes receptor preference from JAK1 to JAK2 recruitment

To delineate the molecular mechanisms which are responsible for the capacity of the murine OSMR to activate STAT5 receptor tyrosine independently, we examined the activation profile of JAK1 and JAK2 in response to stimulation through the human or murine OSMR. While both receptors result in an equivalent tyrosine phosphorylation of JAK1 (Fig. 5A, upper panel), double-tyrosine phosphorylated and therefore activated JAK2 is better detectable when activating the murine receptor (Fig. 5A, lower panel). Likewise, murine OSM activated JAK2 much better in MEF than human OSM in HDF (Fig. 4A, middle panel), whereas JAK1 was activated equally well (lower panel). Furthermore, exchange of F820 to cysteine did not only change the STAT5 activation mode, but additionally allowed a much stronger JAK2 tyrosine phosphorylation than the native human receptor (Fig. 4F, middle panel). The JAK1 activation profile remained unchanged (Fig. 4F, lower panel).

Therefore, we hypothesized that the human and murine OSMR differ in their potential to recruit JAK1 or JAK2, respectively, and that mutation of F820 to cysteine changes the affinity of the human receptor for JAK2. Indeed, we can coimmunoprecipitate endogenous JAK2 with the murine OSMR and with the human OSMR F820C variant (Fig. 5B, lanes 4-7).

Finally, we were interested whether STAT5B is directly recruited by JAK2 and performed coimmunoprecipitation studies between the Janus kinases and the transcription factor. No association of JAK1 and STAT5B can be observed, neither in response to stimulation of the human receptor nor when the murine receptor has been activated (Fig. 5C, upper panel). In contrast, JAK2 could be coimmunoprecipitated with STAT5B in response to stimulation of the murine receptor, but not when activating the human receptor (Fig. 5C, lower panel, compare lanes 3 and 5).

JAK2 deficiency or inhibition strongly reduces STAT5 tyrosine phosphorylation

Phosphorylation of receptor tyrosines is a prerequisite to transform them into docking sites for SH2 domain containing proteins. Since the human OSMR can bind JAK1, JAK2 and TYK2 we examined the involvement of each particular kinase in the activation process of individual STATs in more detail. Therefore, we stimulated human fibrosarcoma cell lines lacking either JAK1 (U4C), JAK2 (γ2A) or TYK2 (U1A) with increasing concentrations of OSM. Parental cells (2C4 or 2fTGH) activate STAT1, STAT3 und STAT5 (Fig. 6A, lane 1-6, 19-24). While 1 ng/ml is sufficient to activate STAT3, a concentration of 2.5 ng/ml is required to activate STAT1 and STAT5. Deficiency in JAK1 abrogates the cells ability to activate STAT5 and
STAT1 and markedly reduces the activation of STAT3 (Fig. 6A, lane 7-12). Interestingly, deficiency of JAK2 differentially affects the STAT activation. STAT1 is activated as well as in the parental cells, but STAT3 and STAT5 tyrosine phosphorylation is strongly reduced (Fig. 6A, lane 13-18). Deficiency in TYK2 expression has no effect on the OSM-induced phosphorylation of STAT1, STAT3 or STAT5 (Fig. 6A, lane 25-30).

Our findings concerning the role of the individual tyrosine kinases for the activation of STAT molecules were confirmed through the use of specific inhibitors against the different Janus kinases. The JAK inhibitor 1, directed against all three kinases, blocks the OSM-mediated activation of STAT1 and STAT3 (data not shown). In contrast to that, the incubation of human fibrosarcoma wild-type cells with AG490, a more selective inhibitor of JAK2, leads to efficient suppression of the OSM-induced STAT5 phosphorylation already at low concentrations (Fig. 6B, upper panel) while STAT3 tyrosine phosphorylation appeared to be affected at higher concentrations of AG490 (Fig. 6B, lower panel).

To further investigate the apparent involvement of JAK2 also in the activation process of STAT5 through the human OSMR complex, we monitored a potential interaction of JAK1 and JAK2 in human cells. Therefore, we immunoprecipitated JAK1 from human and murine fibroblasts and screened for co-precipitated JAK2. Indeed, in HDF, but not in MEF, JAK1 recruits JAK2 (Fig. 6C, lane 2).

**DISCUSSION**

Even though the initial descriptions of the JAK/STAT pathway date back almost 15 years, the precise molecular mechanisms which determine recruitment of JAKs to the receptors, their activation process or their involvement in activation of STATs is only known in parts. So far no structural data on the intracellular region of any JAK-associated cytokine receptor or the complete JAKs are available, which could help in the delineation of the receptor/JAK interaction interface. Doubtless, the highly conserved proline-rich box I region of the receptors as well as the FERM domain of JAKs are essential for the receptor/JAK interaction (1), but particularly how specificity is achieved remains unknown.

Our study demonstrates that a single amino acid exchange in the box 2 region of the human OSM receptor can change the preference of the human receptor for JAK1 binding to the recruitment of JAK2 and subsequently to a receptor tyrosine-independent STAT5 activation mode.

Human and murine OSM differ with respect to their used receptor complexes. Human OSM can signal via the type I receptor complex (gp130/LIFR) as well as via the type II receptor complex (gp130/OSMR) while murine OSM is limited to the type II receptor complex (7,8). Here we find evolutionary distinct mechanisms for the human and murine OSMR to activate STAT5 while the activation mode of STAT1 and STAT3 appears to be conserved.

Due to the facts that murine OSM activates STAT5 in MEFs (Fig. 4A), that the specific LIF inhibitor LIF-05 only suppresses the LIF-induced STAT5 phosphorylation but not the one mediated by OSM (Fig.1C) and that OSM activates STAT5 in the human melanoma cell line A375, which only expresses the type II receptor complex (data not shown), we concluded that the strong OSM-induced activation of STAT5 relies on the involvement of the OSMR. This hypothesis is supported by a study in which homodimerized GCSF-R/OSMR chimeras were able to activate STAT5 (37). We identified the double tyrosine motif Y837/Y839 within the intracellular human OSMR region as recruitment site for STAT5B. These tyrosines are located within the motif PNYLYLLP, that is in accordance with the STAT5-activation motif DXpYΦXΦ described by May et al. (39), differing only in the acidic amino acid N-terminal to the tyrosine residue. Further receptor systems in which a tyrosine-based STAT5 recruitment has been shown include the growth hormone (GH) receptor (40-42), erythropoetin (EPO) receptor (43-45) and the interleukin-2 receptor β-chain (46). Particularly, the similarity with a STAT5-binding motif within the EPOR (Y431) LKYLYLVVS is remarkable (47).

Interestingly, the double tyrosine motif is not conserved between man and mouse. It is found in the human, chimp, rhesus macaque, bovine, equine and canine OSMR sequences, but is absent in the rodents mouse and rat. Rather than being mutated a precise deletion of three amino acids (YLY) is found (see alignment, Fig. 3). Therefore, previous studies trying to elucidate the molecular mechanism how OSM leads to STAT5 activation failed since only tyrosine residues conserved between human and murine OSMR were investigated (37).
In contrast to the human receptor a truncated version of the murine receptor which only comprises the JAK recruiting box1/2 region is sufficient to activate STAT5B (Fig. 4C). This indicated that STAT5 activation via the murine receptor might be directly mediated by the receptor bound Janus kinases. Closer inspection of the JAK activation profile indicated that the human OSMR in combination with gp130 preferentially activated JAK1; only a much weaker activation of JAK2 is detectable. In contrast, stimulation of the murine OSMR/gp130 activated JAK2 much better (Fig. 5A). Since earlier studies suggested a direct JAK2/STAT5 axis, we were wondering whether the human and murine OSMR might differ in their binding preferences for JAK2. Indeed, active endogenous JAK2 could be immunoprecipitated with the chimeric construct containing the murine box1/2 region (Fig. 5B, lane 5), but not when the human box1/2 region was present (Fig. 5B, lane 3). Of note, overexpressed JAK2 can be coimmunoprecipitated with the human OSMR (28) and is also able to upregulate the cell surface expression of the human OSMR (33) which argues for a weaker affinity of JAK2 to the human OSMR rather than a complete disability to bind the box1/2 region.

Mutagenesis analyses of the box1/2 region identified the murine box 2 as the decisive region whether the receptor could activate STAT5 independent of receptor tyrosines (Fig. 4E, lane 4) and within the human box 2 region a single amino acid exchange (F820C) was sufficient to change the STAT5B activation mode (Fig. 4F, lane 6). With regard to our observation that the murine receptor preferentially activates JAK2, we therefore hypothesized that the phenylalanine 820 in the human receptor prevents efficient recruitment of JAK2 to the human receptor. Consequently, the point mutation of this phenylalanine to the respective amino acid in the murine receptor (cysteine) resulted in a human OSMR which efficiently activated and recruited JAK2 (Fig. 5B, lane 7).

Finally, we could show that STAT5B can directly interact with JAK2 in response to ligation of the murine OSMR. No interaction of STAT5B with JAK1 could be observed. The first evidence for a direct binding of STAT5 to one of the Janus kinases came from yeast two-hybrid screens using the kinase-like domain (KLD) (29). Most importantly, a genetic fusion of the kinase domain (KD) of JAK2 to the N-terminal region of the ETS family transcription factor TEL has been shown to activate STAT5 (30). This TEL/JAK2 fusion is the result of a chromosomal translocation and has been identified as the cause of a number of human leukemia (48,49). Additionally, artificial replacement of the EGF receptor intracellular region by the kinase domain of JAK2 resulted in a ligand-induced tyrosine phosphorylation of STAT5 (31). According to structure predictions the kinase-like (KLD) and kinase (KD) domains of JAK2 should have similar folds since only catalytically, but not structurally important amino acids are exchanged between both domains. This could explain why interactions of STAT5 with the KLD (29) or the KD (30,31) were observed.

Our results rather exclude a possible direct JAK2/STAT5 interaction within the human OSM signal transduction. Neither the OSMRΔ1YFFFFFFF (Fig. 2C) nor the OSMRΔ1box1/2 (Fig. 4C) constructs are able to induce a STAT5 phosphorylation via Janus kinases. A supporting role of JAK2 for the human OSMR-mediated signal transduction, however, is evident. In comparison to wild-type cells, a strongly reduced STAT5 tyrosine phosphorylation is detectable in hOSM-stimulated JAK2-deficient human fibrosarcoma cells (γ2A) (Fig. 6A). In accordance with these findings, the STAT5 phosphorylation via JAK2 can be reduced to approx. 50% by the JAK2 inhibitor AG490 at low concentration (Fig. 6B).

As published in previous studies for IL-6 (50), in human cells JAK1 also seems to play the predominant role in STAT activation in response to OSM. No tyrosine phosphorylation of STAT1 or STAT5 and only a strongly reduced phosphorylation of STAT3 can be detected in JAK1 deficient human fibrosarcoma cells (U4C).

We therefore postulate that in the human system both receptors, gp130 and OSMR, preferentially bind and activate JAK1. JAK2, however, plays an important supporting role in the STAT3 and STAT5 activation process and can be recruited to the receptor complex through an interaction with JAK1 (Fig. 6C). In the case of the murine receptor system, gp130 appears to recruit JAK1 while the murine OSMR seems to preferentially bind JAK2, which after activation can directly bind and activate STAT5.
The evolutionary different mechanisms to activate STAT5 by the murine versus the human OSMR are unique and cannot be observed for STAT1 or STAT3. As predicted, STAT3 activation is mediated by the tyrosine motifs Y917 (YVSQ) as well as Y945 (YKMQ) within the OSMR (Fig. 2A and E), both of which are highly conserved and found in all species analyzed so far (Fig. 3). These tyrosine motifs resemble the well defined STAT3-recruiting consensus sequence YXXQ (51,52). These findings fit to the results of Kuropatwinski et al. showing that a truncated OSMR, comprising only the 142 membrane-proximal amino acids and therefore lacking the tyrosines Y917 and Y945, cannot activate STAT3 to the normal extent (37). Interestingly, OSM stimulation of JAK1-deficient fibrosarcoma cells still allows a minor STAT3 activation (Fig. 6A). Taking in consideration that in JAK1-/- cells the cell surface levels of gp130 and the OSMR are significantly reduced (suppl. Fig. 3 and (33)), this remaining STAT3 tyrosine phosphorylation is remarkable. These data are in line with a recent study by Haan et al. (53), in which JAK1-deficient fibrosarcoma cells reconstituted with a kinase-inactive mutant of JAK1 can activate STAT3 in response to OSM.

Making use of phosphopeptides, a characteristic binding motif for STAT1 has been described years ago, as well. In accordance to these data, STAT1 favors peptides with the motif Y-(D/E)-(P/R)-(R/P/Q) (52). Although OSM induces the activation of STAT1, a comparable motif cannot be found within its receptor. The findings shown in Fig. 2B are in line with these postulations and demonstrate that STAT1, as well as the specifically STAT1-regulated gene IRF-1, are activated/induced tyrosine-independent and therefore directly via the OSMR-associated Janus kinases. Even the truncated receptor construct, containing only box1/2, is able to phosphorylate STAT1 in both the murine and the human system (Fig. 4C). The results of Kuropatwinski et al. do also support these findings (37). Within their experiments, the STAT1 activation induced by a chimeric G-CSF/OSMR can only be prevented by the deletion of the JAK-binding box1/2 region. With the help of cells deficient for distinct tyrosine kinases, we could emphasize the crucial role of JAK1 for the OSM-mediated STAT1 phosphorylation (Fig. 6A). These results are in line with data from Gupta et al. showing a direct interaction of the STAT1 SH2 domain with JAK1 in GST pull down assays (54).

Taken together, while the ability of the OSMR to activate STAT1 and STAT3 is evolutionary conserved, two distinct molecular mechanisms for receptor-mediated STAT5 activation have evolved: a receptor-tyrosine dependent and an independent mechanism. Studying the determinants for this difference, we show for the first time that a single amino acid exchange within the cytokine receptor box1/2 region is sufficient to change the receptor’s preference for recruitment of a specific Janus kinase and subsequently changes the mode of STAT activation. This finding contributes to a more detailed understanding how specificity among cytokine receptors can be generated.

ACKNOWLEDGEMENTS

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REFERENCES

FOOTNOTES
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5both senior authors contributed equally to the work

Abbreviations: CIS, cytokine inducible SH2-domain containing protein; HDF, human dermal fibroblasts; IL, interleukin; IRF, interferon regulatory factor; JAK, Janus kinase; LIF, leukemia inhibitory factor; MEF; mouse embryonic fibroblasts; OSM, oncostatin M; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription

FIGURE LEGENDS

Fig. 1: Oncostatin M-induced activation of STAT1, STAT3 and STAT5. A, HDF were stimulated with 20 ng/ml OSM for the indicated time periods and cell fractions were prepared. Western blot analysis was performed using antibodies specific for tyrosine-phosphorylated STAT1, STAT3 and STAT5. Calnexin and Lamin A/C were used as markers for the membrane and nuclear fraction, respectively. B, HDFs were stimulated with indicated amounts of OSM or LIF respectively for 30 min. Aliquots of lysates were separated by SDS-PAGE and the Western blot was stained with an antiserum specific for phospho-STAT5 and restained with an antibody against STAT5. C, HDFs were incubated for 30 min with 10μM LIF-05 before stimulation with 20 ng/ml OSM or 100 ng/ml LIF, respectively. Lysates were prepared and Western blot analysis was carried out as described in B.

Fig. 2: Definition of STAT-recruitment sites within the cytoplasmic region of the Oncostatin M receptor. A and B, stably transfected HEK293 FlpIn cells were induced with doxycyclin (10 ng/ml, 24h) to express the chimeric receptors as indicated. Subsequently cells were stimulated with 10 ng/ml IL-5 for indicated time periods. Western blots of cellular lysates were developed with antisera specific for phospho-STAT3, STAT3 and SOCS3; phospho-STAT1, STAT1, IRF-1. Detection of IRF-1 and SOCS3 mRNA was performed by RT-PCR. C and D, HEK293T cells were transiently transfected with expression vectors for STAT5B and the chimeric receptors as indicated. The cells were stimulated with 10 ng/ml IL-5 for indicated time periods. Whole cellular lysates were prepared and subjected to Western blot analysis using specific antisera against phospho-STAT5 and STAT5. CIS mRNA levels were detected by RT-PCR. E. HEK293T cells were transfected with expression vector encoding STAT5B. Lysates were prepared and subjected to precipitations with biotinylated peptides encompassing the amino acid sequence surrounding the indicated tyrosine residues of the OSMR. Peptides were using NeutrAvidin-coupled agarose, complexes were separated by SDS-PAGE and Western blots were probed with antibodies against STAT1, STAT3 and STAT5.

Fig. 3: Alignment of the OSMR sequences from various species. The amino acid sequences of OSMR from different species (h, human; p, chimp; q, rhesus macaque; b, cow; e, horse; m, mouse; r, rat) were aligned. The box1 and box2 regions as well as the tyrosine motifs are indicated. Residues that are highly conserved among the OSMR sequences and found in at least 6 out of 7 species are highlighted in yellow. Amino acids conserved in their chemical properties are marked in green and the amino acid indicated in magenta is responsible for changes in the JAK binding profile.

Fig. 4: The human and the murine OSMR use distinct mechanisms to activate STAT5. A, HDFs and MEFs were stimulated with 20 ng/ml human or murine OSM, respectively, for the indicated time periods. Proteins were resolved by SDS-PAGE and Western blots were stained with antisera specific for phospho-STAT5, phospho-JAK1 or phospho-JAK2 and restained with a STAT5-, JAK1- or JAK2-specific antibodies. B, HDFs and MEFs were stimulated as mentioned in A. CIS mRNA levels were detected by RT-PCR. C, HEK293T cells were transiently transfected with expression vectors for STAT5B and the chimeric receptors as indicated. The cells were stimulated with 10ng/ml IL-5 for 20 min. Whole cellular lysates were prepared and subjected to Western blot analysis using specific antisera against phospho-STAT1, -STAT3 and -STAT5. Subsequently the blots were stripped and
reprobed with antisera recognizing the proteins irrespectively of their activation. D, Schematic representation of the OSMR box1/2 region. The amino acid sequence is given in one-letter code. Bold letters highlight amino acids in the box1 and box2 region, italic letters represent amino acids in the interbox. Amino acids of the human OSMR are underlined to distinguish them from murine ones. E and F, HEK293T cells were transiently transfected with expression vectors for STAT5B, α/gp130-YFFFFF and a β/OSMR chimera containing one of the box1/box 2 regions shown above or a point-mutation as indicated. The cells were stimulated with 10 ng/ml IL-5 for 20 minutes. Whole cellular lysates were prepared and subjected to Western blot analysis using specific antisera against phospho-STAT5, phospho-JAK1 or phospho-JAK2 and antibodies against STAT5, JAK1 and JAK2.

Fig. 5: The murine OSMR preferentially recruits JAK2. A, HEK293T cells were transiently transfected with expression vectors for the chimeric receptors as indicated and stimulated with 10 ng/ml IL-5 for 20 minutes. Equal amounts of lysates were separated by SDS-PAGE. Western blots were stained with specific antisera against phospho-JAK1 and phospho-JAK2, stripped and reprobed with a polyclonal antiserum against STAT3. B and C, HEK293T cells were transiently transfected with expression vectors for STAT5B and the chimeric receptors as indicated. After stimulation with 10 ng/ml IL-5 for 20 min, equal amounts of lysates were subjected to immunoprecipitations using a monoclonal antibody against IL-5Rβ or a polyclonal antiserum against STAT5. Immunocomplexes were purified using Protein A-sepharose and Western blots were stained with polyclonal antibodies against phospho-JAK1, phospho-JAK2, JAK2, IL-5Rβ and STAT5.

Fig. 6: Involvement of Janus kinases in STAT activation. A, human fibrosarcoma cells were stimulated with increasing amounts of OSM for 15 minutes and Western blots lysates developed with antisera specific for phospho-STAT1, -STAT3 and -STAT5. Subsequently the blots were stripped and reprobed with antisera recognizing the proteins irrespectively of their activation. B, human fibrosarcoma parental cells were preincubated with indicated amounts of AG490 for 45 min, followed by stimulation with 20 ng/ml OSM for 15 minutes. Lysates were prepared and subjected to Western blot analysis using antibodies against phospho-STAT5 or phospho-STAT3. Blots were reprobed with antisera against STAT5 and STAT3. C, HDFs and MEFs were stimulated with 20 ng/ml human or murine OSM, respectively, for 20 min. Equal amounts of lysates were subjected to immunoprecipitations using a polyclonal antiserum against JAK1. Immunocomplexes were purified using Protein A-sepharose and the Western blot was stained with a polyclonal antiserum against JAK2. Equal loading was confirmed by reprobing the blot with an polyclonal antiserum against JAK1.
Figure 1

A. Fractional distribution of phosphorylated Stat1, Stat3, and Stat5 proteins in cytosolic, membrane, and nuclear fractions after treatment with OSM (20 ng/ml) for 60 min.

B. Western blot analysis of whole cellular extracts showing the effects of LIF and OSM on the phosphorylation of Stat5 and Stat1.

C. Comparison of the effects of LIF, OSM, and LIF-05 on the phosphorylation of Stat5 in whole cellular extracts.

Legend:
- pY-STAT1
- pY-STAT3
- pY-STAT5
- Calnexin
- Lamin A/C

Abscisca: Time (min) or concentration (ng/ml)

1. Cytosolic
2. Membrane
3. Nuclear

Whole cellular extracts

1. LIF
2. OSM
3. OSM (20 ng/ml)
4. LIF (100 ng/ml)
5. LIF-05 (10 μM)

Phosphorylation status:
- (-) No phosphorylation
- (+) Phosphorylated

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Figure 2

A

IL-5

20 min IL-5

pY-STAT3

STAT3

SOCS3

GAPDH

12 4 36 5

B

IL-5

20 min IL-5

pY-STAT1

STAT1

IRF1

GAPDH

12 4 36 5

C

IL-5

20 min IL-5

pY-STAT5

STAT5

CIS

GAPDH

12 4 36 5

D

IL-5

1 h IL-5

CIS

GAPDH

12 4 36 5
### Figure 2

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**Whole cellular extracts**
Figure 4

A

HDF

0 0.5 1 3

MEF

0 0.5 1 3

whole cellular extracts

0 2 4 6 8

pY-STAT5

STAT5

pYpY-JAK2

JAK2

pYpY-JAK1

JAK1

B

HDF

0 0.5 1 3

OSM (h)

0 0.5 1 2 3

0.5 1 2 3

total RNA

CIS

GADPH

whole cellular extracts

0 2 4 6 8

pY-STAT5

STAT5

pY-STAT1

STAT1

pY-STAT3

STAT3

20 min IL-5

IL-5

α β

IL-5

α β

IL-5

α β

IL-5

α β

IL-5

α β

gp130

gp130

gp130

gp130

hOSMR

hOSMR-B1/2F

mOSMR

mOSMR-B1/2F

pYpY-JAK2

JAK2

JAK1

pYpY-JAK1

pY-STAT5

STAT5

STAT1

STAT3

 whole cellular extracts

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Figure 4

D

1. hOSMR
2. mOSMR
3. hOSMR (mu Box2)
4. mOSMR (hu Box2)
5. hOSMR (mu Interbox)
6. mOSMR (hu Interbox)

box 1  
box 2  
interbox

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E

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mOSMR-Box1/2

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hOSMR-B1/2  
hOSMR-B1/2

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20 min IL-5  
20 min IL-5

pY-STAT5  
STAT5

pY-JAK2  
JAK2

pY-JAK1  
JAK1
Figure 5

**A**

Whole cellular extracts after 20 min IL-5 treatment, analyzed by western blotting for pYpY-Jak1, Jak1, pYpY-Jak2, and Jak2.

**B**

Seph. control and whole cellular extracts from gp130 knockout cells (HOSMR) and WT cells (mOSMR) after 20 min IL-5 treatment, analyzed for pYpY-JAK1, STAT5, pYpY-JAK2, and STAT5.

**C**

Whole cellular extracts from gp130 knockout cells (HOSMR) and WT cells (mOSMR) after 20 min IL-5 treatment, analyzed for pYpY-JAK1, STAT5, and IL-5Rβ.
Figure 6

A  

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whole cellular extracts

15 min OSM

AG490 (µM)

- 50 - 5 25 50

12 4 3

HDF MEF

C

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20 min OSM

IP: JAK1

1 2 3 4
Box 2 region of the Oncostatin M receptor determines specificity for recruitment of Janus kinases and STAT5 activation

Christoph Hintzen, Christina Evers, Barbara E. Lippok, Rudolf Volkmer, Peter C. Heinrich, Simone Radtke and Heike M. Hermanns

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