Influence of Subunit Combinations on Signaling by Receptors for Oncostatin M, Leukemia Inhibitory Factor, and Interleukin-6*

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Oncostatin M (OSM), leukemia inhibitory factor (LIF), and interleukin-6 (IL-6) induce expression of a similar set of acute phase plasma protein genes in hepatic cells. The redundant action of these cytokines has been ascribed to the involvement of the common signal-transducing receptor subunit, gp130, in combination with cytokine-specific, ligand-binding subunits. To define the specificity of the signal transduction by the LIF/OSM receptor (a heterodimer of gp130 and LIF receptor (LIFR)) and the OSM-specific receptor (a heterodimer of gp130 and OSM receptor (OSMR)), we reconstituted the receptor function by transfection into receptor-negative Hep3B hepatoma cells. Both receptors activate DNA binding activity of STAT1, -3, and -5B and induce gene transcription through IL-6-responsive elements. The signaling-competent cytoplasmic domain regions of OSMR and LIFR were defined by the analysis of progressive carboxyl-terminal deletion constructs. The 36 residue carboxyl-terminal region containing the distal box 3 sequence motif of OSMR is required for signal transduction by the OSM-specific receptor. In contrast, signaling by LIFR did not display the same requirement for receptor domains and was not strictly dependent on the box 3 elements. The signaling by endogenous LIF and OSM receptors differed from that by IL-6R by the prominent activation of STAT5 as shown in the mouse hepatoma cell line, Hepa-1. The data suggest that the signaling specificity of the receptors for the three cytokines is determined by the composition of the cytoplasmic domains associated in the signal-competent receptor complex and that the signaling is not identical among these cytokine receptors.

Members of the hematopoietin receptor gene family are categorized based on similarities in their receptor-protein structure, ability to bind the same cytokines, and use of common subunits for assembly into a signal-competent receptor complex (1, 2). The receptors for the interleukin-6 (IL-6)-type cytokines, comprising IL-6, IL-11, LIF, OSM, CNTF, and carboxy-terminal-1, utilize gp130 as a common subunit in combination with cytokine-specific subunits (3). The current model of receptor action proposes that signal initiation requires minimally a dimerization of signal transducing cytoplasmic domains. IL-6 initiates formation of a signaling receptor complex by binding to a low affinity, ligand binding subunit (IL-6R). A signaling complex is then formed by recruitment and homodimerization of two gp130 molecules, resulting in a proposed hexameric structure that consists of two molecules each of IL-6, IL-6R, and gp130 (4, 5). While IL-11 bound to the Y chain of IL-11 receptor is proposed to also initiate homodimerization of gp130, other members of the IL-6-type cytokines initiate heterodimerization between gp130 and other signaling-competent receptor subunits. LIF, OSM, CNTF, and CT-1 all transduce signaling events through heterodimerization of gp130 and LIFR (first characterized as the low affinity LIF receptor; Ref. 6). While CNTF and possibly CT-1 (7, 8) both utilize a third cytokine-specific subunit, LIF and OSM require only gp130 and LIFR to form signaling receptor complexes. This complex has been described as the LIF/OSM receptor or the type I OSM receptor (6, 9). Additionally a second OSM-specific receptor complex has been identified, consisting of gp130 heterodimerized with an OSM-specific second subunit, OSMR (or OSMRβ), which form the type II OSM receptor (9, 10). The essential role of gp130 for signaling by these receptors is demonstrated by the fact that antibodies to gp130 are capable of neutralizing the cell responses to all IL-6-type cytokines (11). The characterization of a battery of anti-gp130 monoclonal antibodies has also revealed that the various ligand binding (or α-) subunits do not interact at identical sites on gp130 (12).

The signal transduction by IL-6-type cytokine receptors involves activation of the JAK/STAT pathway and, depending upon the cell type, pathways linked to Src- and Btk-related kinases (3, 13). The hallmark of gp130-dependent signaling is the activation of STAT3 and STAT1 through recruitment by binding to box 3 sequences in the cytoplasmic domain of gp130 (14, 15). In reconstitution experiments that utilize G-CSFR-gp130 chimeric constructs, the capability of gp130 to activate STAT5 is also detected (16). The similarity of IL-6, LIF, and OSM action extends beyond the activation of DNA binding activity of STATs and has been recognized by the transcriptional induction of similar sets of genes in particular cell types. The induction of type 2 acute phase plasma proteins in hepatic cells describes best the redundancy of action of IL-6-type cytokines (17). Despite the high degree of similarity in regulation, quantitative differences in the response to LIF, OSM, and IL-6 have been noted (18, 19).

The seemingly equivalent action of IL-6-type cytokines has led to the generally accepted notion that the gp130-dependent receptors execute essentially the same signaling functions (3). To assess whether this similarity in action also includes the

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‡ The abbreviations used are: IL, interleukin; APP, acute phase protein; CAT, chloramphenicol acetyltransferase; CNTF, ciliary neurotrophic factor; EMSA, electrophoretic mobility shift assay; G-CSF, granulocyte colony-stimulating factor; GH, growth hormone; LIF, leukemia inhibitory factor; OSM, oncostatin M; R, receptor; RE, response element; STAT, signal transducer and activator of transcription; PCR, polymerase chain reaction; HR, hematopoietin receptor; SIE, sis-inducible element.
signaling by the recently cloned type II OSM-specific receptor, OSMR, we evaluated its ability to activate STAT proteins and APP gene products by reconstitution. We compared this signaling with that mediated by LIF and IL-6. Analysis of functional domains within the cytoplasmic regions of OSMR and LIFR was facilitated by the use of G-CSF receptor chimeric molecules. G-CSF, a related cytokine, has been predicted to form a signaling receptor complex by homodimerization of two G-CSFR molecules (20). Utilizing chimeric molecules with the G-CSFR extracellular domain and OSMR or LIFR transmembrane and cytoplasmic regions allows signal transduction events initiated following G-CSF-mediated receptor homodimerization (21, 22). Here, we describe that OSMR does signal like LIFR, although the functional elements within the cytoplasmic domain of OSMR differ substantially from those of LIFR and gp130. Moreover, we show that the mechanism of signaling of OSMR and LIFR differs when signaling is carried out by heteromeric or homomeric cytoplasmic receptor domains. One distinguishing feature in the signaling of OSM and LIF from that of IL-6 is the more prominent activation of STAT5.

EXPERIMENTAL PROCEDURES

Cells—COS-1, human Hep3B, and HepG2 cells (23) were maintained in minimal essential medium. Rat H-35 cells (clone T-7;18; Ref. 17), HTC cells (24), and mouse Hepa-1 cells (25) were cultured in Dulbecco’s modified Eagles medium. The media contained 10% fetal calf serum and antibiotics. Cytokine treatments were carried out in serum-free minimal medium that included 100 ng/ml purified human IL-6 (Genetics Institute), LIF, OSM, or G-CSF (Immunex Corp.). For optimal activation of CAT reporter and endogenous APP genes, 1 μM dexamethasone was also added to the medium.

To identify the role of gp130 in OSM signaling, cells were incubated in transfection medium for 24 h, and the pseudotransfected with the following monoclonal antibodies to human gp130 (10 μg/ml): K5 and S1, which inhibit only OSM activity, and 144, which blocks the action of all IL-6-type cytokines (Ref. 12; antibodies generously provided by H. Gascan, Angers, France). After 30 min, the cytokines were added and treatments continued for 24 h.

DNA Constructs—Expression vectors for full-length and progressive carboxyl-terminal truncations of human OSMR, LIFR, and the G-CSFR-domain and OSMR-G-CSFR-LIFR chimeras are illustrated in Fig. 1. The expression vector containing OSMR sequences (Fig. 1A) was pdC409 (26). The full-length coding region for OSMR (10) was subcloned into the SalI and NotI sites. A series of truncated cytoplasmic domains was generated using PCR with 3′-oligonucleotides designed to incorporate in-frame termination codons followed by the recognition sequence for NotI. The G-CSFR-OSMR chimera expression vector was generated from the previously described expression vector pDC402 containing the extracellular domain of G-CSFR (residues 1–601) (21, 22, 27) and inserting a PCR-generated fragment encoding transmembrane and cytoplasmic domains of OSMR. Similarly, a series of chimeric receptors encoding truncated cytoplasmic OSMR domains was generated using PCR with the same 3′-oligonucleotides as used above for truncated OSMR. All sequences were confirmed by DNA sequencing.

Expression vectors for the human gp130 and series of LIFR and G-CSFR-LIFR chimeras are illustrated in Fig. 1B. The expression vector containing OSMR sequences (Fig. 1A) was pdC409 (26). The full-length coding region for OSMR (10) was subcloned into the SalI and NotI sites. A series of truncated cytoplasmic domains was generated using PCR with 3′-oligonucleotides designed as above for truncated OSMR. All sequences were confirmed by DNA sequencing.

Transfection—Hep3B, COS-1, and H-35 cells were transfected with DEAE-dextran (31) using a final concentration of 5 μg/ml plasmid (Hep3B and COS-1 cells) or 10 μg/ml (H-35 cells). To determine STAT activation by receptors, Hep3B and COS-1 cells were transfected with expression vectors for the receptors (2 μg/ml), and STAT isoforms (3 μg/ml), or empty vector yielding final DNA concentrations of 5 μg/ml. The transfected cells were cultured for 36 h, then maintained for an additional 18 h in serum-free medium before a 15-min cytokine treatment. One fifth of the washed and collected cells was solubilized by boiling in SDS buffer and used for Western analysis. The remainder of the cells was used to prepare whole cell extracts for electrophoretic mobility shift assay (EMSA) (32). For determining CAT gene regulation, Hep3B cells were transfected with CAT reporter constructs (3.5 μg/ml), receptor expression vector (0.5 μg/ml), and pIE-MUP (1 μg/ml), whereas H-35 cells received the same combinations but at double concentrations. HepG2 cells were transfected with calcium phosphate (33) using a total concentration of 20 μg/ml DNA. The mixture contained 15 μg/ml CAT reporter construct, 1 μg/ml receptor expression vector, and 2 μg/ml pIE-MUP. The transfected cultures were subdivided and after a 24-h recovery period were treated for 24 h with cytokines. CAT activity was determined in serially diluted cell extracts to ensure measurement in the linear range of the assay. The values were normalized to major urinary proteins produced by the co-transfected marker plasmid pIE-MUP and calculated relative to the control culture in each experimental series (defined as 1.0). The results of individual experimental series are reproduced in the figures and are representative examples of at least three independently performed experiments.

EMSA—Whole cell extracts containing 5 μg of proteins were applied to EMSA as described (32). 32P-End-labeled, double-stranded oligonucleotides of the high affinity SIEm67 (32) served as substrate for STAT1 and STAT3 and the 40-base pair TB-2 (34) as substrate for STAT5 and as low affinity substrate for homodimer STAT1 and STAT6/STAT7 heterodimers (6, 28). The contribution of STAT3 and STAT5 to the gel patterns was identified by supershift using polyclonal rabbit anti-STAT5B (C17; Santa Cruz Biotechnology) that reacts with both STAT5A and STAT5B, anti-STAT5A (C20; Santa Cruz Technology) that reacts specifically to STAT5A isoform, and mouse anti-STAT3 (C23; Santa Cruz Biotechnology).

Western Blot—A1iquots of cell lysate (30 μg of protein) were separa-
ated on SDS-polyacrylamide gels. Proteins were transferred to Immo-
bilon P membrane (Millipore) and reacted with rabbit serum raised against the hematopoietin domain of human OSMR (Ref. 10; Immunex Corp.), or goat antibodies to the extracellular domain of human G-CSF (Springville Laboratories, Roswell Park Cancer Institute). The immune complex was visualized by enhanced chemiluminescence reaction (Amersham).

Radioligand Binding Assays—Human OSM and human G-CSF were labeled with 125I-1ODOGEN (Pierce) to a specific activity of 67.7 and 54.4 Ci/mg, respectively. To determine surface expression of OSMR, Hep3B cells were transfected with the expression vector (2.5 μg/ml) for human gp130 (10, 22) alone (= control) or combined with the expression vector (2.5 μg/ml) for the OSMR. The cells were subdivided into six-well cluster plates. After 24 h of recovery, the cells were incubated for 2 h at 4 °C in binding medium (minimal essential medium containing 25 mM Hepes buffer, pH 7.5, and 1% bovine serum albumin) and increasing concentrations of 125I-OSM. The cells were washed four times with binding buffer and twice with phosphate-buffered saline. After solubilization of the cells with 0.1% SDS in 0.1% NaOH, the cell-associated radioactivity was measured by scintillation counting. G-CSF binding to transiently transfected cell cultures was determined by a single point binding assay using 0.5 nm 125I-G-CSF alone or in the presence of 100-fold excess of cold G-CSF (Agen, Inc.). The amount of competitive G-CSF binding (expressed in fmol bound/105 cells) was used as a measure for cell surface G-CSF activity. Mock-transfected cells served as control, for which no specific G-CSF binding was detected.

OSMR at the cell surface were quantitated by probing with anti-
OSMR. Hep3B cells were transfected with OSMR expression vectors or empty expression vectors (= control). After 36 h of recovery, the cells in six-well cluster plates were blocked by incubation with binding medium containing non-immune goat IgG (100 μg/ml) for 2 h at 4 °C. The cells were then reacted with rabbit anti-OSMR (1:100 dilution) for 1 h at 4 °C. After exhaustive washing with binding medium, the cells were treated for 1 h at 4 °C with 0.1 μg/ml 125I-goat-anti-rabbit IgG (16.1 μCi/mg). The radioactivity associated with the washed cells was measured by scintillation counting To recover surface-exposed OSMR, transfected Hep3B cells, in 6-cm diameter dishes, were treated with rabbit anti-OSMR serum. The washed cells were lysed in 1 ml of 25 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, pepstatin, and apro-
in, 1 mM NaF, and 1 mM sodium orthovanadate on ice for 10 min. The lysates was cleared by centrifugation and reacted with 25 μl of protein G-Sepharose. The bound proteins were eluted with SDS buffer, separated on SDS mini-gels, and analyzed by Western blotting using anti-OSMR.
RESULTS

**OSMR Expression and STAT Activation**—To identify whether the cloned full-length OSMR could initiate signaling, we selected Hep3B cells as an experimental system for receptor reconstitution. Unlike any other hepatoma cell line, these cells do not respond to LIF or OSM, yet have retained normal IL-6 responsiveness (Fig. 2A), indicating a lack of LIFR and OSMR while maintaining a functional gp130 and productive signal transduction mechanism (10, 22). Hep3B cells do not produce OSM; thus, we expected that introduction of OSMR should not encounter any autocrine effect as observed with LIFR (22).

Transfection of the full-length OSMR into Hep3B cells and treatment with OSM did not yield detectable activation of endogenous STATs (Fig. 2A). Considering that the transfection protocol yielded approximately 2–5% of transfected cells in the culture, as judged from expression of the co-transfected lacZ gene (data not shown), we assumed that the amount of STAT proteins available for activation by OSMR in these cells was below the detection of the EMSA. Therefore, we determined the signaling capability of OSMR by providing, through co-transfection, excess amounts of the individual STAT proteins known to be activated by gp130. Under these conditions, full-length OSMR mediated a ligand-dependent activation of the DNA binding activity of STAT1, -3, and -5B (Fig. 2B, OSMR/218).

To determine the functional relevance of the cytoplasmic domain elements box 1, 2, and 3, we introduced OSMR with progressive carboxyl-terminal truncation as shown in Fig. 1A. Removal of two box 3 sequences in OSMR(142) drastically reduced all STAT activation and with truncation beyond 105, STAT activation was no longer detectable. Western blot analysis indicated that the lower signaling activity was not due to reduced expression of the truncated OSMR (Fig. 3A, left panel). In fact, full-length OSMR was expressed at a much lower level than any of the truncated forms, suggesting a high specific signaling activity of the full-length OSMR. The surface display of OSMR was verified by two measurements. In the first measurement, $^{125}$I-OSM binding to transfected Hep3B cells was...
determined (Fig. 3B). Since OSMR does not function as a ligand binding subunit, but serves as an affinity converter for the low affinity OSM binding to gp130 (10), the reconstitution of OSMR function by the transient transfection approach is only recognizable qualitatively by the recovery of high affinity OSM binding. OSMR(218), OSMR(142), and OSMR(6), although not expressed at equal amounts on the cell surface as determined by antibody tagging and Western blotting (Fig. 3A, inset), contributed to a comparable fraction of high affinity OSM binding activity that was not observed in the vector control cultures. This result suggested that each of the transfected OSMR forms functionally interacted with gp130. The second measurement quantitated OSMR on the cell surface by antibody binding (Fig. 3C). The values obtained by this approach were in good agreement with the relative levels of expression detected by Western blotting of whole cell lysates (Fig. 3A, left panel).

**G-CSFR-OSMR Constructs Have Signaling Capability**—Since the OSMR signaling observed in Fig. 2B was attributed, in part, to the action of gp130, we determined whether the OSMR cytoplasmic domain could signal independently of gp130. To test this, we prepared chimeric receptors consisting of the extracellular domain of human G-CSFR linked to the transmembrane and cytoplasmic domains of OSMR (Fig. 1A). Transfection of Hep3B cells with G-CSFR-OSMR(218) carrying the full-length cytoplasmic OSMR domain mediated an activation of STAT1, -3, and -5B essentially the same as seen with OSMR(218) (Fig. 2B, center panel). Progressive truncation of cytoplasmic domains, however, did not yield the same abrupt loss of signaling capacity as with the OSMR. The cytoplasmic domain with 60 residues was sufficient for maintaining sub-
Signaling by OSMR and LIFR

FIG. 4. STAT protein signaling of G-CSFR-LIFR. A. Hep3B were transfected with expression vectors for G-CSFR-LIFR constructs as indicated at the top and for the STAT proteins listed at the right. The cells were treated with G-CSF for 15 min. Equal amounts of cell extracts were used for EMSA. B, six-well cultures of Hep3B cells transfected with the indicated constructs (5 μg/ml) were reacted with 125I-G-CSF and specific G-CSF binding determined (mean ± S.D. of three separate cultures). C, COS-1 cells were transfected with G-CSFR-LIFR constructs, STAT3, and STAT5B and analyzed as Hep3B cells in A. One additional set of COS-1 cell cultures was transfected with receptor constructs but without STAT expression vectors and used to determine activation of the endogenous COS-1 STAT protein (primarily STAT1) and receptor protein in total cell lysate by Western blot with anti-G-CSFR. The band at ~120 kDa above the G-CSFR-LIFR protein bands is an unidentified immunoreactive protein that is also present in non-transfected cells (see also Fig. 3A, right panel). This band serves as internal marker for protein loading and molecular size reference.

G-CSFR-LIFR

238 190 180 170 150 140 90 40 20
STAT3
STAT1α
STAT5B

Western Blot

EMSA

COS-1 STAT6

STAT3

STAT5B

G-CSFR-LIFR Constructs

From these results, we concluded that OSMR is a signal-transducing subunit with a STAT specificity similar to gp130. However, since the OSMR cytoplasmic region between 60 and 142 is capable of activating STATs in a G-CSFR-directed homodimeric form but is much less efficient in a heterodimer form with gp130, the mechanism by which OSMR transduced the intracellular signal appears dependent on the specific combination of cytoplasmic domains.

Signaling by LIFR Differs from OSMR—Since LIFR showed highest sequence similarity with OSMR (10) and LIFR forms a heteromeric complex with gp130 (6–8), we expected that LIFR, when introduced into Hep3B cells, would produce a STAT signaling pattern that is equivalent to that of OSMR. The only complication was that Hep3B cells, although being devoid of LIFR, produced LIF that caused an autocrine stimulation in LIFR reconstituted cells (22). Nevertheless, transfected full-length LIFR(238) was able to activate co-transfected STAT1, -3, and -5B (Fig. 2B, right panel). Progressive truncation of the cytoplasmic domain, however, did not result in a loss of signal capability as noted for OSMR. The STAT signaling action of the truncated LIFR appeared qualitatively similar to that seen with the G-CSFR-OSMR constructs, in that constructs without box 3 sequences (140 and 131) (Fig. 1B) were still effective at activating all three STATs. We could not assess the LIFR protein levels in transfected Hep3B cells, because none of the available anti-LIFR antibody preparations was suitable for detecting the LIFR protein by either immunoprecipitation or Western blotting.

The signaling capacity of the cytoplasmic domain of LIFR independent of gp130 was also determined by using G-CSFR-LIFR chimeric constructs (Fig. 1B). Transfected constructs showed that cytoplasmic domains with at least 140 residues were signaling through the STAT proteins (Fig. 4A). Unexpectedly, the construct with 90 residues, that was comparable to the 71 residue LIFR construct, did not show significant activity. Yet, when reduced to 20 residues, a prominent STAT signaling capability re-appeared. 125I-G-CSF binding verified that the various chimeric receptors were expressed at the cell surface (Fig. 4B).

The G-CSFR-LIFR expression vectors were less effective than the G-CSFR-OSMR vectors in producing protein levels in Hep3B cells detectable by Western blot. Therefore, we repeated the analysis of the same receptor constructs in COS-1 cells, which yielded a higher expression than Hep3B cells (Fig. 4C). Western blot indicated comparable amounts of G-CSFR-LIFR proteins with the exception of full-length (G-CSFR-LIFR(238)) that was severalfold lower. The data were also in agreement with the G-CSF binding values previously reported for some of the G-CSFR-LIFR constructs in CV-1 cells (22) and are also similar to the values from Hep3B cells (Fig. 4B). The transfected receptor forms with cytoplasmic domains from full-length to 150 residues activated endogenous COS-STATs (primarily STAT1) that showed the box 3 sequence specificity that had been reported previously (35). Overexpressed STAT3 and -5B were activated by the same receptor forms but also by the box 3-deficient constructs 140 and 90. With deletion to the last 20 residues, a STAT3 and -5 activation reappeared that was, especially for STAT5B, lower than in Hep3B (Fig. 4A). We conclude from these results that LIFR, like OSMR, has the ability to function as a homodimeric complex signaling through STAT1, -3, and -5 as the native receptor. Unlike the OSMR, the...
dependence on box 3 sequences for significant STAT activation in heteromeric but not homodimeric LIFR complexes was not observed.

**Gene Induction by OSMR**—The effects of the OSMR signals on induction of gene transcription were determined using two types of reporter gene constructs: IL-6RE-CAT and HRRE-CAT. The first construct requires a box 3-dependent signal for induction, and the second construct was independent of such a signal (16). Transfection of these constructs into Hep3B cells indicated that full-length OSMR regulated both constructs, whereas truncation to 142 residues eliminated these regulations (Fig. 5A, left panel). Furthermore, the observation that OSMR(181), which still contains the box 3a motif and was expressed on the cell surface of Hep3B cells (Fig. 3C), was also ineffective in mediating gene induction (data not shown), suggested that the COOH-terminal region including the box 3b motif was essential for OSMR action.

OSMR cytoplasmic domains in the G-CSFR chimera showed differences in signaling functions. The induction of the IL-6RE-CAT constructs was supported only by full-length domain, confirming its strict dependence on the box 3 signal. In contrast, HRRE-CAT induction was mediated by receptor constructs with an intracellular domain as short as 60 residues (Fig. 5A, right panel). The pattern of gene induction correlated with the potential of G-CSFR-OSMR to activate STATs (Fig. 2B). The data also illustrated that the capability of the receptors to activate STAT3 per se was not sufficient to achieve induction through IL-6RE.

To prove that transfected OSMR signaled through interaction with gp130 at specific sites defined by neutralizing monoclonal antibodies (12), OSMR-transfected Hep3B cells were treated with different antibodies during incubation with OSM (Fig. 5B). The antibodies K5 and S1, which had been defined as OSM-restricted anti-gp130 (12), did essentially abolish OSM induction of the reporter genes while minimally affecting IL-6 reaction. As expected, the pan-blocking anti-gp130, 144, reduced both OSM and IL-6 induction. In contrast to the suggested heterooligomer of OSMR and gp130, the G-CSFR-OSMR is predicted, based on the study of the native G-CSFR (20) to function as a homodimer. Since the most truncated G-CSFR-OSMR(6) was signaling-incompetent (Fig. 5A), we expected that this construct would act as a dominant-negative subunit in the context of dimeric G-CSFR complex. The transfection of different ratios of full-length and truncated chimeric receptors (see Fig. 3D for relative expression levels) yielded indeed an inhibition of gene induction (Fig. 5C) that was compatible with the proposed model of ligand-dependent homodimerization of the extracellular domain of G-CSFR and a requirement of two cytoplasmic domains for signaling.

**OSMR Signaling Specificity Is Not Cell Line Restricted**—The OSMR signaling was analyzed in a hepatoma cell line with a very limited repertoire of IL-6-type cytokine receptor systems. It was thus conceivable that the cells had also limitations in cultures were treated with medium alone (−) or containing OSM or G-CSF. CAT activity in equal amounts of cell extract was determined, and the -fold stimulation relative to untreated controls was calculated (numbers above autographic image). B, Hep3B cells were transfected with OSMR(218) and p(8xHRRE)-CAT as listed at the top left and right, respectively. Subcultures were treated with medium alone (−) or containing OSM or G-CSF. CAT activity in equal amounts of cell extract was determined, and the -fold stimulation relative to untreated controls was calculated (numbers above autographic image). C, Hep3B cells were co-transfected with pH(5xIL-6RE)-CAT and the listed amounts of G-CSFR-OSMR constructs. The cells were stimulated with G-CSF and CAT activity were determined relative to the untreated controls. Mean ± S.D. of three separate experimental series are shown.

**Fig. 5. Regulation of CAT reporter gene constructs by OSMR.**

A, Hep3B cells were transfected with expression vectors for the OSMR and G-CSFR-OSMR constructs and pH(5xIL-6RE)-CAT or p(8xHRRE)-CAT as listed at the top left and right, respectively. Subcultures were treated with medium alone (−) or containing OSM or G-CSF. CAT activity in equal amounts of cell extract was determined, and the -fold stimulation relative to untreated controls was calculated (numbers above autographic image). B, Hep3B cells were transfected with OSMR(218) and p(8xHRRE)-CAT as listed at the top left and right, respectively. Subcultures were treated with medium alone (−) or containing OSM or G-CSF. CAT activity in equal amounts of cell extract was determined, and the -fold stimulation relative to untreated controls was calculated (numbers above autographic image). C, Hep3B cells were co-transfected with pH(5xIL-6RE)-CAT and the listed amounts of G-CSFR-OSMR constructs. The cells were stimulated with G-CSF and CAT activity were determined relative to the untreated controls. Mean ± S.D. of three separate experimental series are shown.
intracellular signaling pathways. Hence, we determined the signaling pattern of OSMR in two additional hepatoma cell lines, HepG2 and H-35, which are known for a broader array of IL-6-type cytokine responses (17, 19). HepG2 cells could only be used for testing the G-CSFR\[\text{z}\]OSMR constructs due to the presence of the endogenous OSMR and LIFR (12). The transfected chimeric receptors regulated the two reporter gene constructs HRRE-CAT and IL-6RE-CAT (utilizing the element from the β-fibrinogen gene; Ref. 16) with a specificity that was comparable to that defined in Hep3B cells (Fig. 6). The activation through HRRE was achieved by constructs reduced to 60 residues, while activation through IL-6RE was only detected with the full-length OSMR domain.

The rat hepatoma cell line, H-35, permitted analysis of both G-CSFR\[\text{z}\]OSMR and OSMR constructs because of the relative minor response of these cells to human OSM through the endogenous LIF and OSM receptors (Fig. 7, control in lower panel). Full-length OSMR, but none of the deletion constructs, induced the HRRE-CAT gene above the level of the endogenous receptor (Fig. 7, upper panel). In agreement with the data from the other cell lines, the G-CSFR\[\text{z}\]LIFR chimera regulated the same reporter gene with the intracellular domain reduced to 60 residues (Fig. 7, lower panel). The results from the CAT gene experiments suggested that specificity of OSMR signaling to the gene elements was primarily determined by the OSMR receptor motifs and not by the hepatoma cell line used. We also conclude that this signaling, as well as STAT protein activation, is influenced by the composition of the cytoplasmic receptor domain complex. The signal-competent region (in the context of G-CSFR-OSMR) from 142 to 60 of the OSMR appears ineffective in combination with gp130.

OSMR/LIFR Activate More Prominently STAT5 than IL-6R—The data in Fig. 2B indicated that transfected OSMR and LIFR have the capability to activate overexpressed STAT1, -3, and -5. The analyses of STAT proteins activated by IL-6 in Hep3B (Fig. 2A) and by IL-6, LIF, and OSM in HepG2 or H-35 cells showed the highly prominent DNA binding activity of STAT1 and -3 (16, 32) but none that could be attributed to STAT5 (data not shown). The failure to detect STAT5 was explained in part by the low level of this STAT protein expressed relative to STAT3 (Fig. 8). To confirm that the STAT activation pattern of OSM, LIF, and IL-6 in the context of natural hepatic cells, we screened other hepatoma cell lines for their STAT5 expression level. Mouse Hepa-1 cells proved to contain approximately equal amounts of immunodetectable STAT3 and STAT5. Although reagents are not available to detect murine OSMR, these cells bear IL-6R and LIFR, but fail to respond to IL-6 or LIF by induction of any APP genes.\(^2\) Treatment of Hepa-1 cells with human OSM or IL-6, however, led to the activation of STAT3 detected by binding to SIE (Fig. 9A, upper panel) with a kinetics identical to that in HepG2, H-35 and Hep3B cells (Ref. 36 and data not shown). A prominent TB-2 binding activity was detected in OSM-treated cells and only trace level in IL-6-treated cells (Fig. 9B). Supershift analysis (Fig. 9B) indicated that the OSM-activated TB-2 complex contained primarily STAT5. In contrast, the IL-6-activated TB-2 complex contained STAT3, and only after a longer exposure was a low level of STAT5 detectable. These results demonstrated that in Hepa-1 cells, OSM, possibly by signaling through both LIFR and OSMR, differed in its action from IL-6, by the more prominent recruitment of STAT5. This finding implied that OSM or LIF treatment of hepatic cells would not generate a signaling truly redundant to IL-6.

\(^2\) K. K. Kuropatwinski and H. Baumann, unpublished data.
The ability of OSM and LIF to activate a slightly different STAT pattern from IL-6 might be physiologically relevant in cases where the liver cells are exposed sequentially to IL-6 and then to LIF or OSM. Under such conditions, which might exist during an inflammatory reaction (28, 37), one would predict that STAT5 is activated temporally delayed relative to STAT3. To show that STAT5 activation is not abrogated by IL-6 treatment, we incubated Hepa-1 cells first for 4 h with IL-6 and then challenged the cells with IL-6, LIF, or OSM (Fig. 10). We have shown that activation of STAT5 by OSM and LIF occur at comparable levels when tested in human hepatoma cells. Therefore, differences in the level of STAT5 activation seen in the murine Hepa-1 system (Fig. 10) may be attributable to cross-species receptor activation where human OSM is considerably less effective than human LIF (Fig. 7). Nevertheless, STAT3 activity was somewhat enhanced by all three challenging cytokines, STAT5 was notably activated only with OSM or LIF, indicating that IL-6 pretreatment had reduced but not extinguished that separate signaling pathway utilized by LIF or OSM.

**DISCUSSION**

This study demonstrates that full-length OSMR shares with LIFR a similar pattern of STAT protein activation (Fig. 2) and induces gene transcription (Figs. 5A and 7) through regulatory elements defined previously to be responsive to IL-6 (16, 22, 38). This similarity in cell responses has been explained by the involvement of the common gp130 subunit (3, 13). The fact that OSMR and LIFR have signal transducing capabilities on their own that are similar but not identical to gp130 probably contributes to the observation that the heteromeric combination of OSMR (or LIFR) with gp130 produce a signaling pattern different from IL-6R (Fig. 9; Ref. 18). The most notable differences appears to be the ability of OSMR/LIFR to activate STAT5 (Figs. 9 and 10).

The IL-6R, while equally effective as LIFR or OSMR in activating STAT3, achieves only a trace level of STAT5B. This seems contradictory to previous data that indicated the ability of gp130 to activate STAT5B (16). There, the signaling action of gp130 was determined as part of a G-CSFR/gp130 chimeric construct. The G-CSF-directed homodimer demonstrated strong activation of STAT5B. However, this particular activity is not as prominently realized in the native IL-6R complex. Two aspects need to be considered when interpreting the difference. First, the characterization of STAT activation pattern of the G-CSFR/gp130 subunits was performed in cells overexpressing STAT5; this may have magnified a low level STAT5 activation process. Second, the dimer-

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**FIG. 8.** Western analysis for STAT3 and STAT5 in hepatoma cells. Confluent cultures of untreated HepG2, Hep3B, HTC, Hepa-1, and H-35 cells were lysed directly in SDS buffer. Equal amounts of the cell extracts were separated in duplicate sets on one gel. After transfer of the separated proteins to Immobilon membrane, the left half of the membrane was reacted with anti-STAT3 and the right half with anti-STAT5. Both membrane halves were processed together for chemiluminescent detection. The image after a 10-s exposure is shown. The two lines at the center mark the position of the co-electrophoresed molecular size markers.

**FIG. 9.** STAT activation in Hepa-1 cells. A, monolayer cultures of Hepa-1 cells in 6-cm diameter dishes were treated with OSM or IL-6 for the indicated lengths of time. Equal amounts of cell extract were subjected to EMSA for SIE and TB-2 binding activity. SIE panel was exposed for 6 h, and the TB-2 pattern for 16 h. B, equal amounts of extracts from Hepa-1 cells treated for 15 min with OSM or IL-6 used in A were reacted with TB-2 in the presence of control antibody, anti-STAT5B, anti-STAT3, or anti-STAT5B plus anti-STAT3 and subjected to EMSA. The autoradiograms were exposed for 16 and 72 h as indicated. In a separate experiment (data not shown), extracts from treated cells were reacted with anti-STAT5A, which caused approximately half of the total TB-2 binding activity to supershift.

**FIG. 10.** Sequential activation of STAT proteins in Hepa-1 cells. Hepa-1 cell cultures in 6-cm diameter dishes were treated with 100 ng/ml IL-6 for 4 h (right panel) or with medium alone (left panel). Then, the medium was replaced with fresh medium that contained no cytokines (control) or 100 ng/ml IL-6, OSM, or LIF. After 15 min, cell extracts were prepared and subjected to EMSA for binding to SIE and TB-2. Autoradiograms after 3-h (SIE) and 6-h (TB-2) exposures are shown.

The ability of OSM and LIF to activate a slightly different STAT pattern from IL-6 might be physiologically relevant in cases where the liver cells are exposed sequentially to IL-6 and then to LIF or OSM. Under such conditions, which might exist during an inflammatory reaction (28, 37), one would predict that STAT5 is activated temporally delayed relative to STAT3. To show that STAT5 activation is not abrogated by IL-6 treatment, we incubated Hepa-1 cells first for 4 h with IL-6 and then challenged the cells with IL-6, LIF, or OSM (Fig. 10). We have shown that activation of STAT5b by OSM and LIF occur at comparable levels when tested in human hepatoma cells. Therefore, differences in the level of STAT5 activation seen in the murine Hepa-1 system (Fig. 10) may be attributable to cross-species receptor activation where human OSM is considerably less effective than human LIF (Fig. 7). Nevertheless, STAT3 activity was somewhat enhanced by all three challenging cytokines, STAT5 was notably activated only with OSM or LIF, indicating that IL-6 pretreatment had reduced but not extinguished that separate signaling pathway utilized by LIF or OSM.
Signaling by OSMR and LIFR

The initial functional analysis of LIFR truncation mutants had already indicated that LIFR with deleted cytoplasmic domains, including those missing the box 3 motifs as found in LIFR(131), were still capable of inducing gene expression through IL-6RE by the box 3-deficient LIFR(131) (22). This finding alone was sufficient to question whether LIFR functions simply as a heterodimer with gp130, or as a presumed heterodimer with OSMR. For these two receptors, however, the use of G-CSFR chimeras was not intended to mimic natural receptor combinations (the models predicted only heterodimers with gp130) (3, 6–8, 9, 10), but served as an experimental tool to detect signaling capacity of the receptor domains independent of gp130. The analyses of G-CSFR-OSMR constructs suggest that the G-CSFR-mediated dimerization is leading to the assembly of a cytoplasmic OSMR dimer that signals more effectively than the gp130-OSMR heterodimer (Figs. 2B and 5–7). The observation that cytoplasmic domains ineffective in the native OSMR construct do signal when present in G-CSFR chimeras suggests that these receptor domains are able to recruit part of the signal-transducing machinery of the cells. Since these OSMR sequences are only detectably active as homodimers, we have to conclude that either these elements may not have equivalent counterparts in the gp130, or the dimerization reaction between OSMR and gp130 is structurally different.

A significantly different picture emerges with LIFR. The initial functional analysis of LIFR truncation mutants had already indicated that LIFR with deleted cytoplasmic domains, including those missing the box 3 motifs as found in LIFR(131), were still capable of inducing gene expression through IL-6RE in hepatoma cells (22). This finding alone was sufficient to question whether LIFR functions simply as a heterodimer with gp130, in particular, in view of the model that hematopoietin receptors require minimally a dimer of cytoplasmic signal transducing domains. This study confirms the unusual signaling activity of the LIFR at the level of STAT protein activation. In contrast to findings with OSMR, the LIFR cytoplasmic domain did not indicate any fundamental differences in the signaling capabilities when tested either as a homodimer form as part of G-CSFR chimeras or as a presumed heterodimer with gp130 (Figs. 1 and 2). How can we explain induction of an IL-6RE by the box 3-deficient LIFR(131) (22)? Two possible explanations come to mind. 1) The short cytoplasmic LIFR domains in combination with the gp130 (Fig. 2) or with itself (Fig. 4A) are exceedingly signaling-competent, or 2) the functional LIFR may associate into a larger complex that also includes homodimers of gp130 and/or LIFR.

Although the precise mechanisms of OSMR and LIFR signal initiation need to be elucidated, our data have provided a possible explanation for the difference observed in the responses of hepatic cells to treatment with OSM, LIF, and IL-6 (18, 19, 36). The combinations of cytoplasmic domains of the signal-transducing receptor subunits determine the specificity of the signaling event. The most obvious difference was the engagement of STAT5 by LIFR and OSMR (Fig. 10). Although this feature was only demonstrated here for Hepa-1 cells, which express appreciable amounts of STAT5 (both STAT5A and -5B; data not presented), the activation of STAT5 by OSM and LIF, but not by IL-6, could also be detected in mouse L-cells and NIH 3T3 cells. Considering that the LIFR serves as a signaling subunit for both LIF and OSM, the precise contribution of the OSM-specific receptor for the signaling event is difficult to assess in these mouse cells.

The relevance of STAT5 in cytokine receptor signaling and gene regulation in rodent liver cells has recently been documented in two separate experimental settings: response to treatment with growth hormone (GH) (41, 42) and to adjuvant-induced inflammation (28). STAT5 activation by GH receptor was previously documented in various cell systems (e.g. see Ref. 43, and references therein), and its occurrence in liver cells was thus not unexpected. A role of GH as a mediator of hepatic acute phase response, however, appears unlikely (e.g. see Refs. 44 and 45, and references therein). Considering that cells critical for initiating the systemic inflammatory response, such as macrophages, monocytes, and stromal cells, are producers of LIF and OSM (46), an acute phase-mediated activation of hepatic STAT5 by these cytokines in the circulation seems highly probable. It is uncertain whether the liver cells will always be in the position to deliver a STAT5 signal to the LIF or OSM challenge. This is primarily due to the additional process of GH-controlled STAT5 activation level and subcellular localization, both of which are in part dictated by the pulsative or continuous mode of GH delivery occurring in male and female animals, respectively (47).

The hepatic cell response to IL-6-type cytokines is dominated by the transcriptional activation of APP genes (37). The regulation of these genes requires the concerted action of constitutive and inducible transcription factors. Among these, STAT3 (or APRF) has gained prominence in part by its preferred activation by the cytokine receptors (32, 48, 49) and in part by the findings that the APP genes contain in their cytokine response regions STAT3 binding sites (50) that are inducible by STAT3 (51, 52) or are inhibited by transdominant forms of STAT3 (53, 54). A specific regulatory role for STAT5 in controlling expression of APP genes has not been recognized until recently. Two of the most inflammation-responsive APP genes of the rat, α2-macroglobulin and serine protease inhibitor 3, contain in the 5′-flanking region a STAT-binding element that mediates induction by both STAT3 and STAT5 (28, 36, 45). The specific combination of each STAT protein to the inflammation induction of these genes in acute phase liver is not yet known. It may be recognized in animals with targeted knockout of the STAT genes or STAT function in the liver.

The potential of activating STAT5 by OSM or LIF, separate from activation of STAT3 by IL-6, may also offer an explanation for the delayed presence of nuclear STAT5 DNA binding activity in adjuvant-induced acute phase liver (28). The suggested mechanism proposes a sequential exposure of the liver to IL-6 and OSM or LIF. To verify this possibility, the physiologically relevant changes in the circulating concentrations of the latter two cytokines need to be determined (55). Additionally, it is not yet known whether OSM- or LIF-activated STAT5 significantly contributes to the expression of APP genes, such as α2-macroglobulin or SPI-3. Taken together, the results and conclusions from this study have offered a guide for new and direct analyses of the mechanisms of cytokine induction of APP genes and the specific role of OSMR and LIFR in hepatic acute phase response.

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