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Signaling of Type II Oncostatin M Receptor*

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Oncostatin M (OSM) mediates its bioactivities through two different heterodimer receptors. They both involve the gp130-transducing receptor, which dimerizes with either leukemia inhibitory receptor β or with OSM receptor β (OSMRβ) to generate, respectively, type I and type II OSM receptors. Co-precipitation of gp130-associated proteins, flow cytometry, polymerase chain reaction, and tyrosine phosphorylation analyses allowed the characterization of both types of OSM receptors expressed on the surface of different cell lines. It also allowed the detection of a large size protein, p250, that specifically associates to the type II OSM receptor components and that is tyrosine-phosphorylated after the activation peak of the gp130·OSMRβ heterocomplex. The restricted expression of type I OSM receptor by the JAR choriocarcinoma cell line, and type II receptor by the A375 melanoma cell line, permitted the characterization of their signaling machineries. Both type I and type II OSM receptors activated Jak1, Jak2, and Tyk2 receptor-associated tyrosine kinases. The information is next relayed to the nucleus by the STAT3 transcriptional activator, which is recruited by both types of OSM receptors. In addition, STAT5b was specifically activated through the gp130·OSMRβ type II heterocomplex.

The signaling pathway differences observed between the common type I LIF/OSM receptor and the specific type II OSM receptor might explain some of the bioactivities specifically displayed by OSM.

Oncostatin M (OSM)[1] is a multifunctional cytokine belonging to the interleukin-6 (IL-6) family and that shares many properties with those reported for LIF (1). Both OSM and LIF are able to inhibit the spontaneous differentiation of embryonic stem cells (2). They also induce the terminal differentiation of the M1 murine myeloid cell line (3). Like the other IL-6 family members, OSM has been shown to induce acute phase protein synthesis in hepatocytes (4). Beside these LIF-shared bioactivities, OSM displays some specific properties and can inhibit the growth of a variety of solid tumor cells (5) and triggers *in vitro* the proliferation of Kaposi's sarcoma-derived cell lines (6, 7). In addition, expression of an oncostatin M transgene in the early T cell lineage stimulates a dramatic accumulation of T cells in the mice lymph nodes (8).

The redundancy of OSM and LIF biological properties is in part explained by the shared use of a common heterocomplex receptor composed of the gp130 signal transducing protein associated with the LIF receptor β (LIFRβ) component (9, 10). Binding experiments have pointed out the existence of a second and different high affinity receptor for OSM (also referred to as type II OSM receptor) (9–11). Type II OSM receptor complex binds OSM in a specific manner and is not recognized by LIF (9–11). Type II receptor also involves a gp130-transducing component that associates with a second receptor subunit very recently isolated as OSM receptor β (OSMRβ), which displays an apparent molecular mass of 180 kDa (12). Comparison of OSMRβ to gp130 and LIFRβ shows high homology levels in both domain structure and primary amino acid sequence.

Signaling mediated through the common type I LIF/OSM receptor was studied in detail previously (13–17). Activation of type I receptor by LIF leads to the recruitment of Jak1, Jak2, and Tyk2 receptor-associated kinases, which are in turn relayed by STAT3 transcription factor to transduce the signal to the nucleus.

Until recently the nature of the type II OSM receptor was less well characterized, and very little information regarding its signaling machinery has been reported. It has been shown that the type II OSM receptor recruits the serine-threonine MAP kinase pathway (11), as well as the *src*-related kinase $p62^{yes}$ (18).

In the present study we analyzed the expression of both type OSM receptors in a series of cell lines, and by using the A375 melanoma cell line specifically expressing the type II OSM receptor complex, we studied its signaling transduction pathway.

MATERIALS AND METHODS

Cells and Reagents—A375 melanoma, JAR choriocarcinoma, KB epidermoid carcinoma, HepG2 hepatoma, and SK-N-MC neuroblastoma cell lines were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum. OSM (2×10^6 units/mg) was purchased from Peprotech (Canton, MA), and purified recombinant LIF (10^8 units/mg) produced in the Chinese hamster ovary cell line was kindly provided by Dr. K. Turner (Genetics Institute, Boston, MA). B-T6 (IgG1), B-P4 (IgG1), B-K5 (IgG1), and B-R3 (IgG2a) anti-gp130 mAbs were described in detail elsewhere (19). mAb 32209 (IgG2b) directed against LIFRβ was obtained from R & D Systems (Minneapolis, MN). mAbs recognizing STAT1 (IgG1) and STAT3 (IgG1) were obtained from Transduction Laboratories (Lexington, KY). Rabbit anti-STAT4, anti-STAT5b, and antibodies recognizing the carboxyl-terminal sequence of LIFRβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antibodies directed against Jak1, Jak2, and Tyk2 and 4G10 anti-phosphotyrosine mAb were from Upstate Biotechnology, Inc. (Lake Placid, NY).

Flow Cytometry Analysis—The immunofluorescence studies were performed following the standard protocols. The samples and the appropriate isotype controls were then analyzed on a FACSCAN from

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[1] The abbreviations used are: OSM, oncostatin M; IL-6, interleukin-6; LIF, leukemia inhibitory factor; LIFRβ, LIF receptor β; OSMRβ, OSM receptor β; mAb(s), monoclonal antibody(ies); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter.

Becton Dickinson (Mountain View, CA).

OSMRβ PCR Analysis—cDNA was synthesized from 5 μg of total RNA by using an antisense oligonucleotide located at positions 2050–2073 of OSMRβ sequence as primer (12). For the PCR analysis 40 amplification cycles (93 °C, 30 s; 57 °C, 1 min; 72 °C, 1 min) were carried out by using a second derived primer corresponding to the positions 1586–1609 of the OSMRβ published cDNA. Amplified products were analyzed on a 2% agarose gel.

Tyrosine Phosphorylation Analysis—Tyrosine phosphorylation analysis was performed as described in detail previously (19, 20, 23). After immunoprecipitation the complexes were isolated with beads coupled to protein A, subjected to SDS-PAGE, and transferred onto an Immobilon membrane (Millipore, Bedford, MA). The membranes were subsequently incubated with the relevant primary antibody before being incubated with the appropriate second antibody labeled with peroxidase for 60 min. The reaction was visualized on an x-ray film by using the ECL reagent (Amersham Corp., Les Ulis, France) according to the manufacturer's instructions. The membranes were then stripped in 0.1 M glycine HCl, pH 2.5, for 1–6 h depending on the previously used antibodies, neutralized in 1 M Tris-HCl, pH 7.6. Before reblotting, disappearance of all remaining signal was checked by a 30-min film exposure. For cell surface biotinylation, the A375 cells were starved for a 4–6-h period before being stimulated with 50 ng/ml OSM for 5 min. Then, the cells were washed with PBS and incubated for 20 additional minutes in PBS, 0.1 M Hepes, pH 8, 0.5 mg/ml water-soluble sulfo-NHS-biotin (Pierce) (21). After the contact period the reaction was stopped by adding 0.1 M Tris/HCl, pH 8. The cells were lysed and the proteins immunoprecipitated with the B-T6 anti-gp130 mAb and analyzed as described above. After tyrosine phosphorylation analysis and stripping of the blot, the visualization of the biotinylated proteins was carried out by using a streptavidin/peroxidase solution (1/5,000) from Dako (Trappes, France)

DNA-binding Protein—After stimulation with OSM, whole cell extracts were prepared as described previously (24, 25). Solubilized proteins were incubated for 18 h in the presence of 1 μg of double strand 5′-biotinylated oligonucleotides bound to streptavidin-agarose beads. STAT3 and STAT5b high affinity interacting GAS motifs were derived, respectively, from the c-*fos* gene (*sis*-inducible element, SIEM 67) (5′-CATTTCCCGTAAATCTTGTCG-3′) and from the FcγR gene (*GRR*) (5′-GTATTTCCCAGAAAAGGAAC-3′) as reported before (24, 25). The complexes were precipitated, subjected to SDS-PAGE, and transferred onto an Immobilon membrane (Millipore). The membranes were then stained by using an anti-STAT3 mAb (Transduction Laboratories) or an anti-STAT5 polyclonal antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized with ECL Western blotting reagent as described above. Specificity of the observed signals was controlled by introducing in OSM-activated cell extracts a 100-fold excess of unlabeled double strand DNA as competitor for the biotinylated oligonucleotides.

RESULTS AND DISCUSSION

Biochemical Characterization and Expression of Type II OSM Receptor—In the present work we analyzed the cell surface expression of both gp130 and LIFRβ by flow cytometry in different cell backgrounds. The obtained results and their correlation with the sensitivity of the studied cell lines to the OSM cytolytic activity led us to concentrate our investigation on the A375 melanoma and JAR choriocarcinoma cell lines. Both cell lines expressed the common signaling receptor protein, gp130 (Fig. 1*A*). In contrast the LIFRβ subunit was only detected on the JAR cell surface, but not on the A375 melanoma cell line. A PCR analysis revealed the presence of a very low level of LIFRβ gene transcription in A375 cells, but we could not surface-detect protein (Fig. 1, *A–C*). Analysis of the proliferative responses of the two studied cell lines grown in the presence of OSM showed a strong inhibition of the A375 cell line growth, but not of the JAR choriocarcinoma cells. LIF was without effect in both cultures (Ref. 19 and data not shown). The conclusions of these results were similar to those reported previously by other groups using different approaches (9, 11, 12, 22). The results indicate that despite the fact of a lack of detectable expression of LIFRβ on the A375 cell surface, OSM could mediate a functional response in this cell line.

We recently characterized the B-T6 anti-gp130 mAb (19).

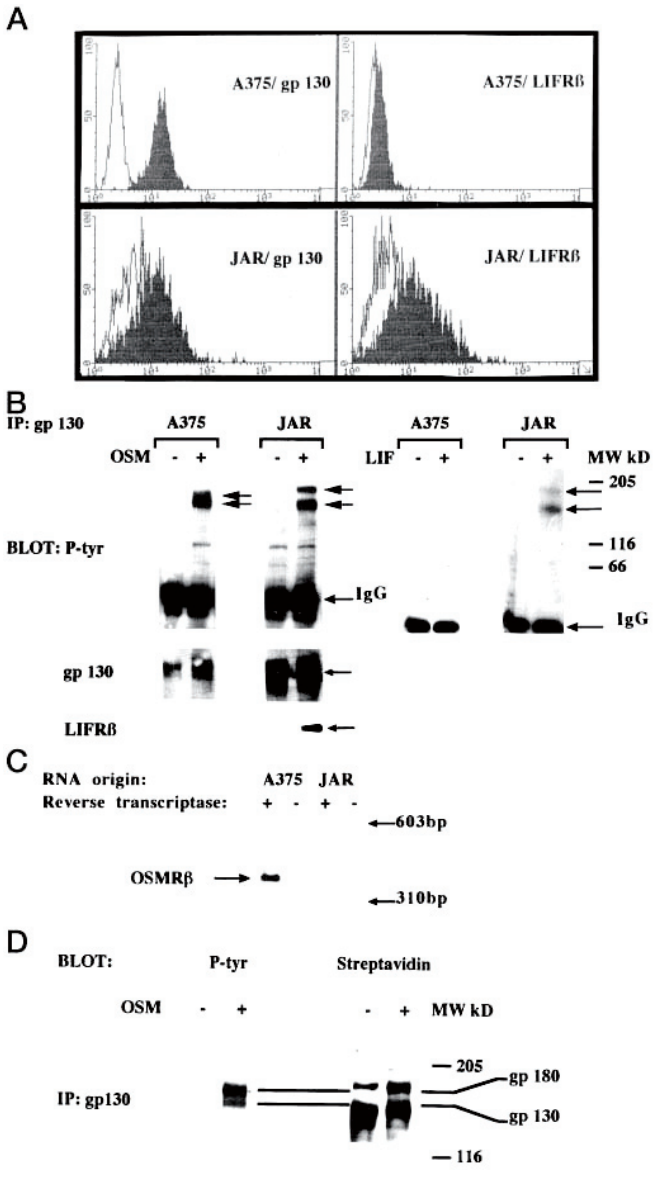

FIG. 1. **Biochemical characterization and activation of type II OSM receptor.** *A*, expression of gp130 (*left panels*) and LIFRβ (*right panels*) was monitored by FACS analysis using the B-K5 anti-gp130 mAb (IgG1) or the 32209 anti-LIFRβ mAb (IgG2b) (*black histograms*). The *white histograms* represent the signals recorded with an isotype matched control mAb. *B*, analysis of tyrosine-phosphorylated proteins associated with gp130 after OSM and LIF activation. Cells were incubated in the presence or in the absence of 50 ng/ml OSM or LIF for 10 min. After cell lysis in 1% Brij 96, proteins were immunoprecipitated with the B-T6 anti-gp130 mAb and their tyrosine phosphorylation level analyzed. Identification of gp130 and LIFRβ was carried out by restaining the blot with B-P4 anti-gp130 mAb and with an anti-LIFRβ polyclonal antibody, respectively. *C*, PCR analysis of OSMRβ expression. cDNAs were synthesized from 5 μg of total RNA by using an antisense oligonucleotide located at positions 2050–2073 of OSMRβ sequence. 40 amplification cycles were carried out by using a second derived primer corresponding to positions 1586–1609. Amplified products were analyzed on a 2% agarose gel. *D*, A375 cells were activated with OSM, and their cell surface was labeled with soluble sulfo-NHS-biotin, as indicated under "Materials and Methods." After immunoprecipitation, proteins were stained with a monoclonal antibody directed against phosphotyrosine (*left panel*) and then with streptavidin coupled to peroxidase (*right panel*).

B-T6 is a mute antibody, which did not interfere with the biological responses mediated by the cytokines of the IL-6 family, but allows the co-precipitation of receptor subunits that associate with gp130. Receptor tyrosine phosphorylation events observed in response to OSM in A375 and JAR cell lines

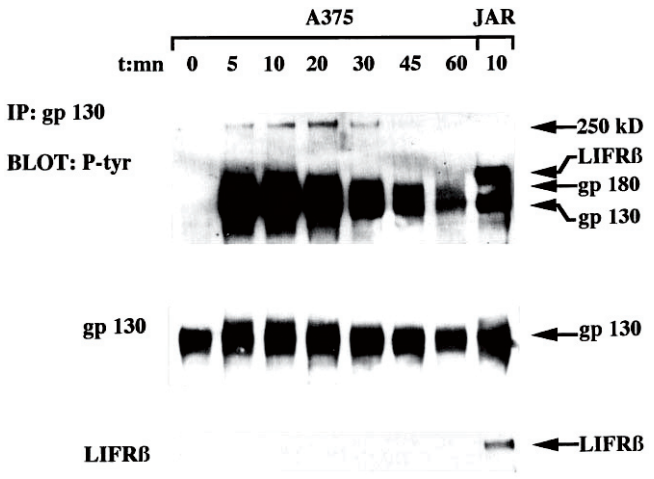

FIG. 2. **Kinetic of A375 cell line activation by 50 ng/ml OSM.** Proteins were immunoprecipitated with the B-T6 mAb, separated by SDS-PAGE, transferred onto an Immobilon membrane, and labeled with 4G10 anti-phosphotyrosine mAb, with the B-P4 anti-gp130 mAb, or with a polyclonal antibody against the LIFRβ. The OSM-activated JAR cell line was used as control (*right lane*).

were studied. Treatment of the JAR cells with OSM for 10 min resulted in the induction of gp130 tyrosine phosphorylation and of an additional protein with a molecular mass of 190–210 kDa, co-precipitating with gp130 (Fig. 1*B*). Reblotting the filter with an antibody directed against LIFRβ identified the associated protein as LIFRβ, in agreement with the FACS patterns.

A similar approach with the A375 cell line allowed the detection of the activated form of gp130 associated with a protein displaying a slightly higher molecular mass of 150–180 kDa (Fig. 1*B*). Membrane reprobing indicated that the LIFRβ subunit was not detectable in the A375 cell line and that the observed 150–180-kDa product very likely corresponded to the recently cloned gp180/OSMRβ (12). This notion was further reinforced by analyzing the presence of OSMRβ mRNA in A375 and JAR cell lines by PCR amplification (Fig. 1*C*). The presence of an OSMRβ transcript was clearly detectable in A375 melanoma cells, but not in JAR choriocarcinoma-derived cell line. The transmembrane expression of OSMRβ was studied by labeling the A375 extracellular surface with a water-soluble biotin ester. After activating the cells with OSM we analyzed the gp130-associated protein(s) that were both tyrosine-phosphorylated and biotinylated (21). The obtained results show the presence in the A375 cells of a 150–180-kDa transmembrane protein very likely corresponding to OSMRβ (Fig. 1*D*). Interestingly, in the absence of activation, a nonphosphorylated form of gp180 could be to some extent co-precipitated with gp130, as detected after streptavidin/peroxidase staining of the membrane. A slight decrease in gp130 electrophoresis mobility was also observed upon OSM activation as noticed previously (23). Treatment of the A375 cells with LIF did not allow the recruitment of either gp130 or gp180/OSMRβ, further reinforcing the specificity of the receptor expressed by this cell line (Fig. 1*B*). Moreover, pretreatment of the A375 cells with a molar excess of B-R3 anti-gp130 mAb, previously reported to inhibit the homo- or heterodimerization of gp130 with its neighboring receptors, abrogated the induction of tyrosine phosphorylation of both receptor subunits (Ref. 19 and data not shown). Altogether, our results corroborate results obtained by performing binding analyses of LIF and OSM and demonstrate a specific expression of type II OSM receptor at the cell surface of the A375 melanoma cell line (9, 11). In contrast, the JAR choriocarcinoma cell line only expressed on its surface the common LIF·OSM receptor complex.

TABLE I
Expression of p250 and OSM receptor components in different cell lines

Cells were activated for 10 and 20 min in the presence of 50 ng/ml OSM. After cell lysis in 1% Brij 96, gp130 and associated proteins were immunoprecipitated with the B-T6 anti-gp130 mAb and their tyrosine phosphorylation level analyzed.

Tissue type	Cell line	gp130	LIFRβ	OSMRβ	p250
Melanoma	A375	+	−	+	+
Epidermoid carcinoma	KB	+	+	+	+
Hepatoma	HepG2	+	+	+	+
Neuroblastoma	SK-N-MC	+	+	−	−
Choriocarcinoma	JAR	+	+	−	−

Type II OSM Receptor Complex Specifically Associates with a 250-kDa Protein—A detailed kinetic study of OSM responses in A375 cells revealed a simultaneous activation of gp130 and gp180 that was maximally recruited after a 5–10-min contact with OSM. JAR cell line was incubated for 10 min with the cytokine and used as control (Fig. 2). Interestingly, in addition to gp130 and OSMRβ, a third product with an apparent molecular mass of 250 kDa was detected in the A375 cell line. Compared with the receptor heterodimer, tyrosine phosphorylation of the 250-kDa protein, or p250, is delayed 10–15 min and also becomes dephosphorylated after 30 min. Staining of the membrane with an anti-LIFRβ-specific antibody failed to detect any signal in the A375 cells, but gave a readily detectable band in the JAR cell line, indicating that p250 was not related to LIFRβ. p250 was not detectable after activation of type I OSM receptor regardless of the contact time with the activating cytokine (Fig. 2, *right lane*, and data not shown). Expression of p250 was not restricted to the A375 cell line as summarized in Table I, and it could be co-precipitated with gp130 in all the cell lines expressing OSMRβ/gp180 we have analyzed so far. In contrast, we could not detect the p250 receptor-associated protein in cell lines expressing solely the type I OSM receptor. Similarly, by using the SK-N-MC neuroblastoma cell line that expresses all the known high affinity receptors for the IL-6 family members (19, 23) (with the exception of OSMRβ), we were not able to co-associate p250 to gp130 after activating the cells by any of the six related cytokines belonging to this family. This result suggests a specific recruitment of p250 in the context of the type II OSM receptor or a lack of p250 expression in the SK-N-MC cells. In line with our observation, it should be noted that a similar association of a 250–300-kDa large size protein to type II OSM receptor has already been observed in some instances (18). These results indicate that activation of the gp130·gp180 heterocomplex by OSM leads to a delayed activation of a p250 protein, which seems to interact in a specific way with the OSM receptor type II complex.

Type OSM II Signaling Pathway Involves Jak1, Jak2, and Tyk2 Tyrosine Kinases and STAT3 and STAT5b Transcription Factors—Receptor activation of the IL-6/LIF family of cytokines results in immediate phosphorylation of the transduction subunits by the Jak family members. Homo- or heterodimerization of the gp130 signal transducer was shown to induce activation and recruitment of Jak1, Jak2, and Tyk2 (13–15). It was demonstrated previously that gp130/LIFRβ type I OSM receptor utilize the same Jak kinases for tyrosine phosphorylation (13–15). This led us to investigate whether the Jak-Tyk kinases were involved in signaling initiated through type II OSM receptor. Fig. 3*A* shows that tyrosine phosphorylation of Jak1, Jak2, and Tyk2 signaling proteins is induced after treatment of A375 cells by OSM. Tyk2 phosphorylation observed in response to OSM is weaker than the signals detected for Jak1 and Jak2 as reported previously for the other members of the IL-6 family (13). Nevertheless, immunoblots of lysates revealed

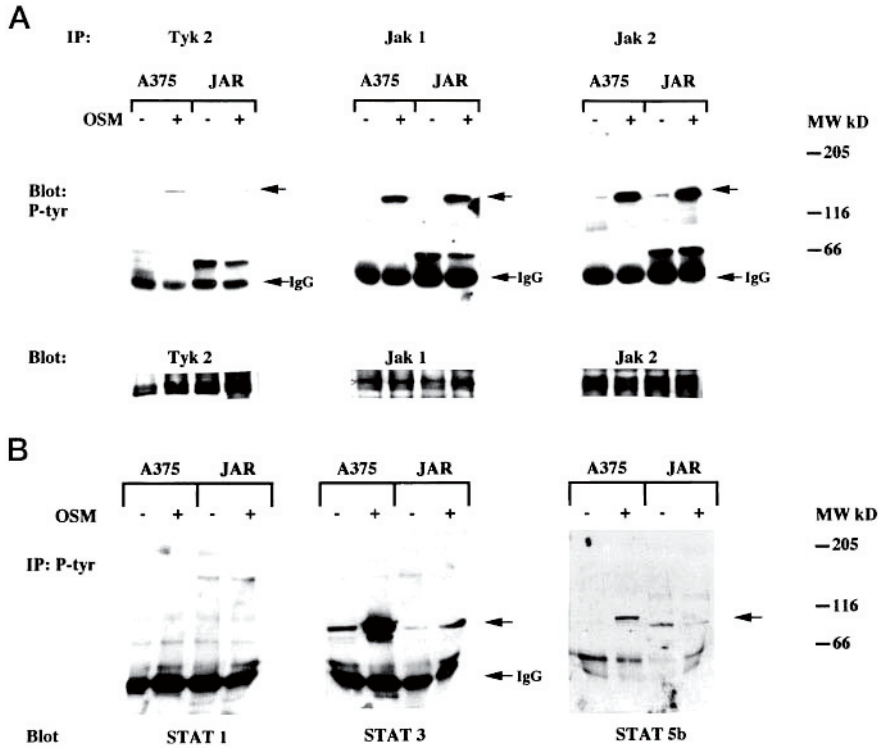

FIG. 3. **Recruitment of the Jak and STAT family members by type I and type II OSM receptors.** *A*, cells were incubated in the presence or in the absence of 50 ng/ml OSM for 10 min. After cell lysis in 1% Brij 96, proteins were immunoprecipitated with the indicated antibodies directed against Jak1, Jak2, or Tyk2 and the tyrosine phosphorylation level of the Jak kinases analyzed by using the 4G10 anti-phosphotyrosine mAb. Sample loading was controlled by restaining the blots with the antibodies used for the immunoprecipitation. *B*, cells were activated as in *A*, but lysed in the presence of 1% Triton X-100. The immunoprecipitations were performed by using the 4G10 anti-phosphotyrosine mAb, since the anti-STAT antibodies used were more efficient for membrane staining rather than for the precipitation step.

a clear expression of Tyk2. Thus, these data indicate that the Jak signaling cascade induced by activation of type II OSM receptor is similar to that induced through the type I OSM receptor as it appears for the JAR cell line (Fig. 3*A*).

Downstream signaling events were further analyzed by studying the transcription factors known as STATs, which are tyrosine-phosphorylated at the cytoplasmic level before being translocated to the nucleus (16, 17). As shown in Fig. 3*B*, stimulation of the A375 cells with OSM elicits tyrosine phosphorylation of STAT3 and STAT5b proteins. Similarly, STAT3 was also recruited by the gp130·LIFRβ heterocomplex, but STAT5b activation level remained unchanged after OSM stimulation of the JAR cell line, sustaining the notion of a specific recruitment of STAT5b by type II OSM receptor. We also looked for a possible induction of STAT1 and STAT4 tyrosine phosphorylation by activating both type receptors with OSM, but no detectable signal could be observed (Fig. 3*B* and data not shown). To further establish the activation and recruitment of STAT3 and STAT5b in response to OSM, interaction of STAT transcription factors with DNA GAS motifs was studied. DNA target sequences were chosen, respectively, in the promoters of c-*fos* and Fcγ receptor genes, as described previously (24, 25). The results presented in Fig. 4 show that activation of type II OSM receptor by its cognate ligand induces a binding of both STAT3 and STAT5b to DNA. Several isoforms, or phosphoforms of STAT proteins, were observed in agreement with the published studies (25). In contrast, and despite a clear expression of STAT5b in the JAR cell line (data not shown), its activation was not observed in response to the stimulation of type I LIF/OSM receptor. The obtained results are in line with the tyrosine phosphorylation study presented in Fig. 3. Altogether these results indicate that OSM type I and II receptors are both able to activate STAT3, whereas STAT5b is specifically recruited by the OSM type II receptor.

Concluding Remarks—Type I OSM receptor involving gp130/LIFRβ preferentially recruits the Jak1, Jak2, and Tyk2 tyrosine kinases as it was reported after its activation by LIF (13). The information is next relayed to the nucleus by STAT3 transcriptional activator (16–17). No variation could be observed in the detection and activation of the STAT1 signaling protein. STAT1 gene inactivation in the mouse has underlined its essential implication in mediating the antiviral properties of interferons (26, 27). No evidence for an alteration of the phys-

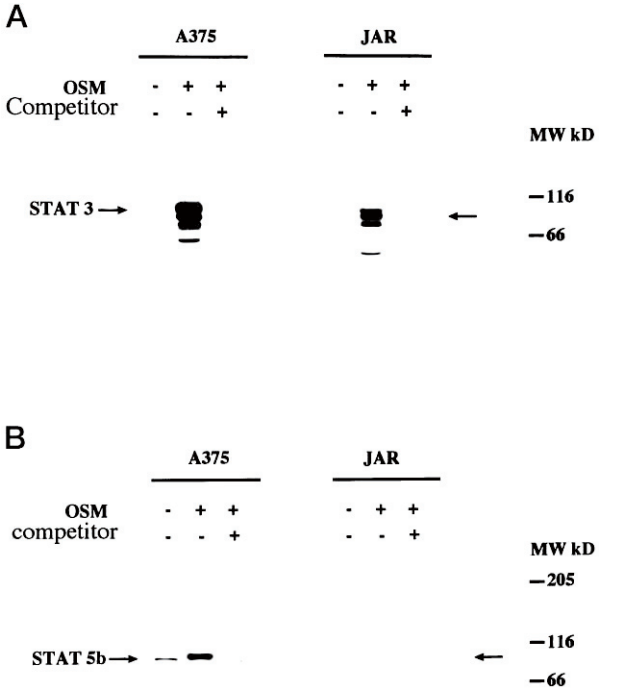

FIG. 4. **STAT binding to GAS oligonucleotides.** *A*, OSM-activated or resting cell extracts were incubated in the presence of a double strand-biotinylated SIEM oligonucleotide and then precipitated with streptavidin-agarose beads. The complexes were subjected to SDS-PAGE and the proteins transferred onto a membrane. Western blot analysis was performed by using an anti-STAT3 mAb and the ECL reagent. Specificity of the observed signal was assessed by introducing a 100-fold excess of nonbiotinylated competitor oligonucleotide in the incubation step. *B*, samples were treated as in *A* except that oligonucleotides were derived from the GRR site and that the membrane was stained by using an anti-STAT5b polyclonal antibody.

iological responses dependent upon the IL-6 cytokine family was detected in the STAT1 deficient mice.

It was demonstrated by Thoma *et al.* (11) that type II OSM receptor strongly activates the MAP kinase pathway. In the present work we observed that the gp130·gp180 heterocomplex was able in addition to STAT3 to also recruit the STAT5b transcription factor. Similarly, it was recently demonstrated that IL-6 can activate STAT5b in rat liver (28). We also pointed out the presence of a large size protein, p250, which can be co-precipitated with the activated gp130/gp180 subunits, but whose tyrosine phosphorylation is delayed when compared with the heterocomplex receptor. Preliminary experiments performed after an external biotin labeling of the A375 cell surface indicate that p250 is a transmembrane protein, but we do not know yet whether it can directly bind to the ligand. It was reported recently that activation of the α-interferon receptor complex could be modulated by a large size receptor-associated protein that was identified to be the CD45 phosphatase (29). We cannot exclude the eventuality that related processes might occur to regulate the activation of some of the IL-6 family receptors. Similarly, it was reported that the phosphotyrosine phosphatase 1D might directly couple the intracytoplasmic portion of gp130 to activation of the gp130·LIFRβ heterocomplex (16).

OSM displays some specific biological properties not shared by LIF and can, for instance, inhibit the growth of solid tumor-derived cell lines (5) and trigger the proliferation of Kaposi's sarcoma-derived cell lines (6, 7) and of some T cells subsets (8). These observed differences might be explained by the tissue distribution of type II OSM receptor and by the specificity of its signaling.

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