Cloning and expression of an alternatively spliced mRNA encoding a soluble form of the human interleukin-6 signal transducer gp130

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Abstract The membrane-bound gp130 glycoprotein acts as an affinity converting and signal transducing receptor (R) for interleukin-6 and several other cytokines. In this work, we RT-PCR amplified gp130 cDNA using primers flanking the sequence encoding the transmembrane domain of gp130. We observed in blood mononuclear cells, in addition to the expected 333-bp length fragment, a second major band of 418 bp. Sequencing of the 418-bp fragment and its genomic counterpart showed a new 85-bp exon located in the sequence encoding the extracellular region of the gp130 protein. This exon is most likely due to alternative splicing and leads to a frame-shift resulting in a stop-codon 1 bp before the transmembrane coding region. Correspondingly, supernatants from chinese hamster ovary cells transfected with this cDNA contained 4–5 times more soluble (s) gp130 than supernatants from cells transfected with a cDNA encoding the membrane-bound gp130 protein. Both gp130 and alternatively spliced sgp130 were also transcribed by the myeloma cell lines XG-1, XG-2, XG-4, XG-4CNTF XG-6, XG-7, XG-9, XG-10, U266 and RPMI 8226. However, XG-4A cells derived from XG-4 cells, but growing independently of exogenous IL-6, did not transcribe sgp130 mRNA. A possible interference with intracrine stimulatory factors by alternatively spliced sgp130 needs to be further investigated.

Key words: gp130; Soluble receptor; Alternative splicing

1. Introduction

The membrane bound glycoprotein gp130 was initially demonstrated to be a high-affinity converter and signal transducer for IL-6 [1]. Later, it was also shown to be part of the receptor complexes involved in the binding of leukemia inhibitory factor (LIF) [2,3], oncostatin M (OSM) [3,4], IL-11 [5], ciliary neurotrophic factor (CNTF) [2], and cardiotrophin-1 (CT-1) [6]. This would explain why these cytokines share several effects [5–7]. For some of the cytokines in this group, a specific α-chain receptor component has been identified [8–11]. Thus, the IL-6 receptor (R) complex consists of at least two glycoproteins, the α component (gp80/IL-6R) and the β component (gp130) [1]. gp130 does not by itself bind IL-6 but associates with the complex of IL-6 and gp80/IL-6R. Both gp80/IL-6R and gp130 belong to the hematopoietic growth factor family of cytokines [12,13]

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Soluble (s) forms of many cytokine receptors have been described (reviewed in [14,15]). For example, sgp130 is found in normal human serum at concentrations of 390 ± 72 ng/ml. The serum sgp130 consists of two different forms with M_r of around 90 and 110 kDa [16].

Whereas the sIL-6R, the sCNTFR and the sIL-11R have been shown to have a ligand agonistic effect in several in vitro systems [17–19], most soluble cytokine receptors, among these sgp130, act as antagonists to their respective ligands [16]. However, soluble receptor function in cytokine transport or protection against degradation have also been suggested [15].

Soluble cytokine receptors are generated by either proteolytic cleavage (shedding) of their membrane-bound counterparts, or by alternative mRNA splicing, resulting in a transcript encoding a soluble receptor. A combination of these two mechanisms has been described for sIL-6R generation [20,21].

Investigating a cell line transfected with the cDNA encoding the membrane-bound gp130, Müllberg et al. reported that sgp130 is generated by a shedding mechanism, but the extent of gp130 shedding was much lower than that of gp80/IL-6R [22]. Here, we report the identification and cloning of a gp130 generated by alternative splicing of a new 85-bp exon. In addition we demonstrate that this mRNA encodes a sgp130 molecule when transfected into chinese hamster ovary (CHO-K1) cells.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells were isolated from normal individuals using Lymphoprep (Nycomed Pharma, Oslo, Norway) according to the manufacturer's instructions. Multiple myeloma cell lines XG-1, XG-2, XG-4, XG-4CNTF, XG-4A, XG-6, XG-7, XG-9, XG-10, U266 and RPMI 8226 were grown as previously described [23,24]. By growing XG-4CNTF cells in decreasing concentrations of recombinant (r) human (h) IL-6, a cell line (XG-4A) growing independently of exogenously added IL-6 was established. This cell line was grown in RPMI 1640, supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Roskilde, Denmark). CHO-K1 cells were grown in RPMI 1640+10% (FCS)

2.2. Primers

Primers were selected for PCR analysis using the gp130 sequence submitted to Genbank by Hibi et al. (GenBank accession No. M57230) [12] (Fig. 1). Primers were purchased from Pharmacia Biotech (Allerød, Denmark).

2.3. RNA extraction, nuclease treatment and poly-A mRNA purification

Cells, 2×10^7, were harvested by centrifugation in 50-ml polypropylene tubes (Nunc, Roskilde, Denmark) and total RNA was extracted as described by Chomczynski et al. [25]. RNA integrity was

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investigated by running a sample on a 1% TBE gel followed by ethidium bromide staining. In some experiments, the RNA was treated with RNAse and/or DNAse before reverse transcription. In a total volume of 20 μl, 10 μg total RNA was incubated with 2 μl 10×RQ1 buffer (Promega Madison, WI, USA), 0.5 μg RNAse, (DNAse-free, Boehringer Mannheim, Mannheim, Germany) and/or 1 u RQ1 DNAse, RNAse-free (Promega). After incubation for 30 min at 37°C, samples were heated to 65°C for 10 min. Poly-A mRNA was purified using a Dynabeads mRNA direct kit (Dynal, Skøyen, Norway). In an Eppendorf tube, 100 μl $(dT)_{25}$ Dynabeads were washed in binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol (DTT)), and 20 μl pretreated RNA solution was added. After 5 min, the tube was placed in a magnetic particle concentrator (MPC) and the supernatant removed. After washing 2 times with washing buffer (10 mM Tris-HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA) with 0.1% LiDS and once with washing buffer without LiDS, the poly-A mRNA was eluted in 20 μl elution buffer (2 mM EDTA, pH 8.0) at 65°C for 2 min.

2.4. cDNA synthesis

Synthesis of cDNA was carried out using Superscript II reverse transcriptase (RT) (Gibco BRL). After denaturation at 75°C for 5 min and cooling to 4°C, RNA (1 μg total RNA or 5 μl poly-A mRNA) was incubated with 4 μl 5×reaction buffer, 2 μl DTT (0.1 M), 2 μl rRNasin (40 u/μl), 2 μl dNTP (10 mM), 1 μl oligo$(dT)_{15}$ (50 μM), 1 μl Superscript II RT (50 u/μl) and incubated at 42°C for 1 h followed by 5 min at 95°C.

2.5. PCR

In 0.2-ml PCR tubes (Perkin Elmer, Allerød, Denmark) a master mix consisting of 3 μl 10×PCR buffer II (Perkin Elmer), 9.5 μl H_2O, 1 μl dNTP (10 mM of each), 5 μl upper primer (10 μM), and 5 μl of lower primer (10 μM). An Ampliwax PCR gem (Perkin Elmer) was added and the tube heated to 75°C for 5 min. After cooling and 10 min incubation at room temperature, a master mix consisting of 20 μl H_2O, 10×PCR buffer II, 3 μl $MgCl_2$, 1 μl of template cDNA and 0.5 μl Taq polymerase (2.5 u/μl) was added. Samples were placed in a PTC 200 thermocycler (MJ Research, Watertown, MA, USA) and denatured for 4 min at 96°C. After denaturation, 35 cycles of PCR were run. Each cycle consisted of denaturation at 96°C, for 20 sec, annealing at 60°C for 30 sec, and extension at 72°C for 80 sec. After the last cycle, extension was performed for 5 min followed by cooling to 4°C.

2.6. Southern blotting

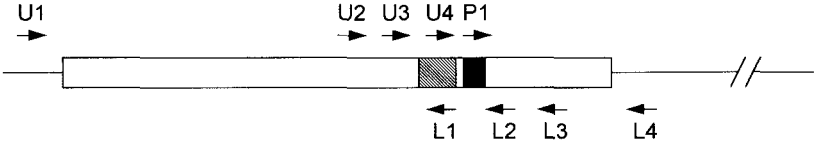
PCR samples were analyzed on an 1% TAE agarose gel. Amplified DNA was detected by ethidium bromide staining and capillary transferred to Zetaprobe blotting membranes according to the manufacturers instructions (Bio-Rad, Hercules, CA, USA). Membranes were hybridized overnight at 45°C with γ-^{32}P-ATP (Amersham, Little Chalfont, UK) 5′-end kinase-labeled oligonucleotide probes in hybridization solution (9×SSC, 0.015 M EDTA, 3×Denhardt's solution, 0.15 mg/ml denatured salmon sperm DNA, 0.015% SDS). Membranes were washed at room temperature 6×20 min followed by 3×10 min at 45°C in 2×SSC, 0.05% SDS. After washing, the membranes were exposed to radiosensitive film.

2.7. Cloning and DNA sequencing

PCR products were gel purified using the Wizard PCR purification kit (Promega) and cloned in the pCRII vector supplied in the TA-cloning kit (Invitrogen, Leek, The Netherlands). Sequencing was done on a automatic DNA sequencer (ALFexpress, Pharmacia Biotech) using the dideoxynucleotide sequencing method using a thermosequenase cycle sequencing kit (Amersham).

2.8. Cloning and expression of the alternatively spliced sgp130

The TA cloned U2-L2 PCR product containing an 85-bp insertion was digested with *Eco*RI and *Bst*EII and the resulting 269-bp fragment was gel purified using the Wizard PCR purification kit (Promega). The *Xho*I/*Xba*I fragment of gp130 cDNA was excised from the gp130 mammalian expression vector pRcg10-gp130 [26]. After gel purification, the *Xho*I/*Xba*I insert was further digested with *Eco*RI and *Bst*EII resulting in 2 fragments of approx. 1 and 2 kb. The 3 fragments were cloned back into the expression vector pRcg10 resulting in pRcg10-sgp130. The expression vectors were transfected in CHO-K1 cells using the DOTAP lipofection reagent (Boehringer Mannheim). Five μg DNA+30 μl DOTAP reagent were transfected to approx. 0.5×10^6 cells in Nunc culture dishes (60 mm in diameter). The medium was changed after 24 h. Supernatants were harvested 24 h later and sgp130 concentrations measured using a sgp130 ELISA with a detection limit of 80 pg/ml (R and D Systems, Oxon, UK). The expression of membrane-bound gp130 by transfected CHO-K1 cells



Name	Sequence (5′-3′)	Position on gp130 cDNA*
U1	aatggaaaaagcatgacatttagaa	-94 - -70
U2	aaatgaaactgctgtgaatgtggatt	1689 - 1714
U3	tggcagcatacacagatgaaggtgg	1775 - 1799
U4	gcttgagtcttgccaacgaggacctt	--
L1	aaggtcctcgttggcaagactcaag	--
L2	tgccttggaggagtgtgaggtgacca	1996 - 2021
L3	gccgctcctctgaatctaac	2358 - 2377
L4	tagctttactttataggtactgctga	2783 - 2808
P1	tcgtgcctgtttgcttagcattcct	1865 - 1899

* Primer bp positions are numbered according to the gp130 sequence published by Hibi et al. [12].

Fig. 1. Primers used for gp130 PCR amplification. The gp130 coding region is boxed. The transmembrane coding region is shown in solid and the hatched section represents the new exon described in this paper. *Primer bp positions are numbered according to the gp130 cDNA sequence published by Hibi et al. [12].

was investigated by flow cytometry, using the GPX7 anti-gp130 antibody (ab), as described previously [27].

3. Results

3.1. Characterization of a splice variant of the gp130 mRNA

cDNA was prepared from polyA$^+$ mRNA extracted from BMNC. A 333-bp fragment containing the transmembrane coding region of the gp130 cDNA was PCR amplified by use of the primer pair U2-L2 (Fig. 1). Besides the expected 333-bp fragment, several fragments with higher MW were also detected (Fig. 2). The fact that all PCR products disappeared after RNAse treatment before RT suggests, that they were generated from different gp130 mRNA variants. DNAse treatment before RT had no effect (Fig. 2). TA-cloning and complete sequencing of the most abundant of the PCR products with a higher MW than expected, revealed that it corresponds to the sequence of gp130 mRNA with an 85-bp insertion located just before the transmembrane coding domain of the gp130 cDNA. The predicted amino-acid sequence generated by this mRNA showed that the 85-bp insertion results in a frame-shift (Fig. 3A). The sequence following the insertion encodes 4 amino acids followed by a stop codon placed 1 bp before the sequence encoding the transmembrane part of gp130, strongly suggesting that the cDNA encodes a truncated soluble form of gp130.

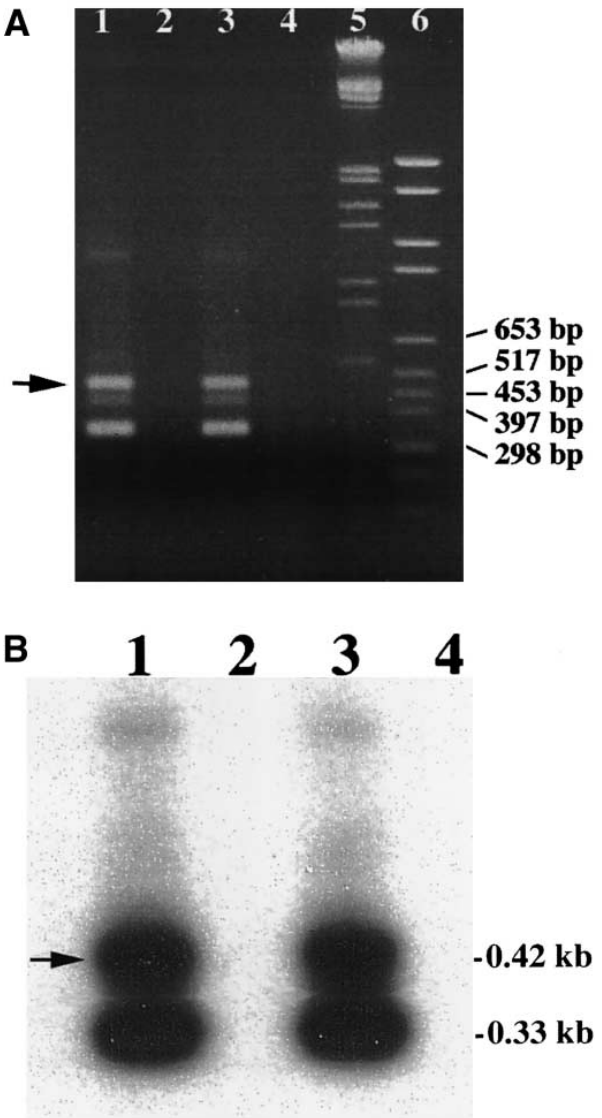


Fig. 2. A) Ethidium bromide staining of an RT-PCR run in 1% agarose. Normal BMNC cDNA was PCR amplified with primers U2 and L2. mRNA purification and cDNA synthesis were carried out with untreated RNA (lane 1), RNAse treated RNA (lane 2), DNAse treated RNA (lane 3), or with both DNAse and RNAse treatment (lane 4). MW markers: λ DNA×*Eco*RI+*Hin*dIII (lane 5) and pBR328DNA×*Bgl*I+pBR328 DNA×*Hin*fI (lane 6). B) Southern blot of the same gel as in A hybridized with γ-^{32}P-ATP 5′-end kinase-labeled P1 oligonucleotide. Arrows indicate the sgp130 PCR product.

Two primer pairs U1-L1 and U4-L4, were used to PCR amplify the 5′- and 3′-ends, respectively, of the cDNA containing the 85-bp insertion. Complete sequencing of these showed that, apart from the 85-bp insertion, they were identical with the gp130 sequence published by Hibi et al. [12] (not shown).

3.2. Genomic PCR

To investigate the exon-intron junctions of the newly discovered exon, the primer pairs U3-L1 and U4-L2 were used to PCR amplify genomic DNA isolated from normal BMNC. Sequence analysis of the fragments, demonstrated the presence of an intron flanking each side of the sgp130 specific exon. In accordance with the known consensus sequences for exon-intron junctions [28], both intron sequences began with GT at their 5′-end and ended with AG at their 3′-end (Fig. 3B),

3.3. Expression of sgp130 mRNA in CHO-K1 cells

The sgp130 cDNA was cloned in the mammalian expression vector pRcg10 containing a cytomegalovirus promoter [26]. This construct was transiently transfected into CHO-K1 cells. As controls, untransfected CHO-K1 cells, CHO-K1 cells transfected with pRcg10-gp130 or pRcg10 without insert were used. The concentrations of sgp130 protein in transfected CHO-K1 cell supernatants were determined by an sgp130 specific ELISA. As shown in Fig. 4, no sgp130 protein was detected in untransfected or CHO-K1 cells transfected with the pRcg10 vector without insert. The sgp130 concentration in supernatants from pRcg10-sgp130 transfected cells was 0.63 ng/ml, whereas pRcg10-gp130 transfected cell supernatants contained 0.14 ng/ml.

Flow cytometry of the transfected CHO-K1 cells, using the GPX7 anti-gp130 ab, showed that only cells transfected with pRcg10-gp130 expressed membrane-bound gp130. No membrane-bound gp130 was detected on untransfected cells or cells transfected with pRcg10-sgp130 (results not shown).

3.4. Alternative sgp130 mRNA in MM cell lines

To investigate if the alternative sgp130 mRNA was expressed by MM cell lines, RNA isolated from eleven different MM cell lines and BMNC from three normal individuals were reverse transcribed. The cDNA was PCR amplified using the primer pair U4-L3. As shown in Fig. 5A, the expected 591-bp band, containing the 85-bp insertion, was amplified from all cDNA samples, except from the MM cell line XG-4A. Several other bands were also seen, both with higher and lower MW than the expected. All PCR amplified bands were unaffected by DNAse treatment of the RNA before RT, whereas pre-

A.

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   1800                                                    1840
5′  ggg aag gat ggt cca gaa ttc act ttt act acc cca aag ttt gTC
    Gly Lys Asp Gly Pro Glu Phe Thr Phe Thr Thr Pro Lys Phe Val

    TCA TGG ATC CAT TAT GGC TTC TTC ACA TGG CTT GAG TCT TGC CAA
    Ser Trp Ile His Tyr Gly Phe Phe Thr Trp Leu Glu Ser Cys Gln

                                                       1841
    CGA GGA CCT TAC AGC TTA GTT GAA GAA ATT TTG CAT AAc tca agg
    Arg Gly Pro Tyr Ser Leu Val Glu Glu Ile Leu His Asn Ser Arg

          1856
    aga aat tga  3′
    Arg Asn ***
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B.

5′ **taccccaaagtttg**gtaagaaata.....tctgtag**TCTCATGGATCCATT**

ATGGCTTCTTCACATGGCTTGAGTCTTGCCAACGAGGACCTTACAGCTTAGTTG

AAGAAATTTTGCATAAgtaaatcaga.....ttttaag**ctcaaggagaaat** 3′

C.

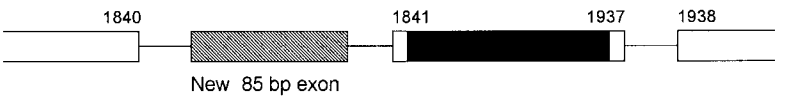

Fig. 3. (A) cDNA and predicted amino acid sequence of sgp130 cDNA. The new 85-bp exon nucleotide sequence is written in capital letters. (B) Exon/intron junctions flanking the new 85-bp exon (capital letters). Consensus GT-AG sequences are underlined. Exons are written in bold. (C) Schematic representation of part of the gp130 genomic sequence. The coding regions are boxed. The hatched region represents the new 85-bp exon and the solid section represents the transmembrane coding region. The size is not representative of the actual exon/intron length in bp. Bp numbering is according to Hibi et al. [12].

treatment with RNAse resulted in the disappearance of all amplified products (results not shown).

To further investigate the levels of sgp130 mRNA by the cell lines XG-4, XG-4CNTF and XG-4A, cDNA was amplified both with primers specific for both gp130 and sgp130 (U2-L3) and primers specific for sgp130 (U4-L3). Blotting of the PCR products followed by hybridization with a γ-^{32}P-labeled oligonucleotide (P1) demonstrated that all three cell lines transcribed gp130 mRNA, whereas sgp130 mRNA was only detected in XG-4 and XG-4CNTF cells (Fig. 5B). The 0.35 kb band seen in lanes 3, 4, 6, 7 and 13 in Fig. 5A was not detected by the hybridization (Fig. 5B), indicating that this band was unrelated to gp130. Sequencing of the 0.35-kb band confirmed this and showed that it was amplified by the 5′ primer alone. In contrast, the 1.9 and 1.4-kb bands found after RT-PCR of mRNA isolated from XG-4 and XG-4CNTF were also detected by the oligonucleotide hybridization (Fig. 5B).

4. Discussion

We have demonstrated the presence of an alternatively spliced mRNA encoding a soluble form of gp130 of 624 aa. The mRNA has an 85-bp exon inserted in the part encoding the extracellular part of the receptor. This exon gives rise to a frame-shift resulting in a stop-codon, 1 bp before the beginning of the transmembrane coding part of the mRNA. The genomic sequence of gp130 showed that the 85-bp exon was flanked by introns and an intron was also found between cDNA bp 1937 and 1938.

Several other soluble cytokine receptors, belonging to the hematopoietic growth factor superfamily of receptors, are also

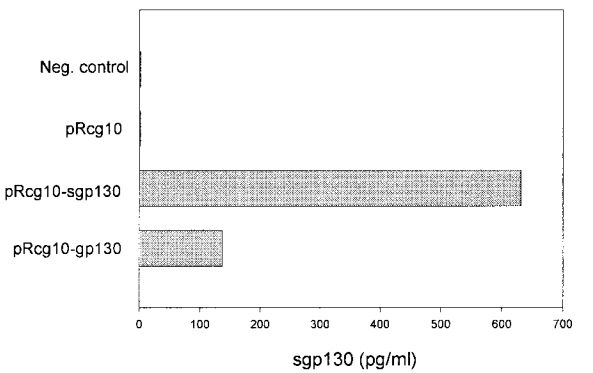

Fig. 4. Soluble gp130 in supernatants harvested from transiently transfected CHO-K1 cells. Cells, 0.7×10^6, were transfected with 5 μg of each plasmid.

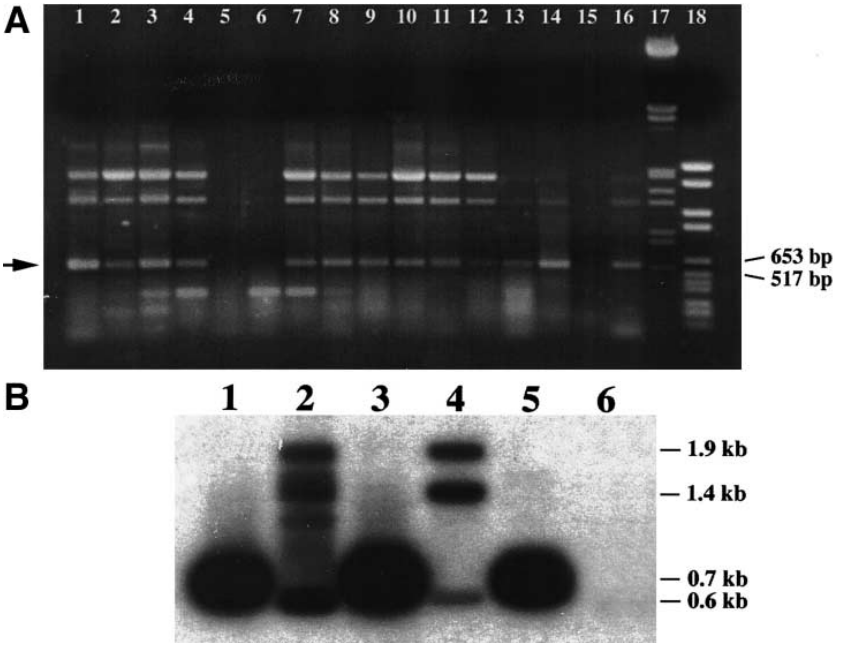

Fig. 5. A) sgp130 RT-PCR using primer pair U4-L3 of RNA isolated from normal BMNC and MM cell lines. RNA from normal BMNC (lanes 1, 14 and 16), from MM cell lines XG-1 (lanes 2 and 11), XG-2 (lane 3), XG-4 (lane 4), XG-4CNTF (lane 5), XG-4A (lane 6), XG-6 (lane 7), XG-7 (lane 8), XG-9 (lane 9), XG-10 (lane 10), U266 (lane 12), RPMI 8226 (lane 13), negative control with no RNA added in cDNA synthesis (lane 15). MW markers: λ DNA×*Eco*RI + *Hin*dIII (lane 17) and pBR328DNA×*Bgl*I + pBR328 DNA×*Hin*fI (lane 18). Arrow indicates PCR product of the expected 591 bp. (B) Southern blot of RT-PCR products from MM cell lines. cDNA from XG-4 (lanes 1 and 2), XG-4CNTF (lanes 3 and 4 and XG-4A (lanes 5 and 6) was PCR amplified using the primers U2-L3 (lanes 1, 3 and 5) or U4-L3 (lanes 2, 4 and 6). The blot was hybridized with γ-^{32}P-ATP 5′-end kinase-labeled P1 oligonucleotide.

generated by mRNA splice variants, adding an exon that causes protein chain termination before the transmembrane domain. This is the case for the soluble forms of the murine (m) IL-4R [29], the human IL-5R [30], the mLIFR [31], and the mouse and rat leptin receptor [32,33].

During morula to blastocyst transition, human embryos have been shown to produce a mRNA encoding a truncated gp130, in which the cDNA sequence between bp 1840 and 1938 is missing. This leads to a frameshift resulting in 45 new amino acids before a stop-codon. Consequently, this mRNA does not encode the transmembrane and intracellular part of the protein [34]. This is in good accordance with our findings of two introns being present between gp130 cDNA bp. 1840/1841 and 1937/1938. Although protein expression studies of this cDNA were not reported, it is predicted that it encodes another form of soluble gp130.

Using a cell line stably expressing both membrane-bound gp130 and gp80/IL-6R, Müllberg et al. showed that both molecules were proteolytically cleaved and released as soluble receptor proteins. Compared to the gp80/IL-6R, the extent of gp130 shedding was almost negligible, and was not stimulated by the phorbol ester PMA [22]. In accordance, we also found that CHO-K1 cells transfected with pRcg10-gp130 released sgp130, although at a concentration 4–5 times lower than that of CHO-K1 cells transfected with pRcg10-sgp130. The sgp130 thus seems to be generated both by a shedding and an alternative splicing mechanism. This might explain the presence of two different species of sgp130 as described by Narazaki et al. [16]. Which mechanism of sgp130 generation that is quantitatively the most important in non-transfected cells is not answered by the present studies. Antibodies generated against the terminal end of either form of sgp130 should be able to discriminate between them and provide the means of answering this question.

Several cytokines using gp130 as a signal transducer, such as IL-6, LIF, OM, IL-11 and CNTF, are important growth factors for myeloma cells [24,35,36]. In some myeloma cell lines, IL-6 sensitivity is augmented by the addition of sIL-6R [27,37]. The myeloma cell responsiveness to IL-6 depends on the relative amounts of IL-6, sIL-6R, membrane-bound gp80/IL-6R and membrane-bound gp130 [27]. RT-PCR analysis using the primer pair U4-L3, detected the alternatively spliced sgp130 mRNA in both normal BMNC and 10 of 11 different myeloma cell lines. Furthermore, this RT-PCR detected 2 bands of higher MW than expected (Fig. 5). Their size and the disappearance of all bands after RNAse pretreatment of the RNA showed that they were not due to genomic DNA contamination of the RNA preparations. The 1.9 and 1.4-kb bands further hybridized to a gp130 specific oligonucleotide probe, indicating that these bands could be other gp130 splice variants. This is presently being investigated.

It has been shown that proliferation of several transformed cell lines is stimulated in an autocrine way, i.e. the cells produce their own growth factor(s). For instance, the myeloma cell lines U266 and RPMI 8226 both produce IL-6, IL-6R and gp130. Levy et al. demonstrated that the cellular proliferation of these cell lines was not inhibited by neutralizing antibodies against IL-6. However, the use of antisense technology to specifically inhibit the expression of IL-6 inhibited cellular proliferation [38]. Similar results have been found in cultures of non-transformed human fibroblasts [39].

The above findings are believed to be due to the presence of an intracrine stimulatory mechanism, in which ligand receptor binding and signal induction takes place inside the cells most

likely in secretory granules containing both the growth factors and their receptors [40]. This, and the fact that most soluble receptors seem to act as inhibitors of their respective ligands indicates a possible functional difference of alternatively spliced and proteolytically cleaved soluble receptors. Soluble receptors generated by proteolytic cleavage of their membrane-bound counter-parts would thus only function extracellularly, inhibiting their paracrine or endocrine ligands. In contrast, alternatively spliced soluble receptors might also have an intracellular function inhibiting self-stimulatory effects induced by intracrine factors. This hypothesis might explain the differences in IL-6 sensitive growth and expression of alternatively spliced sgp130 by the cell lines XG-4, XG-4CNTF and XG-4A. In contrast to XG-4 and XG-4CNTF, XG-4A cells grow independent of added IL-6 and other gp130 related cytokines. We hypothesize that an intracrine growth promoting effect of gp130-stimulating cytokines produced by the three cell lines, may be inhibited by alternatively spliced sgp130 in XG-4 cells and XG-4CNTF cells. On the other hand, in XG-4A cells, lacking alternatively spliced sgp130, the production of intracrine gp130 related cytokines suffices to stimulate cell proliferation. Experiments to substantiate this hypothesis are currently underway.

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