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Differential shedding of the two subunits of the interleukin-6 receptor

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cDNAs coding for the two receptor subunits of the interleukin-6 receptor have been stably expressed in Madine Darby canine kidney (MDCK) cells. The fate of the IL-6 binding protein (IL-6R) and of the signal transducing protein gp130 was studied independently. Both proteins were proteolytically cleaved from cells metabolically labeled with [^{35}S]methionine/cysteine leading to the release of soluble receptor proteins of 55 kDa and 100 kDa, respectively. In contrast to the shedding of the IL-6R gp130 was inefficiently released from the cells and the process was not significantly stimulated by the phorbolester PMA. In addition we show that the soluble forms of the IL-6R and gp130 released by transfected cells can form a ternary complexe with interleukin-6 indicating that such complexes also may occur in vivo.

gp130; Interleukin-6; Interleukin-6-receptor; Protein kinase C; Shedding

1. INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine involved in the regulation of the immune response, hematopoiesis and acute phase response [1–5]. It has recently been recognized to be a member of the α-helical cytokine family [6]. IL-6 shares its activities with leukemia inhibitory factor (LIF) [7], oncostatin M (OSM) [8], interleukin-11 (IL-11) [9], and ciliary neurotrophic factor (CNTF) [10].

IL-6 exerts its action via a cell surface receptor which consists of two subunits, an 80 kDa ligand binding protein (IL-6R) and a glycoprotein of 130 kDa (gp130) which is essential for the generation of an intracellular signal. Both proteins belong to the family of hemopoietic receptors characterized by conserved cysteines and a Trp-Ser-X-Trp-Ser motif in the extracellular part of the receptor proteins [11]. Interestingly the cytokines LIF, OSM, IL-11 and CNTF also use the gp130 protein for signaling which may explain their overlapping biological activities [12–17,26].

Many if not all cytokine receptors have been described to exist not only as membrane-bound but also as soluble forms which can be generated by two distinct mechanisms. Soluble receptors for IL-4, IL-7 and granulocyte macrophage colony stimulating factor (GM-CSF) are translated from differentially spliced mRNAs [18–20] whereas the soluble receptors for IL-1, tumor necrosis factor (TNF), and nerve growth factor (NGF) are produced by limited proteolysis of the membrane-bound receptors [21,22]. We have recently shown that a soluble form of the IL-6R is generated by shedding and that this process is regulated by PKC [23,24].

For the soluble form of the IL-6R and for a genetically engineered form of the soluble CNTF receptor it has been shown that in the presence of the ligand they act agonistically on cells expressing the signal transducing receptor subunit gp130 [25–28]. This fact raises the important question whether also the gp130 receptor subunit is released from the cell. Quantitative shedding of gp130 would lead to a cell which is not responsive to the cytokines IL-6, IL-11, LIF, OSM and CNTF.

Here we show that the second subunit of the interleukin-6 receptor, the signal transducer gp130, is released from the cell as a 100 kDa soluble protein. The extent of shedding of gp130, however, is almost negligible when compared with shedding of the IL-6R. Furthermore we demonstrate that shedding of gp130 is not significantly induced by PMA suggesting that the process is not regulated by PKC. IL-6 and the soluble forms of gp130 and the IL-6R released by the cell form a complex suggesting that such complexes can occur in body fluids in vivo. Indeed, soluble gp130 has recently been detected in human serum [29].

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Abbreviations: CNTF, ciliary neurotrophic factor; gp, glycoprotein; IL, interleukin; IL-6R, IL-6 binding subunit of the IL-6 receptor; LIF, leukemia inhibitory factor; mAb, monoclonal antibody; MDCK, Madine Darby canine kidney; OSM, oncostatin M; PKC, protein kinase C; PMA, 4β-phorbol-12-myristate-13-acetate

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2. MATERIALS AND METHODS

2.1. *Reagents*

Restriction enzymes, calf intestinal phosphatase, T4-DNA ligase and protease inhibitors were purchased from Boehringer Mannheim (Mannheim, Germany). Tran[^{35}S]label (44 TBq/mmol) was obtained from ICN (Meckenheim, Germany). DMEM was from GIBCO (Eggenstein, Germany). Recombinant human (rh) IL-6 was prepared as described by Arcone et al. [30]. The specific activity was 1.5×10^6 B-cell stimulatory factor 2 units/mg protein [31]. The IL-6R-cDNA was isolated as described [32]. The polyclonal monospecific antiserum against the IL-6R was prepared by injecting the extracellular domain of the IL-6R expressed in *E. coli* into rabbits [33]. Culture supernatants from transfected COS-7 cells containing soluble IL-6R have been described [23]. The generation and characterization of monoclonal antibodies against human gp130 are described in [34]. Rhodamine conjugated anti mouse IgG antibody was obtained from Dakopats (Hamburg, Germany).

2.2. *Cell Cultures*

MDCK cells were grown in DMEM at 5% CO_2 in a water saturated atmosphere. All cell culture media were supplemented with 10% fetal calf serum (Seromed, Berlin, Germany), streptomycin (100 mg/l) and penicillin (60 mg/l).

2.3. *Construction of expression plasmids and transfection of cells*

The construction of the IL-6R expression vector pExIR1 has been described [35]. The human gp130 expression vector pZipNeo130BAS has been used by Hibi et al. [26]. Transfections were carried out as described [36] using the calcium-phosphate precipitation method [37]. Cell clones stably expressing the IL-6R or gp130 were selected in the presence of 500 μg/ml G418 and analyzed by Northern blotting [38] and immunofluorescence. Expression of the IL-6R was induced by stimulation of transfected MDCK cells with 100 μM $ZnCl_2$ for 8–12 h.

2.4. *Immunoprecipitation of proteins*

Cells were metabolically labeled with [^{35}S]methionine/cysteine under conditions indicated in the legends to figures. Cell lysis was performed in 10 mM Tris-HCl, pH 7.4, 60 mM EDTA, 1% Nonidet P40 and 0.4% sodium deoxycholate in the presence of a standard cocktail of protease inhibitors (Boehringer Mannheim). Cell lysates and media were pretreated with pansorbin (Calbiochem, La Jolla, CA). SDS was added to a final concentration of 0.3%, Nonidet P40 to a final concentration of 1%. Subsequently cell lysates and media were incubated with the appropriate antibodies for 2 h at 4°C. The immune complexes were precipitated with protein A-Sepharose, separated on 10% SDS PAGE [39] and visualized by fluorography [40]. Immunoprecipitation of IL-6/IL-6R/gp130 complexes was performed in the absence of SDS at a final concentration of 0.5% Triton X-100.

2.5. *Indirect immunofluorescence*

Approximately 10^5 MDCK cells grown on cover glasses for 24 h were fixed with 2% paraformaldehyde as described [23]. Cells were treated with a 1/200 dilution of a mAb against the IL-6R (MT18) or against gp130 (AM64) for 20 min. Detection of the mAbs was carried out with a 1/200 dilution of a rhodamine-conjugated anti murine IgG antibody for 20 min. All buffers used contained 0.1% Triton X-100. Cover glasses were mounted on slides with moviol and analyzed using fluorescence microscopy. A 500-fold magnification was used to photograph the cells.

3. RESULTS

Since we wanted to compare shedding of both subunits of the interkeukin-6 receptor (Fig. 1A) we stably transfected MDCK cells with expression plasmids coding for the human IL-6R and human gp130. Fig. 1B shows that surface as well as intracellular expression of the IL-6R and gp130 on transfected MDCK cells could be detected by indirect immunofluorescence using antibodies directed against the IL-6R and gp130.

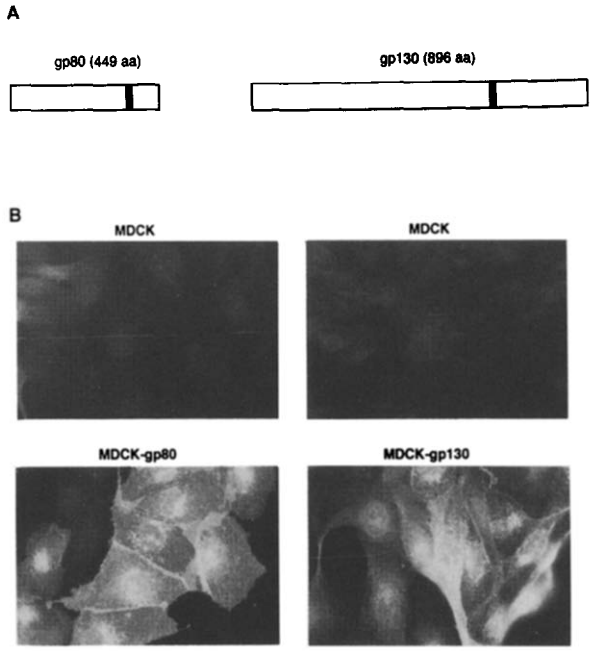

Fig. 1. Expression of the IL-6R and gp130 in MDCK cells. (A) Schematic representation of the two interleukin-6 receptor subunits expressed in transfected MDCK cells. The black box represents the transmembrane domain. (B) Expression of the IL-6R on MDCK cells (MDCK-gp80, left) and of gp130 on MDCK (MDCK-gp130, right) was assayed by indirect immunofluorescence staining using an IL-6R or gp130 specific antibody, respectively, followed by rhodamine conjugated anti mouse IgG antibodies. As a control untransfected MDCK cells were treated with the same antibodies. A 240-fold magnification was used to photograph the cells.

Cell lysates and media from transfected MDCK cells which had been metabolically labeled with a pulse of [^{35}S]methionine/cysteine in the absence or presence of PMA were immunoprecipitated with antibodies directed against the IL-6R and gp130. As shown in Fig. 2A the IL-6R could not be immunoprecipitated from untransfected cells (lane 1). In transfected cells an 80 kDa protein was immunoprecipitated from cell lysates but was undetectable in cells which had been treated with PMA. In the supernatants of the same cells a 55 kDa protein could be immunoprecipitated with an IL-6R specific antiserum. The generation of this soluble form of the IL-6R was strongly induced by the activator of PKC. Interestingly, after PMA treatment we repeatedly observed a shift of the cell-associated IL-6R protein to a higher molecular weight form. A possible explanation would be that a PKC-mediated phosphorylation of IL-6R occurs (Fig. 2A, lanes 2 and 3). Interestingly, such a mobility shift is not observed when shedding of an IL-6R protein devoid of the cytoplasmic domain is analyzed (J. Müllberg, unpublished results).

A protein of 130 kDa was immunoprecipitated from

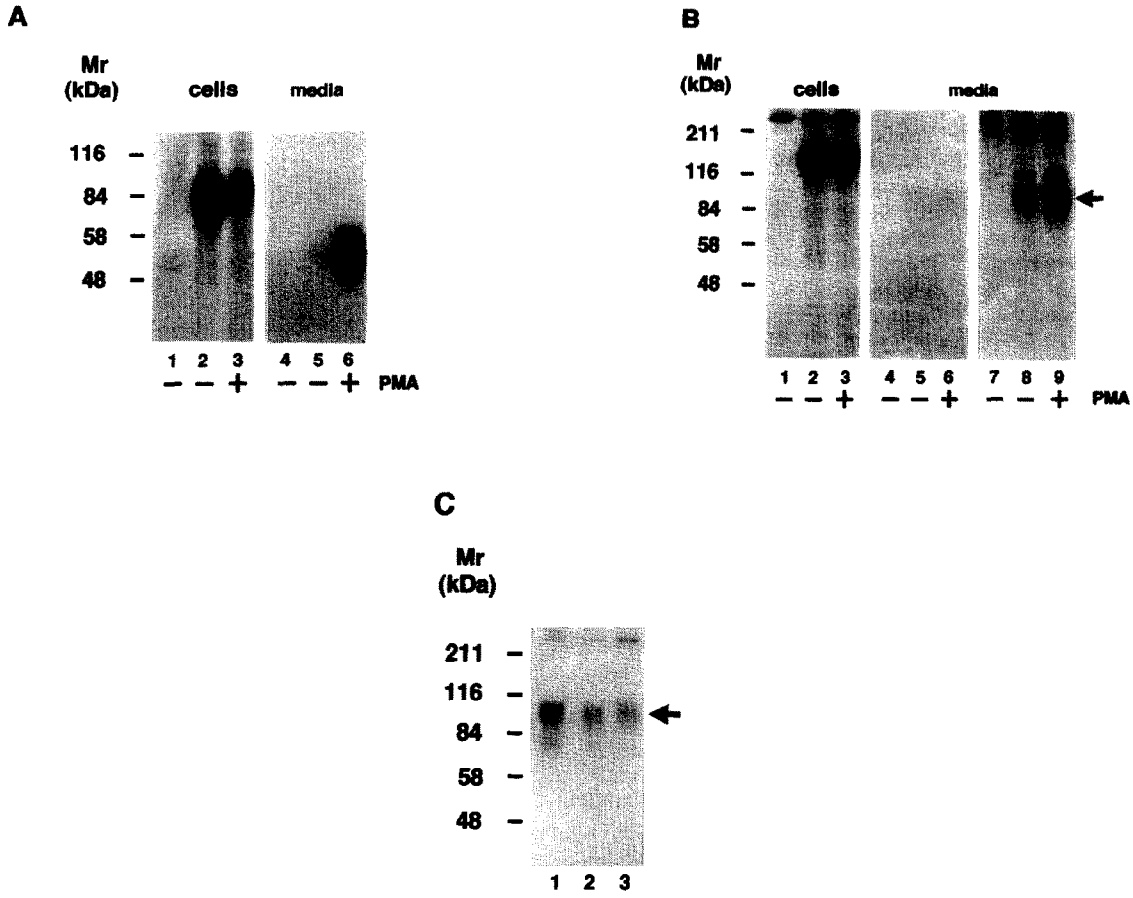

Fig. 2. Generation of soluble forms of the IL-6R and gp130 by limited proteolysis. (A) Expression of the IL-6R in MDCK cells (MDCK-gp80) was stimulated by treatment with 100 μM $ZnCl_2$ for 12 h. 2×10^6 cells were labeled with 50 μCi Tran[^{35}S]label in methionine/cysteine-free medium for 2 h. After 1 h of chase cells were incubated with 10^{-7} M PMA for 1 h as indicated in the figure. The IL-6R was immunoprecipitated from cell lysates (lanes 2 and 3) and media (lanes 5 and 6) with an IL-6R specific antiserum and analyzed by SDS-PAGE and fluorography. Untransfected MDCK cells were used as a control (lanes 1 and 4). (B) MDCK-gp130 cells constitutively expressing gp130 were radioactively labeled and immunoprecipitated in the same way as described for MDCK-gp80 cells in (A). Lanes 7–9 are identical with lanes 4–6 except that they were exposed ten times longer. Immunoprecipitations were performed with the gp130 specific mAb AM64. Soluble gp130 is marked by an arrow. (C) 2×10^7 MDCK-gp130 cells were labeled with 500 μCi Tran[^{35}S]label in methionine/cysteine-free medium for 2 h. After 16 h of chase soluble gp130 protein was immunoprecipitated from aliquots of the conditioned medium using various mAbs against gp130: AM64 (lane 1), GPX7 (lane 2) and GPZ35 (lane 3). Soluble gp130 is marked by an arrow. Exposure time: (A) 48 h, (B) 48 h (lanes 1–6) and 3 weeks (lanes 7–9), (C) 2 weeks.

cells transfected with gp130 cDNA (Fig. 2B). A faint band of 100 kDa was detected when supernatants of the same cells were immunoprecipitated (Fig. 2B, lanes 5 and 6). The fluorography shown in Fig. 2B, lanes 7–9 is the same as the one in lanes 4–6 except that exposure time was ten times longer. In contrast to the PMA-induced release of a soluble form of the IL-6R , the generation of the 100 kDa soluble form of the gp130 protein was only slightly increased when transfected cells were treated with PMA (compare Fig. 2A, lanes 5 and 6 and Fig. 2B, lanes 8 and 9). Since the protein which migrates above the soluble form of gp130 is also seen in untransfected cells and is not always observed (Fig. 2C), we believe that it is only unspecifically precipitated. The soluble form of gp130 generated from transfected MDCK cells was immunoprecipitated with three different mAbs directed against gp130. As shown in Fig. 2C the same 100 kDa protein was recognized by all mAbs indicating that the soluble 100 kDa protein is indeed a soluble form of gp130.

Since both proteins, the IL-6R and gp130 were released from cells by limited proteolysis we asked whether these soluble receptor proteins could form a complex in the presence of IL-6. Fig. 3 shows an experiment in which supernatants of metabolically labeled MDCK cells stably transfected with gp130 cDNA were immunoprecipitated with antibodies against gp130, the IL6-R and IL-6 in the presence or absence of IL-6 and soluble IL-6R. The mAb AM64 which is directed against the extracellular part of gp130 recognizes a 100 kDa protein (lanes 1, 8 and 10). The same protein is coprecipitated with an IL-6R specific antiserum only in the presence of IL-6 and soluble IL-6R (compare lane 2 and lanes 4 and 7). An IL-6 antiserum does not coprecipitate the soluble gp130 (lanes 3, 5 and 6). This is in accordance with the fact that this antiserum neutralizes

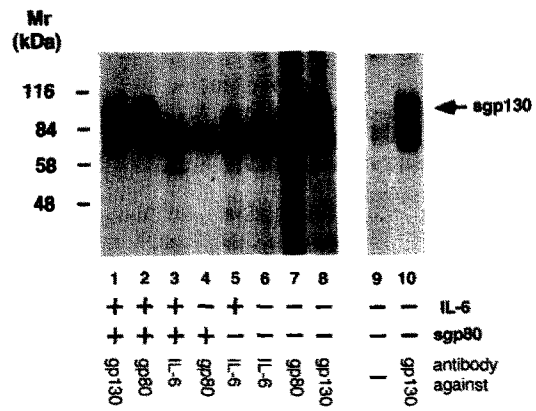

Fig. 3. Formation of a ternary complex of IL-6, soluble IL-6R and soluble gp130. 2×10^7 MDCK-gp130 cells were labeled with 500 μCi Tran[^{35}S]label in methionine/cysteine-free medium for 2 h and subsequently chased for 16 h. To aliquots of labeled MDCK-gp130 culture medium 100 ng recombinant human IL-6 and 500 ng soluble IL-6R protein in conditioned cell culture medium were added as indicated in the figure. Immunoprecipitations were carried out using antibodies against IL-6 (polyclonal antiserum), the IL-6R (polyclonal antiserum) and gp130 (mAb AM64) as indicated in the figure.

biological effects of IL-6 (data not shown). In all co-precipitations a protein of 80 kDa is recognized due to the non stringent precipitation conditions (compare Fig. 2 and Fig. 3; see Section 2). Since such a protein band is also seen when no antibody was used (lane 9) or when untransfected cells were analyzed (data not shown) co-precipitation could be explained by non-specific adsorption to protein A Sepharose.

4. DISCUSSION

We have previously shown that a soluble form of the IL-6R is generated by limited proteolysis and that this process is strongly induced by activation of cellular PKC [23,24]. We investigated in this study whether this is also true for gp130 the signal transducing subunit of the interleukin-6 receptor.

Metabolically labeled MDCK cells transfected with a gp130 expression vector release a 100 kDa soluble protein which is recognized by three different gp130 mAbs. The cytoplasmic domain plus the transmembrane domain of gp130 consist of 299 amino acids which when subtracted fom 130 kDa results in a protein size of around 100 kDa. This indicates that cleavage of the protein must have occured close to the membrane. The fact that we used a gp130 cDNA to transfect MDCK cells makes it unlikely that the soluble form of gp130 we detected has been synthesized from an alternatively spliced mRNA. We conclude that at least in MDCK cells the soluble form of the gp130 protein is generated by limited proteolysis. The mechanism of the formation of the soluble gp130 in vivo remains to be elucidated. The extent to which the soluble form of gp130 is released is negligible when compared with the shedding of the IL-6R. In addition, this process seems not to be regulated by PKC since it can not be induced by the addition of PMA.

The gp130 also serves as a signal transducing subunit of the receptors for IL-11, LIF, CNTF and OSM. Shedding of gp130 would render cells unresponsive to all these cytokines. In view of this fact it is not surprising that gp130 is not quantitatively released from the membrane as has been shown for the IL-6R. It can, however, not been excluded that there are stimuli which lead to more efficient shedding of gp130.

Genetically engineered soluble forms of the IL-6R and gp130 have been shown to form a complex in the presence of IL-6 [41]. Our finding that this is also true for the the soluble forms of the two interleukin-6 receptor subunits which are released by cells might have interesting implications. The soluble form of the IL-6R together with IL-6 initiates IL-6 specific signals on cells which only express gp130 on the cell surface, i.e. which do not by themselves bind IL-6. It can be speculated that the soluble form of gp130 might act as an antagonist of soluble IL-6R/IL-6 complexes. The serum concentrations of the IL-6R, gp130 and IL-6 and their affinity constants will determine whether agonistic or antagonistic activities are obtained. Therefore it is of great interest that in a recent study soluble gp130 has been detected in human serum and has been shown to form complexes with the soluble IL-6R and IL-6 [29]. Soluble gp130 even seems to have the potential to inhibit signals through the membrane-anchored gp130 signal transducing protein [29].It will be important to measure the exact concentrations of soluble IL-6R, gp130 and IL-6 in body fluids in normal and inflamed states and to determine the respective binding affinities. Only the knowledge of all these parameters will allow to understand and to predict biological effects of the cytokine IL-6.

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REFERENCES

[1] Kishimoto, T. (1989) Blood 74, 1–10.
[2] Sehgal, P.B., Grieninger, G. and Tosata, G. (1989) Ann. NY Acad. Sci. 557, 1–583.
[3] Heinrich, P.C., Castell, J.V. and Andus, T. (1990) Biochem. J. 265, 621–636.
[4] Van Snick, J. (1990) Annu. Rev. Immunol. 8, 253–279.
[5] Hirano, T. and Kishimoto, T. (1990) in: Handbook of Experimental Pharmacology 95/1, Peptide Growth Factors and their Receptors (Sporn B. and Roberts, A.B. Eds.) pp. 633–665, Springer, Berlin.
[6] Bazan, F. (1990) Immunology Today 11, 350–354.
[7] Baumann, H. and Wong, G.G. (1989) J. Immunol. 143, 1163–1167.

[8] Richards, C.D., Brown, T.J., Shoyab, M., Baumann, H. and Gauldie, J. (1992) J. Immunol. 148, 1731–1736.
[9] Baumann, H. and Schendel, P. (1991) J. Biol. Chem. 266, 20424–20427.
[10] Schooltink, H., Stoyan, T., Roeb, E., Heinrich, P.C. and Rose-John, S. (1992) FEBS Lett. 314, 280–284.
[11] Bazan, F. (1990) Proc. Natl. Acad. Sci. USA 87, 6934–6938.
[12] Gearing, D.P., Comeau, M.R., Friend, D.J., Gimpel, S.D., Thut, C.J., McGourty, J., Brasher, K.K., King, J.A., Gillis, S., Mosley, B., Ziegler, S.F. and Cosman, D. (1992) Science 255, 1434–1437.
[13] Liu, J., Modrell, B., Aruffo, A., Marken, J.S., Taga, T., Yasukawa, K., Murakami, M., Kishimoto, T. and Shoyab, M. (1992) J. Biol. Chem. 267, 16763–16766.
[14] Ip, N.Y., Nye, S.H., Boulton, T.G., Davis, S. Taga, T., Li, Y., Birren, S.J., Yasukawa, K., Kishimoto, T., Anderson, D.J., Stahl, N. and Yancopoulos, G.D. (1992) Cell 69, 1121–1132.
[15] Kishimoto, T., Akira, S. and Taga, T. (1992) Science 258, 593–597.
[16] Yin, T., Taga, T., Lik-Shing Tsang, M., Yasukawa, K., Kishimoto, T., Yang, Y.-C. (1993) J. Immunol., in press.
[17] Taga, T. and Kishimoto, T. (1992) FASEB J. 6, 3387–3396.
[18] Mosley, B., Beckmann, M.P., March, C.J., Idzerda, R.L., Gimpel, S.D., Vanden Bos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J.M., Smith, C., Gallis, B., Sims, J.E., Urdal, D., Widmer, M.B., Cosman, D. and Park, L.S. (1989) Cell 59, 335–348.
[19] Goodwin, R.G., Friend, D., Ziegler, S.F., Jerzy, R., Falk, B.A., Gimpel, S., Cosman, D., Dower, K.S., March, C.J., Namen, A.E. and Park, L. (1990) Cell 60, 941–951.
[20] Raines, M.A., Liu, L., Quan, S.G., Joe, V., DiPersio, J.F. and Golde, D.W. (1992) Proc. Natl. Acad. Sci. USA 88, 8203–8207.
[21] Fernandez-Botran, R., FASEB J. 5, 2567–2574.
[22] Ehlers, M.R.W. and Riordan, J.F. (1991) Biochemistry 30, 10065–10074.
[23] Müllberg, J., Schooltink, H., Stoyan, T., Günther, M., Graeve, L., Buse, G., Mackiewicz, A., Heinrich, P.C. and Rose-John, S. (1993) Eur. J. Immunol. 23, 473–480.
[24] Müllberg, J., Schooltink, H., Stoyan, T., Heinrich, P.C. and Rose-John, S. (1992) Biochem. Biophys. Res. Commun. 189, 794–800.
[25] Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Matsuda, T., Hirano, T. and Kishimoto, T. (1989) Cell 58, 573–581.
[26] Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T. and Kishimoto, T. (1990) Cell 63, 1149–1157.
[27] Mackiewicz, A., Schooltink, H., Heinrich, P.C. and Rose-John, S. (1992) J. Immunol. 149, 2021–2027.
[28] Taga, T., Narazaki, M., Yasukawa, K., Saito, T., Miki, D., Hamaguchi, M. Davis, S., Shoyab, M., Yancopoulos, G.D. and Kishimoto, T. (1992) Proc. Natl. Acad. Sci. USA 88, 10998–11001.
[29] Narazaki, M., Yasukawa, K., Saito, T., Ohsugi, Y., Fukui, H., Koishihara, Y., Yancopoulos, G.D., Taga, T. and Kishimoto, T. (1993) Blood, in press.
[30] Arcone, R., Pucci, P., Zappacosta, F., Fontaine, V., Malorni, A, Marino, G. and Ciliberto, G. (1991) Eur. J. Biochem. 198, 541–547.
[31] Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyoma, K., Iwamatu, A., Tsunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T. and Kishimoto, T. (1986) Nature 324, 73–76.
[32] Schooltink, H., Stoyan, T., Lenz, D., Schmitz, H., Hirano, T., Kishimoto, T., Heinrich, P.C. and Rose-John, S. (1991) Biochem. J. 277, 659–664.
[33] Stoyan, T., Michaelis, U., Schooltink, H., Van Dam, M., Rudolph, R., Heinrich, P.C. and Rose-John, S. (1993) Eur. J. Biochem., in press.
[34] Saito, T., Taga, T., Miki, D., Futatsugi, K., Yawata, H., Kishimoto, T. and Yasukawa, K. (1993) J. Immunol. Methods, in press.
[35] Rose-John, S., Hipp, E., Lenz, D., Legrès, L., Korr, H., Hirano, T., Kishimoto, T. and Heinrich, P.C. (1991) J. Biol. Chem. 266, 3841–3846.
[36] Rodriguez-Boulan, E., Salas, P.J., Sargiacomo, M., Lisanti, M., Lebivic, A., Sambuy, Y., Vega-Salas, D. and Graeve, L. (1989) Methods Cell Biol. 32, 37–56.
[37] Graham, F.L. and Van der Eb, A.J. (1973) Virology 52, 456–467.
[38] Rose-John, S., Rincke, G. and Marks, F. (1988) Gene 74, 465–471.
[39] Laemmli, U.K. (1970) Nature 227, 680–685.
[40] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–86.
[41] Yasukawa, K., Futasugi, K., Saito, T., Yawata, H., Narazaki, M., Suzuki, H., Taga, T. and Kishimoto, T. (1992) Immunol. Lett. 31, 123–130.