Overlapping and distinct signals through leptin receptor (OB-R) and a closely related cytokine signal transducer, gp130

Kinichi Nakashima[a], Masashi Narazaki[a], Tetsuya Taga[a,b,*]

[a]*Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565, Japan*
[b]*Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kanda-surugadai, Chiyoda-ku, Tokyo 101, Japan*

Received 11 November 1996

Abstract The structure of leptin receptor (OB-R) is highly homologous to that of gp130, the common signal transducing receptor component for the interleukin-6 family of cytokines. Based on this structural similarity, we examined signaling processes initiated by OB-R in comparison with those by gp130. Stimulation of either a long form of OB-R or gp130 led to tyrosine phosphorylation of STAT3, whereas stimulation of the truncated form of OB-R that is predominantly expressed in *db/db* mice failed to do so. Stimulation of the long form OB-R did not induce tyrosine phosphorylation of a Src homology domain 2 containing protein tyrosine phosphatase, SHP-2, while stimulation of gp130 did. In contrast, activation of p42^{ERK2} is mediated by either the long form OB-R or gp130. Two closely related molecules, OB-R and gp130, thus appear to mediate overlapping but distinct signaling procedures.

Key words: Leptin; OB-R; gp130; STAT3; p42^{ERK2}; SHP-2

1. Introduction

Leptin (OB) is an adipose tissue-derived protein which exerts weight-reducing effects in mice when administrated in vivo [1–4]. Recently, the receptor for this factor, OB-R, has been cloned and revealed to be a member of the class I cytokine receptor family. OB-R shows the highest similarity to gp130 that is a common signal transducer for interleukin 6 (IL-6) family of cytokines [5,6].

The extracellular regions of OB-R, gp130 and leukemia inhibitory factor receptor (LIFR) are closely related to each other and are composed of either six or eight fibronectin type III modules. They contain a set of four conserved cysteine residues and the tryptophan-serine-X-tryptophan-serine (X represents any amino acid) motif that are characteristic of the class I cytokine receptor family [5–7]. The cytoplasmic regions of OB-R, gp130 and LIFR possess three conserved motifs, box1, box2 and box3, in the membrane-proximal-to-distal order [5–8]. In the case of gp130, mutational analyses have indicated that the box1 motif is important for the association with Janus kinases (JAKs) and that the box2 motif plays a critical role, in concert with box1, in mediating DNA synthesis [8–11]. Box3 contains a tyrosine-X-X-glutamine (Y-X-X-Q) motif [12] which is proposed as a docking site for signal transducer and activator of transcription 3 (STAT3) [13–16].

*Corresponding author. Fax: (81) (3) 5280-8065.
E-mail: tagamcb@mri.tmd.ac.jp

Abbreviations: OB-R, *obese* receptor; *db*, *diabetes* mutant; IL-6, interleukin-6; STAT, signal transducer and activator of transcription; SHP-2, Src homology domain 2 containing protein tyrosine phosphatase 2; p42^{ERK2}, extracellular signal-regulated protein kinases 2

IL-6, LIF, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11 and cardiotrophin-1 (CT-1) are a subset of cytokines with structural and functional similarity [17,18]. All these IL-6 family cytokines signal through gp130 protein dimerized by itself or with closely related LIFR [17–20]. Formation of gp130 homodimer or gp130/LIFR heterodimer triggers the activation of JAKs, and activated JAKs phosphorylate tyrosine residues in the cytoplasmic region of gp130 and LIFR [10,11,19,20]. STAT3 is then recruited to the phosphorylated Y-X-X-Q motif through its Src-homology 2 (SH2) domain and subsequently phosphorylated on tyrosine by activated JAKs [12,21]. Besides STAT3, STAT1 is tyrosine-phosphorylated when a high dose of IL-6 is used for gp130 stimulation [14], although direct binding between STAT1 and gp130 has not yet been demonstrated. SHP-2, a SH2 domain-containing protein tyrosine phosphatase [22,23], is also tyrosine-phosphorylated following stimulation of gp130 [12,24]. After recruitment, SHP-2 undergoes tyrosine phosphorylation, which enables this molecule to bind with Grb2, an adapter molecules for activation of the Ras-ERK (extracellular signal-regulated protein kinases) cascade ([25,26]; Narazaki, Nakashima and Taga, unpublished result). Indeed, gp130 stimulation leads to activation of p42^{ERK2} [27].

In leptin-nonresponsive obesity mutant mice (*db/db* mice), the short form of OB-R which lacks most of the cytoplasmic region but retains box1 is predominantly expressed. In such mice the long, complete form of OB-R is hardly expressed because of a guanine to thymine base substitution, which generates an aberrant splice donor site [28–30]. Recent reports showed that STAT1, 3, 5 and 6 are activated through the long form of OB-R but not through the short one and suggested that the lack of the long form of OB-R is responsible for the *db/db* mouse phenotype [31,32].

Based on the similarity between OB-R and gp130, it has been predicted that similar cytoplasmic molecules undergo modification and activation after stimulation of these two membrane receptors. Actually, similar kinds of STAT proteins have been reported to be activated following OB-R and gp130 stimulation [17,31,32]. In this study, we have examined leptin-induced modification or activation of SHP-2 and p42^{ERK2} as well as STAT3.

2. Materials and methods

2.1. Materials

Recombinant human IL-6 and soluble IL-6R (sIL-6R) were kindly provided by Dr. K. Yasukawa, Tosoh Corp., Kanagawa. Recombinant human leptin expressed in *E. coli* was generously provided by Dr. F. de Sauvage, Genentech Inc., CA [33]. Anti-STAT3 antibody was generously provided by Dr. S. Akira, Hyogo College of Medicine, Hyogo. Anti-SHP-2 and p42^{ERK2} antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phosphotyrosine

PII S0014-5793(96)01430-5

(4G10) antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

2.2. Plasmid construction

Long and short forms of human OB-R cDNAs were obtained from Genentech Inc. [34,35]. These two OB-R cDNAs and STAT3 cDNA [15] were subcloned into mammalian expression vector pEF-BOS (generous gift from Dr. S. Nagata, Osaka University, Osaka).

2.3. Transfection of COS7 cells and immunoblot analysis

COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and transfected with cDNAs by a DEAE-dextran method. After 2 days culture, the cells were starved for 4 h in medium without serum and then stimulated with cytokines for 10 min at 37°C. Cells were solubilized with Nonidet P-40 (NP-40) lysis buffer (0.5% NP-40/10 mM Tris-HCl, pH 7.6/150 mM NaCl/5 mM EDTA/2 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride/5 μg of aprotinin per ml), and then subjected to immunoprecipitation and immunoblotting.

2.4. Binding assay

Preparation of [^{125}I]leptin and binding assay were carried out as described previously [36]. The specific binding was calculated based on the counts obtained in the absence or presence of 200-fold excess unlabeled leptin.

2.5. p42^{ERK2} kinase assay

Anti-p42^{ERK2} immunoprecipitates were subjected to immune complex kinase assay, using a consensus peptide (the ERK kinase assay system; Amersham, Buckinghamshire, UK) according to the manufacturer's protocol.

3. Results

3.1. The long form of OB-R is functional for STAT3 modification

As for cytokines that utilize gp130 as a signal transducer, STAT3 is predominantly activated [15–17]. Hence, we tested whether STAT3 was tyrosine-phosphorylated in response to leptin stimulation. COS7 cells were transfected with expression constructs for STAT3 along with either a long or short form of OB-R. Transfected cells were therefore incubated with or without leptin, and cell lysates were subjected to immunoprecipitation with anti-STAT3 antibody and subsequent immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 1A (upper panel), leptin induced STAT3 tyrosine phosphorylation through the long form of OB-R, but not through the short one which is predominantly expressed in *db/db* mice. In each immunoprecipitate, comparable amounts of STAT3 were present (Fig. 1A, lower panel). In each transfectant, comparable amounts of OB-R protein were present, as examined by binding of ^{125}I-labeled leptin (Fig. 1B).

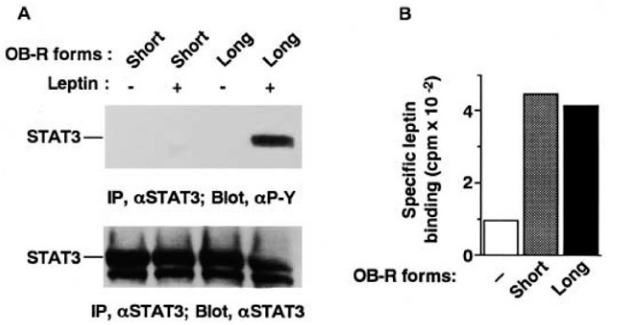

Fig. 1. The long form of OB-R is functional for tyrosine phosphorylatin of STAT3. A: COS7 cells were transfected with cDNA for either the short or the long form of OB-R together with that for STAT3. Transfected cells were incubated with (+) or without (−) 50 ng/ml of leptin and lysed. STAT3 was immunoprecipitated with anti-STAT3 antibody and subjected to Western blotting using anti-phosphotyrosine (upper panel) or anti-STAT3 (lower panel) antibody. B: COS7 cells were untransfected (white column) or transfected with either the short (shaded column) or the long (black column) form of OB-R with STAT3. The cells were incubated with [^{125}I]leptin with or without a 200-fold excess of unlabeled leptin. The specific binding was calculated by subtracting the cell-associated counts obtained with the competitor from those without it.

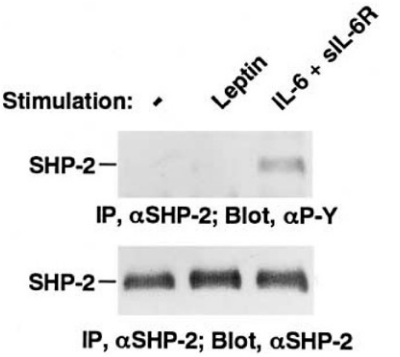

Fig. 2. Tyrosine phosphorylation of SHP-2 is induced by stimulation of gp130 but not OB-R. COS7 cells were co-transfected with the long form of OB-R and STAT3, and treated with medium alone, 50 ng/ml each of leptin or a combination of IL-6 and sIL-6R. Cell lysates were immunoprecipitated with anti-SHP-2 antibody followed by Western blotting using either anti-phosphotyrosine (upper panel) or anti-SHP-2 (lower panel) antibody.

3.2. OB-R stimulation does not induce tyrosine phosphorylation of SHP-2

It has been shown that gp130 stimulation leads to tyrosine phosphorylation of SHP-2 [12,24]. We therefore assessed whether this occurs after stimulation of OB-R. COS7 cells were co-transfected with expression constructs for the long form of OB-R and STAT3, and treated with either medium alone, leptin or a complex of IL-6 and sIL-6R which is known to homodimerize gp130 [19,37]. Endogenously expressed SHP-2 was immunoprecipitated and immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 2 (upper panel), tyrosine phosphorylation of SHP-2 was induced after stimulation of gp130 but not OB-R. The same filter was reprobed with anti-SHP-2 antibody to confirm that comparable amounts of SHP-2 were present in the immunoprecipitates (Fig. 2, lower panel).

3.3. p42^{ERK2} is activated through OB-R stimulation

We examined whether leptin induces p42^{ERK2} activation, which was known to occur by gp130 stimulation. COS7 cell were transfected with expression constructs for the long form of OB-R and STAT3, and treated with either medium alone, leptin or a complex of IL-6 and sIL-6R. Cell lysates were then subjected to immunoprecipitation with anti-p42^{ERK2} antibody. The kinase activities associated with each immune complex were assayed using [γ-^{32}P]ATP and an ERK substrate peptide. As shown in Fig. 3, leptin stimulation through the long form of OB-R led to p42^{ERK2} activation.

4. Discussion

The long form of OB-R is highly homologous to gp130 and

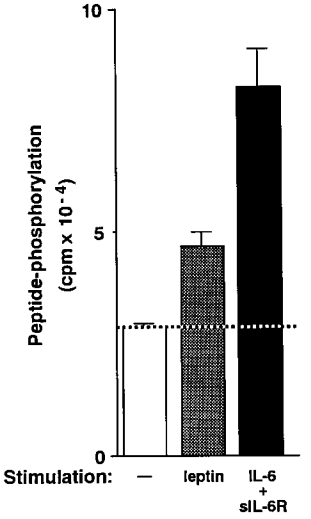

Fig. 3. $p42^{ERK2}$ activation is induced following leptin stimulation. COS7 cells were transfected and treated as in Fig. 2. $p42^{ERK2}$ was immunoprecipitated with anti-$p42^{ERK2}$ antibody, and the immune complex was subjected to in vitro kinase assay, using a peptide substrate (see Section 2). Columns show the mean ± range of two reactions.

contains a conserved box3 motif which has been suggested to be sufficient for recruitment of STAT3, in addition to box1 and 2 motifs which are supposed to be important for activation of JAKs [5–12]. As demonstrated in this and previous papers, the long form of OB-R indeed induced tyrosine phosphorylation of STAT3 following leptin stimulation, while the short form of OB-R was not functional [31,32]. The *db/db* mutant mice hardly express the long form of OB-R because of the mutation in an intron that leads to an aberrant splice choice. This mutation brings about the non-functional short form of OB-R lacking most of the cytoplasmic region. The deleted region includes box2 and box3 [28–30]. This deletion may account for the defect in reception of the leptin signal in *db/db* mice.

Both OB-R and gp130 contain a Y-X-X-V motif (Y^{1079}-L-G-V^{1082} for the former, Y^{759}-S-T-V^{762} for the latter) which is suggested for SHP-2 binding [5,12,24]. In the case of gp130 stimulation, SHP-2 is actually recruited to gp130 and subsequently tyrosine-phosphorylated ([12,24]; Fig. 2). However, this is not the case in leptin stimulation. Amino acid residues in, or around, the Y-X-X-V motif may influence the actual binding of SHP-2 to this motif.

gp130 stimulation induces $p42^{ERK2}$ activation, and so does OB-R stimulation although the extent in the latter case seems smaller at least in COS7 cells (Fig. 3). There are reports indicating that SHP-2 functions as an adapter for activation of the Ras-ERK cascade [25,26], and SHP-2 is indeed recruited to gp130 and tyrosine-phosphorylated after gp130 stimulation [12,24]. Grb2 then is proposed to bind to SHP-2, and is suggested to switch the Ras-ERK cascade on ([25,26]; Narazaki, Nakashima and Taga, unpublished result). In contrast, OB-R stimulation leads to activation of $p42^{ERK2}$ but does not induce tyrosine phosphorylation of SHP-2. The two closely related molecules gp130 and OB-R appear to utilize distinct pathways to activate $p42^{ERK2}$. Taken together, gp130 and OB-R appear to mediate overlapping but distinct cytoplasmic signals. It will be intriguing to investigate what actually accounts for the divergence of the biological outcomes induced by OB-R and gp130.

Acknowledgements: We wish to thank Dr. F. de Sauvage (Genentech Inc., CA) for leptin materials. K.N. and M.N. were supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (K.N., M.N., T.T.) and Human Frontier Science Program (T.T.).

References

[1] Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J.M. (1994) Nature 372, 425–432.
[2] Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T. and Collins, F. (1995) Science 269, 540–543.
[3] Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K. and Friedman, J.M. (1995) Science 269, 543–546.
[4] Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R. and Burn, P. (1995) Science 269, 546–549.
[5] Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Woolf, E.A., Monroe, C.A. and Tepper, R.I. (1995) Cell 83, 1263–1271.
[6] Taga, T. and Kishimoto, T. (1996) Annu. Rev. Immunol. (in press).
[7] Taga, T. and Kishimoto, T. (1992) FASEB J. 6, 3387–3396.
[8] Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. and Kishimoto, T. (1991) Proc. Natl. Acad. Sci. USA 88, 11349–11353.
[9] Baumann, H., Symes, A.J., Comeau, M.R., Morella, K.K., Wang, Y., Friend, D., Ziegler, S.F., Fink, J.S. and Gearing, D.P. (1994) Mol. Cell. Biol. 14, 138–146.
[10] Stahl, N., Boulton, T.G., Farruggella, T., Ip, N.Y., Davis, S., Witthuhn, B.A., Quelle, F.W., Silvenoinen, O., Barbieri, G., Pellegrini, S., Ihle, J.N. and Yancopoulos, G.D. (1994) Science 263, 92–95.
[11] Narazaki, M., Witthuhn, B.A., Yoshida, K., Silvennoinen, O., Yasukawa, K., Ihle, J.N., Kishimoto, T. and Taga, T. (1994) Proc. Natl. Acad. Sci. USA 91, 2285–2289.
[12] Stahl, N., Farruggella, T.J., Boulton, T.G., Zhong, Z., Darnell Jr., J.E. and Yancopoulos, G.D. (1995) Science 267, 1349–1353.
[13] Yuan, J., Wegenka, U.M., Lütticken, C., Buschmann, J., Decker, T., Schindler, C., Heinrich, P.C. and Horn, F. (1994) Mol. Cell. Biol. 14, 1657–1668.
[14] Sadowski, H.B., Shuai, K., Darnell Jr., J.E. and Gilman, M.Z. (1993) Science 261, 1739–1744.
[15] Akira, S., Nishio, Y., Inoue, M., Wang, X.J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, T., Naruto, M. and Kishimoto, T. (1994) Cell 77, 63–71.
[16] Zhong, Z., Wen, Z. and Darnell Jr., J.E. (1994) Science 264, 95–98.
[17] Taga, T. (1996) J. Neurochem. 67, 1–10.
[18] Pennica, D., Wood, W.I. and Chien, K.R. (1996) Cytokine Growth Factor Rev. 7, 81–91.
[19] Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, T., Yasukawa, K., Yamanishi, K., Taga, T. and Kishimoto, T. (1993) Science 260, 1808–1810.
[20] Davis, S., Aldrich, T.H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N.Y. and Yancopoulos, G.D. (1993) Science 260, 1805–1808.
[21] Heim, M.H., Kerr, I.M., Stark, G.R. and Darnell Jr., J.E. (1995) Science 267, 1347–1349.
[22] Adachi, M., Fischer, E.D., Ihle, J., Imai, K., Jirik, F., Neel, B., Pawson, T., Shen, S.-H., Thomas, M., Ullrich, A. and Zhao, Z. (1996) Cell 85, 15.
[23] Neel, B.G. (1993) Semin. Cell Biol. 4, 419–432.
[24] Fuhrer, D.K., Feng, G.-S. and Yang, Y.-C. (1995) J. Biol. Chem. 270, 24826–24830.
[25] Bennett, A.M., Tang, T.L., Sugimoto, S., Walsh, C.T. and Neel, B.G. (1994) Proc. Natl. Acad. Sci. USA 91, 7335–7339.
[26] Li, W., Nishimura, R., Kashishian, A., Batzer, A.G., Kim,

W.J.H., Cooper, J.A. and Schlessinger, J. (1994) Mol. Cell. Biol. 14, 509–517.
[27] Daeipour, M., Kumar, G., Amaral, M.C. and Nel, A.E. (1993) J. Immunol. 150, 4743–4753.
[28] Chen, H., Charlat, O., Tartaglia, L.A., Woolf, E.A., Wen, X., Ellis, S.J., Laket, N.D., Culpepper, J., Moore, K.J., Breitbart, R.E., Duyk, G.M., Tepper, R.I. and Morgenstern, J.P. (1996) Cell 84, 491–495.
[29] Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I. and Friedman, J.M. (1996) Nature 379, 632–635.
[30] Chua Jr., S.C., Chung, W.K., Wu-peng, X.S., Zhang, Y., Liu, S.-M., Tartaglia, L. and Leibel, R.L. (1996) Science 271, 994–996.
[31] Ghilardi, N., Ziegler, S., Wiestner, A., Stoffel, R., Heim, M.H. and Skoda, R.C. (1996) Proc. Natl. Acad. Sci. USA 93, 6231–6235.
[32] Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.-F. and Tartaglia, L.A. (1996) Proc. Natl. Acad. Sci. USA 93, 8374–8378.
[33] Levin, N., Nelson, C., Gurney, A., Vandlen, R. and de Sauvage, F. (1996) Proc. Natl. Acad. Sci. USA 93, 1726–1730.
[34] Luoh, S.-M., Di Marco, F., Levin, N., Armanini, M., Xie, M.-H., Nelson, C., Bennett, G.L., Williams, M., Spencer, S.A., Gurney, A. and de Sauvage, F. (1996) J. Mol. Endocrinol. (in press).
[35] Bennett, B.D., Solar, G., Yuan, J., Mathias, J., Thomas, R. and Matthews, W. (1996) Curr. Biol. 6, 1170–1180.
[36] Taga, T., Kawanishi, Y., Hardy, R.R., Hirano, T. and Kishimoto, T. (1987) J. Exp. Med. 166, 967–981.
[37] Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. and Kishimoto, T. (1989) Cell 58, 573–581.