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14. Although the function of receptor tyrosine phosphorylation in subsequent events has not been established, its occurrence is a marker of the activation of receptor-mediated intracellular signaling. The induced tyrosine phosphorylations of gp130 and LIFRβ observed in COS cells were qualitatively similar to those previously observed in neuronal cell lines (*8*)—that is, they displayed the same pattern of inhibition by kinase inhibitors (they are inhibited by staurosporine but not by H7) and they were accompanied by the tyrosine phosphorylation of the same set of additional, intracytoplasmic proteins.
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16. Although the extent and possibly the stability of this association was variable in different experiments, two other lines of independent evidence support the existence of an intermediate complex containing gp130 and CNTFRα. (i) In the presence of soluble CNTFRα (but not in its absence), we could detect the binding of CNTF to COS cells that overexpressed gp130 alone (S. Davis, unpublished results). (ii) Although CNTF could be cross-linked to all three components in COS cells that overexpressed all three components, cross-linking to only gp130 and CNTFRα was observed either in COS cells that overexpressed only these two components (*20*) or in naturally occurring cell lines that expressed gp130 and CNTFRα but not LIFRβ (*25*).
17. These experiments are consistent with previous observations that cells such as BAFh130 (*10*) or B9 (*15*), which express gp130 but not LIFRβ, can transduce signals initiated by treatment with IL-6 but not those initiated by treatment with CNTF or LIF.
18. In the absence of gp130, we did not detect the CNTF-induced association of CNTFRα and LIFRβ or the tyrosine phosphorylation of LIFRβ alone.
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IL-6–Induced Homodimerization of gp130 and Associated Activation of a Tyrosine Kinase

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The biological functions of interleukin-6 (IL-6) are mediated through a signal-transducing component of the IL-6 receptor, gp130, which is associated with the ligand-occupied IL-6 receptor (IL-6R) protein. Binding of IL-6 to IL-6R induced disulfide-linked homodimerization of gp130. Tyrosine kinase activity was associated with dimerized but not monomeric gp130 protein. Substitution of serine for proline residues 656 and 658 in the cytoplasmic motif abolished tyrosine kinase activation and cellular responses but not homodimerization of gp130. The IL-6–induced gp130 homodimer appears to be similar in function to the heterodimer formed between the leukemia inhibitory factor (LIF) receptor (LIFR) and gp130 in response to the LIF or ciliary neurotrophic factor (CNTF). Thus, a general first step in IL-6–related cytokine signaling may be the dimerization of signal-transducing molecules and activation of associated tyrosine kinases.

When bound to IL-6, the IL-6R [referred to by Davis *et al.* as IL-6Rα (*1*)] associates with gp130 to initiate cytoplasmic signaling processes (*2*, *3*). The binding of IL-6 to its receptor results in the rapid tyrosine phosphorylation of gp130, but this has not been shown to cause its association with other signal-transducing molecules (*2*–*6*). Other cytokines related to IL-6 (*7*), such as LIF or CNTF, cause formation of heterodimers between gp130 and the structurally related LIFR [referred to by Davis *et al.* as LIFRβ (*1*)] and induce tyrosine phosphorylation of both molecules (*1*, *5*, *6*, *8*, *9*).

To examine more closely the potential induction of biochemical changes in gp130 by IL-6, we transfected cells with an expression vector that overexpresses gp130. The entire coding region of human cDNA encoding gp130 (*3*) was inserted into the genome of the vaccinia virus strain LC16mO under the control of a strong promoter, which normally expresses the 7.5-kD viral polypeptide (*10*), and a recombinant virus Vac130 was obtained. Hep 3B cells infected with Vac130 were cultured with [^{35}S]methionine and stimulated with a complex of IL-6 and soluble IL-6R (sIL-6R), which associates with and stimulates gp130 (*2*, *11*). Cell lysates were subjected to immunoprecipitation with monoclonal antibody (mAb) to human gp130 (anti-gp130). As analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions, gp130 was the major protein precipitated from the unstimulated cells (Fig. 1), whereas a protein of 260 kD was detected with gp130 from the cells stimulated with the IL-6–sIL-6R complex. When the same set of immunoprecipitates were treated with a reducing reagent, 2-mercaptoethanol (2-ME; 5%), only the gp130 protein was detected in either unstimulated or stimulated cells. The fact that the 260-kD protein was not present and no major proteins other than gp130 were detected under reducing conditions implied that the 260-kD protein might be a disulfide-linked homodimer of gp130.

To examine whether the 260-kD protein was composed solely of gp130, we performed peptide mapping analysis. The gp130 protein from unstimulated and stimulated cells and the 260-kD protein from stimulated cells were individually subjected to proteolysis with *Staphylococcus aureus* V8 protease. The peptide maps obtained with these three materials were identical (Fig. 1B). This result, taken together with the molecular size of the protein (260 kD, nonreduced; 130 kD, reduced) and its immunoreactivity with polyclonal antibody to human gp130 (*12*), indicated that the 260-kD protein was a disulfide-linked gp130 homodimer. The formation of gp130 homodimers after stimulation by IL-6 alone was also observed in [^{35}S]methionine-labeled IL-6–responsive cells not infected with Vac130, but the amounts observed were very small (*13*), presumably because of the very small amounts of both IL-6R and gp130 protein present in these cells. Thus, gp130 homodimerization may also occur upon binding of IL-6 to membrane-anchored IL-6R as well.

We examined the phosphorylation state of the dimerized gp130 protein. Hep 3B cells infected with Vac130 were incubated with [^{32}P]orthophosphate and stimulated with IL-6 and sIL-6R. Analysis by SDS-PAGE of the anti-gp130 immunoprecipitates under nonreducing conditions revealed that both dimerized and monomeric forms of gp130 protein were phosphorylated in response to stimulation (Fig. 2A). When reduced, the phosphorylated gp130 homodimers dissociated into monomers.

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Stimulation of cells with IL-6 and sIL-6R also induced phosphorylation of dimerized gp130 protein in parental Hep 3B cells not infected with Vac130, but the amount of phosphorylated gp130 was smaller than that in infected cells.

The gp130 monomers from unstimulated and stimulated cells and dimers from stimulated cells were extracted and subjected to phosphoamino acid analysis (Fig. 2B). Monomeric gp130 protein from unstimulated cells was phosphorylated on serine and threonine but not on tyrosine residues. On the other hand, dimerized or monomeric gp130 protein from stimulated cells exhibited increased phosphorylation on serine and threonine residues and was also phosphorylated on tyrosine residues. Tyrosine phosphorylation of gp130 became apparent only upon stimulation, whereas serine-threonine phosphorylation was observed in both unstimulated and stimulated cells, albeit in greater amounts in the latter. The presence of tyrosine-phosphorylated gp130 monomers in stimulated cells may seem inconsistent with the idea that it is the dimer species that is subject to tyrosine phosphorylation. We imagine that these tyrosine-phosphorylated monomers may arise from the dissociation of gp130 dimers, perhaps because of the action of a cellular redox mechanism acting on gp130.

Hep 3B cells infected with Vac130 were stimulated with IL-6 and sIL-6R. Proteins from cell extracts were immunoprecipitated with anti-gp130, and the immunoprecipitates were subjected to an immune complex–kinase assay. Only the dimerized gp130 was phosphorylated (Fig. 3A). These gp130 homodimers phosphorylated in vitro were dissociated into monomers after treatment with a reducing reagent (5% 2-ME). The dimerized gp130 was phosphorylated in vitro on tyrosine as well as serine-threonine residues. These results suggested that the dimerized gp130 protein, not the monomeric protein, was associated with a tyrosine kinase. The additional association of serine-threonine kinase activity with dimerized gp130 protein, as suggested by this assay, is reminiscent of the observation that epidermal growth factor (EGF) stimulates association, and kinase activity, of Raf-1 with the EGF receptor (*14*). The possible role of gp130-associated serine-threonine kinase remains to be clarified.

In the cytoplasmic region of various members of the cytokine receptor family, including gp130, a region, proximal to the membrane, of about 60 amino acid residues is conserved (*4*). In this region, a shorter, eight–amino acid stretch adjacent to the membrane, which we termed box 1, is strongly conserved. Box 1 contains a Pro-X-Pro (where X represents any amino acid) motif that is present in almost all members of this family. This motif in gp130 is critical for the transduction of the IL-6–mediated signals that result in DNA replication; substitution of the two Pro residues at positions 656 and 658 in this motif with serine residues abolished the signaling capability of gp130 when it was assayed in mouse pro-B cell transfectants (*4*). A cDNA encoding the human gp130 that contains these two amino acid substitutions was inserted into the vaccinia virus genome, and a recombinant virus, Vac130PP, was obtained.

Hep 3B cells were infected with Vac130, Vac130PP, or the original virus strain LC16mO as a control and then examined for their production of haptoglobin, one of the acute phase proteins whose expression is regulated by IL-6 (*15*) (Fig. 4A). The basal amounts of haptoglobin produced from Hep 3B cells infected with any of the three viruses were the same. In the presence of IL-6 and sIL-6R, control Hep 3B cells produced an increased amount of haptoglobin as a result of stimulation of their intrinsic gp130 protein. Expression of extra amounts of wild-type gp130 resulted in enhanced responsiveness of the cells to IL-6, whereas expression of the mutant gp130PP by Vac130PP had no effect. These results suggested that the conserved Pro-X-Pro motif is also critical for IL-6–induced haptoglobin synthesis in hepatocytes. Infection with larger titers of virus or for longer infection times resulted in the failure of Hep 3B cells to induce haptoglobin production in response to IL-6 no matter which of the three viruses was tested. Thus, we were unable to further increase the ratio of gp130PP to endogenous gp130. This may explain why we did not observe a dominant negative effect of gp130PP on endogenous gp130 (Fig. 4A), as has been observed with EGF receptor mutants (*16*).

In cells stimulated with IL-6 and sIL-6R, the mutant gp130PP protein also formed a 260-kD dimer that could be dissociated into monomers in the presence of a reducing reagent (Fig. 4). We therefore examined whether the gp130PP protein also became phosphorylated on tyrosine in response to IL-6 and sIL-6R. Hep 3B cells infected with Vac130PP and labeled with [^{32}P]orthophosphate were stimulated with or without IL-6–sIL-6R. Overall, phosphorylation of gp130PP protein from either unstimulated or

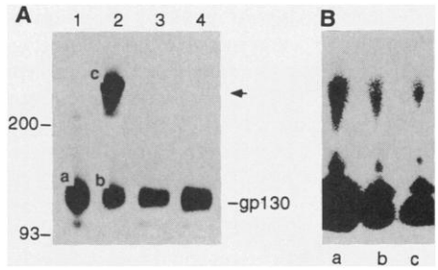

Fig. 1. Formation of homodimers of gp130 in cells stimulated with IL-6 and sIL-6R. (**A**) Hep 3B cells (10^6 cells for each lane) were cultured with Vac130 virus [5 × 10^6 plaque-forming units (PFU)] for 15 hours and stimulated with medium alone (lanes 1 and 3) or IL-6 (1 μg/ml) and sIL-6R (2.5 μg/ml) (lanes 2 and 4) for 10 min. Cell lysates were prepared as described (*4*, *6*) except the NP-40 concentration was 0.5%. Proteins immunoprecipitated from the lysates with anti-gp130 (mAb AM277) (*3*) were analyzed by SDS-PAGE under nonreducing (lanes 1 and 2) or reducing (5% 2-ME; lanes 3 and 4) conditions. Molecular size markers (in kilodaltons) and the position corresponding to gp130 are indicated. The arrow indicates the 260-kD protein. (**B**) Sections of the polyacrylamide gel containing the bands a through c shown in (A) were excised and treated with *S. aureus* V8 protease and analyzed by SDS-PAGE as described (*22*).

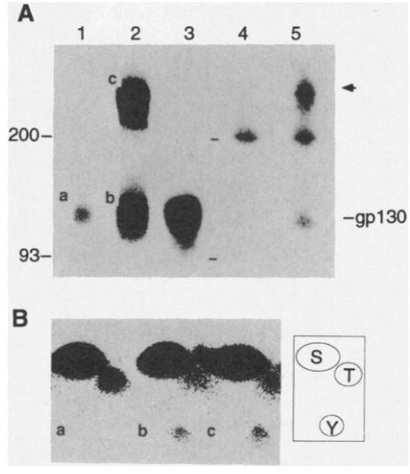

Fig. 2. Tyrosine phosphorylation of homodimerized gp130. (**A**) Hep 3B cells cultured for 15 hours with Vac130 virus (10^6 cells per 5 × 10^6 PFU per lane; lanes 1 through 3) or without virus (1 × 10^7 cells per lane; lanes 4 and 5) were incubated with [^{32}P]orthophosphate and medium alone (lanes 1 and 4) or IL-6 and sIL-6R (lanes 2, 3, and 5; same concentrations as in Fig. 1) for 10 min. Proteins immunoprecipitated from cell lysates with anti-gp130 were analyzed by SDS-PAGE under nonreducing (lanes 1, 2, 4, and 5) or reducing (lane 3) conditions. Exposures: lanes 1 through 3, 5 hours; lanes 4 and 5, 24 hours. The arrow indicates the gp130 homodimer. A band at around 200 kD corresponds to a nonspecifically precipitated protein. (**B**) Sections of the polyacrylamide gel containing the bands a through c in (A) were excised. Proteins containing equal amounts of extracted radioactivity were subjected to phospho-amino acid analysis (panels a through c, respectively) as described (*23*) and analyzed by an imaging analyzer (Fuji, Tokyo, Japan). Approximate positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated.

stimulated cells was similar to phosphorylation of the wild-type gp130 protein (Fig. 2A). However, the mutant gp130PP protein was not tyrosine-phosphorylated in response to stimulation (Fig. 4D). In contrast, the enhancement of serine-threonine phosphorylation of the gp130PP protein in response to IL-6 and sIL-6R was still observed, although the extent of this enhancement was somewhat smaller than that observed for the wild-type gp130 protein (Figs. 2 and 4). Thus, the Pro-X-Pro motif in the cytoplasmic region of gp130 appears to be important for tyrosine kinase activation and signal transduction but not for homodimerization of gp130. Because Pro is considered a helix-breaker and is thought to be important in protein structure (*17*), it is possible that substitution of the two prolines in the cytoplasmic conserved motif may have changed the tertiary structure of the cytoplasmic region of gp130, which may be important for activating a downstream molecule. Our results suggest the importance of gp130 homodimerization in the IL-6 signaling processes but also show that dimerization by itself is not sufficient for signaling. Other results support the latter idea; it has been shown that oligomerization of cell-surface gp130 protein by gp130 mAbs does not produce an agonistic effect (*6*, *12*).

The structure of the gp130 homodimer formed in cells treated with IL-6 stands in contrast to that of the heterodimer formed between gp130 and its relative LIFR in cells treated with LIF or CNTF (*1*, *5*, *6*, *8*, *9*). However, these two types of dimerized receptor components appear to function in an analogous manner, associating with a cytoplasmic tyrosine kinase molecule. Thus, in the case of cytokines sharing gp130, dimerization of the receptor components may be a common mechanism that triggers the cytoplasmic signaling cascades. Granulocyte colony-stimulating factor (G-CSF) also induces homodimerization of the G-CSF receptor (*18*). A constitutively active (hormone-independent) mutant of the erythropoietin receptor is changed at a single amino acid, creating a new cysteine residue in the extracellular region, and forms a disulfide-linked homodimer (*19*). Although none of the receptor components (including signal transducers) that belong to the cytokine receptor family have a tyrosine kinase domain (*20*), they have functions similar to those of receptors that are tyrosine kinases. Whereas ligands cause the dimerization and activation of tyrosine kinase receptors (*21*), the binding of cytokines appears to cause the homo- or heterodimerization of signal-transducing receptor subunits and subsequent association with tyrosine kinases.

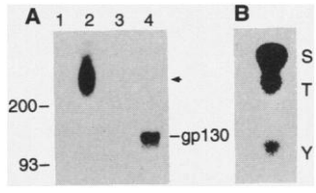

Fig. 3. In vitro phosphorylation of dimerized gp130 protein. (**A**) Hep 3B cells infected with Vac130 were incubated with medium alone (lanes 1 and 3) or IL-6 and sIL-6R (lanes 2 and 4; same concentrations as in Fig. 1). To reduce contaminating proteins, glycoproteins reactive to a lectin, RCA120, were first purified from cell lysates with RCA120-coupled agarose (Seikagakukogyo, Tokyo, Japan) according to the manufacturer's instructions. Proteins immunoprecipitated with anti-gp130 from the purified glycoprotein fraction were labeled in vitro with [γ-^{32}P]adenosine triphosphate (*24*) and analyzed by SDS-PAGE under nonreducing (lanes 1 and 2) or reducing (lanes 3 and 4) conditions. The arrow indicates the gp130 homodimer. The presence of both dimerized and monomeric gp130 in the precipitates from stimulated cells was confirmed with [^{35}S]-labeled cells. (**B**) [^{32}P]-labeled dimeric gp130 protein in (A) (lane 2) was extracted from the gel and subjected to phospho-amino acid analysis. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

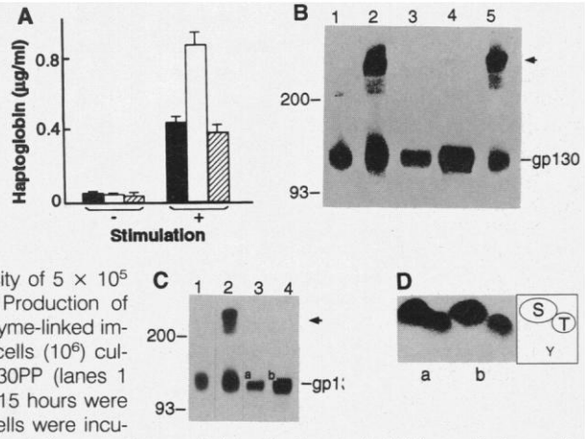

Fig. 4. Effects of mutations in the conserved Pro-X-Pro motif in gp130. (**A**) Hep 3B cells that had been infected with LC16mO (closed columns), Vac130 (open columns), or Vac130PP (hatched columns) for 2.5 hours (1×10^6 PFU of each virus with 1×10^6 cells in 1 ml of medium) were incubated with medium alone (−) or IL-6 (0.5 μg/ml) and sIL-6R (1 μg/ml) (+) for 24 hours at a density of 5×10^5 cells per well in 24-well plates. Production of haptoglobin was measured by enzyme-linked immunosorbent assay. (**B**) Hep 3B cells (10^6) cultured with 5×10^6 PFU of Vac130PP (lanes 1 through 4) or Vac130 (lane 5) for 15 hours were incubated with [^{35}S]methionine. Cells were incubated with medium alone (lanes 1 and 3) or medium containing IL-6 and sIL-6R (lanes 2, 4, and 5; same concentrations as in Fig. 1) for 10 min and solubilized with lysis buffer containing NP-40. Proteins immunoprecipitated with anti-gp130 were analyzed by SDS-PAGE under nonreducing (lanes 1, 2, and 5) or reducing (lanes 3 and 4) conditions. (**C**) Hep 3B cells cultured with Vac130PP for 15 hours were labeled with [^{32}P]orthophosphate and incubated with medium alone (lanes 1 and 3) or medium containing IL-6 and sIL-6R (lanes 2 and 4) for 10 min. Proteins immunoprecipitated with anti-gp130 from the cell lysates were analyzed by SDS-PAGE under nonreducing (lanes 1 and 2) or reducing (lanes 3 and 4) conditions. Exposure was for 10 hours. (**D**) Phosphorylated gp130PP protein [bands a and b in (C)] containing radioactivity equal to that of the protein shown in Fig. 2B were subjected to phospho-amino acid analysis. S and T represent phosphoserine and phosphothreonine, respectively. The position where phosphotyrosine (Y) should appear is also indicated. Molecular size markers in (B) and (C) are in kilodaltons. The arrows in (B) and (C) indicate the dimerized gp130.

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