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16. The same 89-kD band was immunoprecipitated with anti-p91,p84 from an APRF preparation purified by specific DNA–affinity chromatography, proving its identity with APRF (*13*).
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18. Coprecipitation of Jak1 and gp130 was enhanced by a cleavable cross-linker but could also be observed without cross-linking. Tyk2 also coprecipitated with gp130 from HepG2 lysates, albeit to a smaller extent than Jak1 (*15*).
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26. Because APRF is not detected on immunoblots by anti-p91,p84, only the amount of p91 protein could be analyzed on the blot.
27. The HepG2 cells were labelled with [^{32}P]orthophosphate (1 mCi/ml) for 1 hour in phosphate- and serum-free medium. After stimulation with IL-6 or IFN-γ, the cells were lysed, immunoprecipitated with anti-p91,p84, separated by SDS-PAGE, and blotted to PVDF membrane. The APRF band was excised and hydrolysed for 1 hour at 110°C in 6 N HCl. Phosphoamino acid analysis was performed by two-dimensional thin-layer electrophoresis (*22*).
28. Gel retardation assays were done as described (*12*). The ^{32}P-labeled synthetic oligonucleotide 5′-GATCCTTCTGGGAATTCCTA-3′ (upper strand) representing the proximal APRF binding site of the rat α_2-macroglobulin promoter (*12*) was used as a probe.
29. Antiserum to Tyk2 was raised and affinity-purified against a glutathione-S-transferase fusion protein containing a portion of human Tyk2.
30. We used FAO cells to measure the effect of LIF, CNTF, and IL-11 on Jak1, p91, and APRF tyrosine phosphorylation because HepG2 cells responded poorly to these cytokines (*15*).
31. In several lanes of Fig. 2C, two APRF bands (89 and 87 kD) are observed. These are caused by a different serine phosphorylation status of APRF (*15*).
32. Cross-linking was performed by incubation of pelleted cells in phosphate-buffered saline with 100 μM sodium orthovanadate and the cleavable cross-linker dithiobis-succinimidyl-propionate (DSP, 0.5 mM) for 30 min at 4°C as described (*23*).
33. The monoclonal antibodies to gp130 used for immunoprecipitation did not recognize gp130 in immunoblot experiments. Therefore, direct detection of gp130 on the blot was not possible.

34. Supported by grants from the Deutsche Forschungsgemeinschaft, Bonn, Germany, the Fonds der Chemischen Industrie, and the Swiss National Science Foundation. We thank A. Nordheim and J. Krieg for help with phosphoamino acid analysis.

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Association and Activation of Jak-Tyk Kinases by CNTF-LIF-OSM–IL-6 β Receptor Components

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A recently defined family of cytokines, consisting of ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and interleukin-6 (IL-6), utilize the Jak-Tyk family of cytoplasmic tyrosine kinases. The β receptor components for this cytokine family, gp130 and LIF receptor β, constitutively associate with Jak-Tyk kinases. Activation of these kinases occurs as a result of ligand-induced dimerization of the receptor β components. Unlike other cytokine receptors studied to date, the receptors for the CNTF cytokine family utilize all known members of the Jak-Tyk family, but induce distinct patterns of Jak-Tyk phosphorylation in different cell lines.

Although they have different biological activities, CNTF, LIF, OSM, and IL-6 make up a cytokine family on the basis of their predicted structural similarities (*1, 2*) and shared β signal-transducing receptor components (*3–8*). Receptor activation by this cytokine family results from either homo- or heterodimerization of these β components (*6, 8, 9*). IL-6 receptor activation involves homodimerization of gp130 (*9*), a protein initially identified as the IL-6 signal transducer (*10*). CNTF, LIF, and OSM receptor activation requires heterodimerization between gp130 and a second gp130-related protein known as the LIF receptor β (LIFRβ) (*6*), initially identified for its ability to bind LIF (*11*). Both CNTF and IL-6 also require specificity-determining α components that are more limited in their distribution than the β components and thus determine the cellular targets of the particular cytokines (*8*). Both the CNTF binding protein CNTFRα (*12*) and the IL-6 receptor IL-6Rα (*10, 13*) can function as soluble proteins, consistent with the notion that they do not interact with intracellular signaling molecules but that they serve to help their ligands interact with the appropriate signal-transducing β subunits (*8*).

Receptor activation by the CNTF family of cytokines results in the immediate tyrosine phosphorylation of the β components and other cellular proteins (*3, 5, 14–17*). Because the β receptor components do not contain intrinsic tyrosine kinase domains, tyrosine phosphorylation was hypothesized to result from the activation of one or more receptor-associated tyrosine kinases, which appear to be essential for downstream events because tyrosine kinase inhibitors were found to block the early tyrosine phosphorylations and subsequent physiological responses (*3, 14*). We report here that gp130 and LIFRβ associate with the Jak-Tyk family of cytoplasmic protein tyrosine kinases (*18–21*) in the absence of ligand and that Jak-Tyk kinase activity is induced after cytokine binding. Unlike other cytokines studied to date (*21–24*), the CNTF family of factors utilizes all known members of the Jak-Tyk family but elicits distinct profiles of Jak-Tyk activation in different cells.

Stimulation of responsive cells with CNTF or LIF induces tyrosine phosphorylation of both gp130 and LIFRβ, which form a complex that can be recovered after cell

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lysis with antibodies to either β subunit (*5*, *6*). Stimulation of cells with IL-6 [along with soluble IL-6Rα (sIL-6Rα) for cells that do not express their own IL-6Rα] results in tyrosine phosphorylation and complex formation involving gp130 but not LIFRβ (*6*, *9*). After lysis of cells in Brij 96 detergent, immunoprecipitation of the receptor complexes formed upon addition of CNTF, LIF, OSM, or IL-6 + sIL-6Rα resulted in the copurification of a tyrosine-phosphorylated 130-kD protein (pp130) along with the tyrosine-phosphorylated β subunits (Fig. 1, A and B). In vitro kinase assays with these receptor complexes revealed prominent tyrosine phosphorylation of the associated pp130 as well as the β components (Fig. 1A), which suggests that pp130 might be a receptor-associated tyrosine kinase. Supporting this possibility, neither pp130 nor associated tyrosine kinase activity was recovered when receptor complexes were isolated after lysis of the cells in a different detergent, NP-40 (Fig. 1C); similar detergent-dependent association of a kinase with its target has been reported (*25*). In vitro tyrosine phosphorylation of LIFRβ, but not gp130, was observed before complex formation (Fig. 1, A and C, lanes 5), which suggests that pp130 might be constitutively associated with LIFRβ but incapable of autophosphorylation before β component dimerization. Consistent with the notion that the receptor-associated tyrosine kinase that accounts for the in vitro kinase activity is identical to the kinase that functions in vivo, the tyrosine kinase inhibitor staurosporine was similarly effective at inhibiting pp130 tyrosine phosphorylation in vitro or in vivo (Fig. 1D).

The three known members of the Jak-Tyk kinase family (Jak1, Jak2, and Tyk2) are all equally related to each other and range from 130 to 134 kD in size (*18*–*21*). Jak1 and Tyk2 are required for interferon α (IFN-α) signaling (*22*, *26*), Jak1 and Jak2 are required for IFN-γ signaling (*27*), and erythropoietin (EPO), growth hormone (GH), and IL-3 activate only Jak2 (*21*, *23*, *24*). The similarity in size between pp130 and members of the Jak-Tyk family of kinases, together with the recent findings that other cytokines use these kinases, led us to investigate whether any of the Jak-Tyk kinases are involved in signaling initiated by the CNTF family of factors. Tyrosine phosphorylation of Jak1 and Jak2, but not Tyk2, was observed after stimulation of EW-1 cells with CNTF, IL-6 + sIL-6Rα (Fig. 2A), LIF, and OSM (*28*). In contrast, stimulation of these cells with IFN-α induces phosphorylation of Tyk2 and Jak1 but not of Jak2 (Fig. 2A). Tyrosine-phosphorylated Jak1 precisely comigrated with the gp130-LIFRβ–associated pp130, whereas Jak2 comigrates with a fainter gp130-LIFRβ–associated tyrosine-phosphorylated protein that migrated slightly faster than pp130 (Fig. 2B) (*29*). Stimulation of EW-1 cells with CNTF also increased the in vitro kinase activity of both Jak1 and Jak2 (Fig. 2E).

Surprisingly, the pattern of Jak phosphorylation induced by members of the CNTF family differed in various cell lines. SK-MES cells incubated with IL-6 + sIL-6Rα (Fig. 2C) or OSM (*28*) revealed tyrosine phosphorylation of primarily Jak2, with only minor phosphorylation of Tyk2 and Jak1. On the other hand, stimulation of U266 cells with IL-6 resulted in tyrosine phosphorylation of Tyk2 and Jak1 with no apparent change in the phosphorylation of Jak2 (Fig. 2D). These results are not explained by differential expression of the kinases, because immunoblots of lysates from U266 and EW-1 cells revealed equal amounts of Jak2 and Tyk2 (*28*). In contrast, EPO, GH, or IL-3 cause prominent tyrosine phosphorylation of only Jak2 in all responsive cell lines analyzed (*21*, *23*, *24*).

An attractive model for cytokine receptor function suggests that receptor β com-

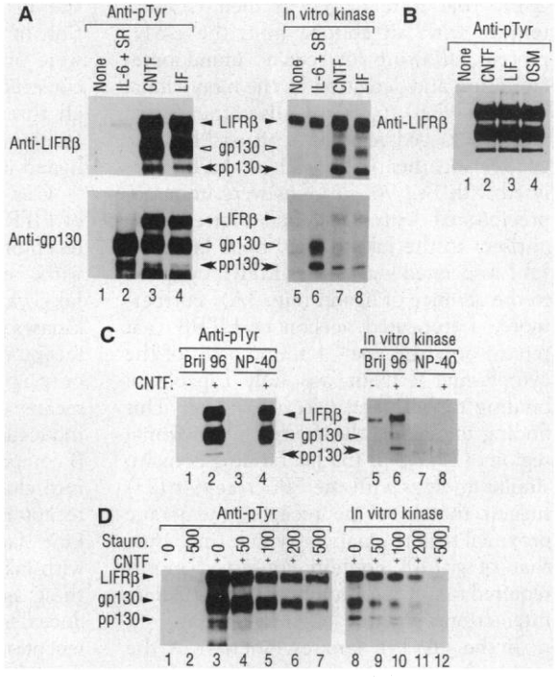

Fig. 1. Copurification of a tyrosine-phosphorylated 130-kD protein and tyrosine kinase activity with CNTF, LIF, OSM, and IL-6 receptor complexes. EW-1 cells were starved in serum-free medium for 2 to 4 hours, then stimulated for 5 min without (None) or with the indicated factors (50 ng/ml) or with IL-6 (50 ng/ml) supplemented with soluble human IL-6Rα (1 μg/ml) (designated SR; R & D Systems, Minneapolis, Minnesota). The cells were then lysed (*5*), in 1% Brij 96 detergent, except in (C), where 1% NP-40 detergent was used as indicated. Proteins from lysates were immunoprecipitated with antiserum specific for LIFRβ (Anti-LIFRβ) (*5*) except in (A) where antiserum specific for gp130 (Anti-gp130) (*6*) was used as indicated. The samples were either immunoblotted (*5*) with antibodies to phosphotyrosine (Anti-PTyr) (**A**, **B**, **C**, and **D**, left panels) or subjected to an in vitro tyrosine kinase assay (*36*) (A, C, and D, right panels). Cells were incubated as described (*3*) with staurosporine (Stauro.) (D) [concentration (nM) used is indicated].

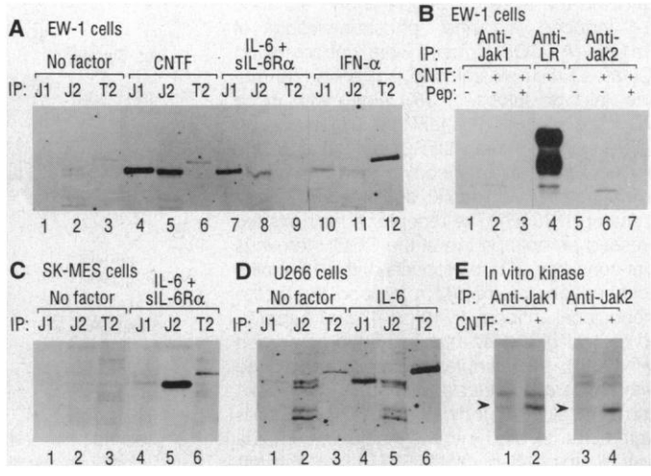

Fig. 2. Tyrosine phosphorylation and activation of Jak1, Jak2, and Tyk2 in response to the CNTF family of factors. Either EW-1 (**A**, **B**, and **E**), SK-MES cells (**C**), or U266 cells (**D**) were stimulated with the indicated factor and lysed as in Fig. 1, except that the buffer contained 1% Triton X-100 or 1% Brij 96 (B, lane 4 only). Proteins from lysates were then immunoprecipitated (IP) with antisera to LIFRβ (LR), Jak1 (J1), Jak2 (J2), or Tyk2 (T2) as indicated (*37*) and then immunoblotted with antibodies to phosphotyrosine (A through D) or subjected to an in vitro kinase reaction (E) as described (Fig. 1). In (B), "Pep" indicates the presence or absence of cognate peptide used for competition during immunoprecipitation. The arrowheads in (E) indicate the position of Jak1 (left panel) or Jak2 (right panel).

ponents are constitutively associated with the Jak-Tyk kinases, which then become activated upon ligand-induced β subunit homo- or heterodimerization (*8*). This model is consistent with the observation that kinase activity is associated with LIFRβ before ligand binding (Fig. 1, A and C). An alternative model would entail recruitment of Jak kinases into the complex after ligand binding (*24*). We therefore investigated whether Jak-Tyk kinases could associate with LIFRβ and gp130 before receptor activation by co-expressing these proteins in COS cells. We used expression constructs encoding forms of LIFRβ and gp130 that were tagged at their COOH-termini with an epitope from the c-Myc protein, allowing for specific immunoprecipitation and detection by the monoclonal antibody 9E10 (*6*, *30*). Cells cotransfected with the tagged versions of either LIFRβ or gp130, together with Jak1 or Jak2, were lysed with Brij 96. Proteins were immunoprecipitated with 9E10 and blotted with antisera to the Jak kinases. Both Jak1 and Jak2 associated with either LIFRβ or gp130 in the absence of ligand (Fig. 3A). Furthermore, a truncated version of LIFRβ that retains only the first 74 amino acids of the cytoplasmic domain was fully capable of binding to Jak1 and Jak2 (Fig. 3A). This finding implicates the membrane proximal region of LIFRβ as the Jak binding domain; similar findings with the EPO receptor (*23*) suggest that cytokine receptor membrane proximal regions, many of which (including that of gp130) contain conserved motifs required for receptor function (*16*), mediate interactions with the Jak-Tyk kinases.

If the Jak-Tyk kinases function as the proximal kinases activated in response to the CNTF family of factors, overexpression of β receptor components together with these kinases should enhance the ligand-induced increase in β subunit tyrosine phosphorylation. We expressed an epitope-tagged form of gp130, either alone or with Jak1 or Jak2 or both, and examined responses to IL-6 + sIL-6Rα. Upon stimulation with IL-6 + sIL-6Rα, neither mock-transfected nor gp130-transfected COS cells revealed substantial tyrosine phosphorylation of gp130 at the exposure shown (Fig. 3B). In contrast, cotransfection with either Jak1, Jak2, or both Jak1 and Jak2 (*31*) gave rise to a substantial increase in the ligand-induced tyrosine phosphorylation of gp130 (Fig. 3B); analogous results were obtained with Tyk2 (*32*). These reconstruction experiments demonstrate that all three known members of the Jak-Tyk family are equally capable of mediating ligand-induced phosphorylation responses.

Our findings emphasize the equivalency of LIFRβ and gp130 as signal-transducing receptor components that can associate with, and mediate the activation of, the Jak-Tyk family of kinases. The Jak-Tyk kinases are constitutively associated with β receptor components but become activated only upon ligand stimulation, perhaps by means of transphosphorylation as ligand-induced hetero- or homodimerization of the β components brings the bound Jak kinases into close apposition (*8*). We suggest that receptor components used by other cytokine families are similarly preassociated with Jak-Tyk kinases and that activation of these associated kinases follows ligand-induced homo- or heterodimerization of the receptor components (*8*). The affinity of the Jak-Tyk kinases for cytokine receptors may vary, and some receptors may require dimerization for optimal binding, perhaps explaining a failure to note an association between the GH receptor and Jak2 before ligand stimulation (*24*).

The β receptor components for the CNTF family of cytokines share the ability to utilize all the known members of the Jak-Tyk family; they may, by extension, also utilize as yet undiscovered members of this kinase family. Whereas the overlapping activities of different classes of cytokines may be explained by convergence at the level of Jak-Tyk kinases, it is also true that different cytokines can, in some cases, elicit qualitatively different responses from the same cells. These differences may result from the use of distinct combinations of the Jak-Tyk kinases by the different cytokine receptors; alternatively, the different cytokine receptor β components may serve as docking proteins to recruit distinct sets of potential substrates for the Jak-Tyk kinases or may interact with other kinases.

Another unexpected feature of the gp130-LIFRβ receptor systems is the finding that a particular cytokine induced distinct patterns of Jak-Tyk phosphorylation in different cell lines. Although this is somewhat reminiscent of Src family members, it differs because the choice of the Src kinase used largely depends on which members are expressed (*33*, *34*). In contrast, cell lines that yield distinct patterns of Jak-Tyk phosphorylation in response to the same factor display similar profiles of Jak-Tyk expression. If the amount of Jak-Tyk kinases is limiting, it is conceivable that the cell-specific expression of one cytokine receptor that preferentially associates with and thus depletes one Jak-Tyk family member may in fact regulate which family members are available for use by another cytokine receptor in that cell. Regardless of mechanism, a single cytokine may induce phosphorylation of distinct repertoires of intracellular substrates in different cells through activation of different combinations of the Jak-Tyk kinases.

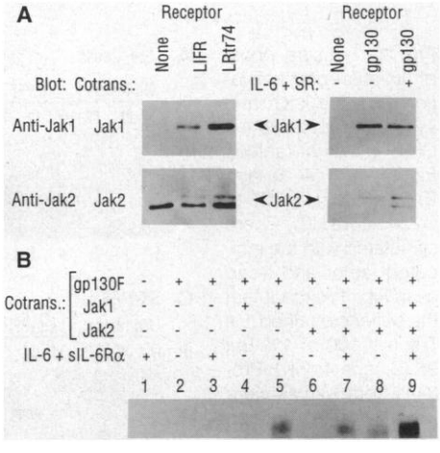

Fig. 3. Jak1 and Jak2 associate with gp130 and LIFRβ in the absence of ligand and enhance IL-6–induced tyrosine phosphorylation of Gp130. (**A**) COSM5 cells were cotransfected (Cotrans.) with plasmids (5 μg) encoding murine Jak1 or Jak2 (*21*, *36*), along with those encoding either human LIFRβ (LIFR), a truncated version of human LIFRβ (truncated at the unique Esp I site) encoding only 74 amino acids of the cytoplasmic domain (LRtr74), or human gp130 (*6*). The receptor constructs expressed an epitope tag at the COOH-terminus that consisted of three repeats of the 10–amino acid sequence from c-Myc [recognized by the monoclonal antibody 9E10 (*38*)], each separated by two glycines. After immunoprecipitation with 9E10, the samples were immunoblotted with antisera to either Jak1 or Jak2 (anti-Jak1 and anti-Jak2), as indicated. (**B**) COSM5 cells were cotransfected with the indicated combinations of plasmids encoding Jak1 (0.5 μg), Jak2 (0.5 μg), or gp130-flag (gp130F) (10 μg); the latter is modified with the flag epitope at the COOH-terminus as described (*6*). After ~40 hours, the cells were serum-starved and stimulated with IL-6 + sIL-6Rα as indicated, lysed in buffer containing 1% Brij 96 detergent, immunoprecipitated with the flag monoclonal antibody M2 (IBI, New Haven, Connecticut), and immunoblotted with phosphotyrosine antibody. Longer exposures revealed induced phosphorylation of gp130 in the absence of cotransfected Jaks (lane 3).

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29. The ability to detect the fainter gp130-LIFRβ–associated protein in receptor complexes varied between experiments. Under different lysis conditions than those depicted for lanes 2 and 6 in Fig. 2B (with Brij 96 instead of Triton X-100), it was possible to detect the associated β components co-immunoprecipitating with the antiserum to Jak1.
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31. The increase in phosphorylation observed upon co-expression of Jak1 and Jak2 is most likely an additive effect of a higher total amount of kinase (Fig. 3B). Further overexpression of either Jak1, Jak2, or Tyk2 (achieved by increasing the amount of plasmid transfected) resulted in constitutive phosphorylation of both the Jak kinases and the co-expressed β subunit (Fig. 3B).
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36. We tested immunoprecipitates for in vitro tyrosine kinase activity by incubating the washed beads for 15 min at room temperature in 20 mM Hepes (pH 7.2), 10 mM $MnCl_2$, 30 μM sodium orthovanadate, 0.1% Brij 96, and 10 μCi of [γ-^{32}P]adenosine triphosphate (6000 Ci/mmol; NEN Dupont). The kinase assays were stopped with electrophoresis sample buffer, and the samples were boiled, subjected to SDS–polyacrylamide gel electrophoresis, and electroblotted to Immobilon P (Millipore). The membrane was then incubated in 1 M NaOH at 65°C for 60 min to eliminate serine and threonine phosphorylation before autoradiography.
37. The Jak1 (Fig. 2, A through D) and Jak2 antisera were as described (*21*); the Jak1 antiserum used for Fig. 2E was raised in rabbits against an NH_2-terminal peptide (*35*). Rabbit antiserum to Tyk2 was raised and affinity-purified against an engineered glutathione-S-transferase fusion product containing a portion of human Tyk2.
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Targeting of $G\alpha_{i2}$ to the Golgi by Alternative Spliced Carboxyl-Terminal Region

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Heterotrimeric guanosine triphosphate (GTP)–binding proteins (G proteins) may participate in membrane traffic events. A complementary DNA (cDNA) was isolated from a mouse pituitary cDNA library that corresponded to an alternatively spliced form of the gene encoding the G protein alpha subunit $G\alpha_{i2}$. The cDNA was identical to that encoding $G\alpha_{i2}$ except that the region encoding for the carboxyl-terminal 24 amino acids was replaced by a longer region encoding 35 amino acids that have no sequence similarity with $G\alpha_{i2}$ or other members of the G protein family. This alternative spliced product and the corresponding protein (sG_{i2}) were present in several tissues. Specific antibodies revealed that sG_{i2} was localized in the Golgi apparatus, suggesting a role in membrane transport. Thus, alternative splicing may generate from a single gene two G protein alpha subunits with differential cellular localization and function.

Proteins that bind GTP participate in the control of many cellular events (*1*). GTP-binding proteins are divided into two classes: the small Ras-related GTP-binding proteins and the heterotrimeric GTP-binding proteins (G proteins). Genetic and biochemical studies in yeast have shown that the first class participates in membrane trafficking (*2*). In mammalian cells this role has been assigned to members of the Arf and Rab families of small GTP-binding proteins (*2*). Heterotrimeric G proteins have classically been defined as the intracellular transducers of signals at the cell surface. However, they also appear to participate in the regulation of membrane trafficking events (*3–5*). A nonhydrolyzable analog of GTP, GTP-γ-S, stimulates GTP-binding proteins and inhibits membrane transport. This effect was thought to result from activation of monomeric GTP-binding proteins. However, AlF_4^-, a compound that activates heterotrimeric but not monomeric GTP-binding proteins, has the same effect as GTP-γ-S on membrane trafficking (*3*). These and other results suggest that heterotrimeric GTP-binding proteins can also influence transport in both secretory and endocytic pathways (*4–7*). Two G proteins, $G\alpha_{i3}$ (*8, 9*) and $G\alpha_s$ (*10*), have been shown to be associated with the Golgi apparatus and their activation modifies the transport of proteins (*9, 10*).

The data presented in this paper demonstrate the existence of a protein generated by alternative splicing of the gene encoding $G\alpha_{i2}$ (G_{i2}). The alternatively spliced transcript encodes a protein with a different COOH-terminus that confers localization in the Golgi apparatus. The alternative localization of this form of G_{i2}, which we term sG_{i2}, suggests that this protein may function in the control of membrane transport events.

We screened a mouse pituitary cDNA library (*11*) at low stringency (40% formamide, 42°C) with a G_{i2} cDNA fragment including sequences conserved between the three inhibitory G protein α subunits (amino acids 200 to 320) (*12*) and isolated several cDNAs. Three isoforms of G_{i2} were obtained. Two of the cDNAs incorporated an alternative 3′ untranslated region and corresponded to previously described cDNAs (Fig. 1) (*12, 13*). The third isoform is identical to G_{i2} through the coding region until amino acid position 331, where the remainder of the COOH-terminal coding region is substituted by a different sequence that encodes 35 amino acids, resulting in an open reading frame of 366 amino acids (Fig. 1). The nucleotide and amino acid sequences of this segment are not similar to those of other G proteins.

To determine the genomic structure of the 3′ region of the mouse G_{i2} gene, a genomic library was screened with an Xba I–Xho I fragment (Fig. 1) containing the divergent COOH-terminal fragment of sG_{i2}. This fragment contains the sequences from the Xba I site of sG_{i2}, 3′ to the end of the cDNA. In the human genome the G_{i2} gene is encoded by nine exons (*14*). The eighth and ninth exons contain the COOH-terminal portion of the coding region and the 3′ untranslated sequences, respectively (*14*). Accordingly, we isolated two phages containing the corresponding region from the mouse genome and determined the sequences of exons 8 and 9 and the boundaries of intron 8 (Fig. 1C). By comparison with the cDNA sequences, we established that the alternative 3′ region present in sG_{i2} is derived from exon 9 and so results from alternative splicing. Both the predicted donor and acceptor splice sites that generate this isoform are noncanonical.

We confirmed the existence of sG_{i2} by

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