THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 38, Issue of September 20, pp. 23374–23379, 1996
Printed in U.S.A.

Activation of the JAK/STAT Pathway Leads to Proliferation of ST14A Central Nervous System Progenitor Cells*

(Received for publication, January 23, 1996, and in revised form, June 5, 1996)

Elena Cattaneo‡, Claudio De Fraja, Luciano Conti, Benedetta Reinach, Liana Bolis, Stefano Govoni§, and Elio Liboi¶

From the Institute of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milano, Italy, §Institute of Pharmacology, University of Pavia, Pavia, Italy, and ¶Institute of Biological Chemistry, University of Verona, Verona, Italy

We were interested in whether central nervous system progenitor cells possess the signal transduction machinery necessary to mediate cytokine functions and whether this machinery can become activated upon stable expression of a particular cytokine receptor. For this purpose we utilized a previously obtained conditionally immortalized striatum-derived nestin-positive cell line (ST14A). We found that ST14A cells express Jak2, but not Jak1 or Tyk2. An identical pattern of expression was found in embryonic striatal tissue. To evaluate the susceptibility of these cytokine specific cytoplasmic transducers to activation, ST14A cells were stably transfected with the α and β (AIC2A) chains of the murine interleukin-3 receptor. Four independent lines expressing both the α and β receptor subunits were obtained. We found that cells from each of these lines were induced to proliferate upon exposure to interleukin-3. Dose response curve, antibody blocking experiments and binding studies revealed that the response was mediated by the reconstituted high affinity interleukin-3 receptor. Immunoprecipitation studies on these cells showed that Jak2 and Stat5 were being phosphorylated after stimulation of the reconstituted receptor. These results indicate that members of the JAK/STAT family of proteins are expressed in central nervous system progenitor cells and are susceptible to activation through stimulation of an exogenously expressed cytokine receptor, ultimately leading to cell proliferation.

In the hematopoietic system, early stem cells generate secondary progenitors which, through successive cell divisions, give rise to the dazzling array of cell types found in blood (1). During the development of the nervous system a similar series of events generates the various neurons and glia constituting the mature brain (2). In recent years, it has become evident that the pyramidal hierarchy of cell maturation peculiar to these two cell systems is controlled by a variety of soluble molecules (1, 3) and that functionally and/or biochemically homologous proteins operate in the two systems (4–6).

The idea of a functional and structural link between the hematopoietic and nervous systems is further supported by the findings that cytokines and cytokine receptors are expressed in both the developing and mature mammalian central nervous system (CNS)[1] (7–10). In addition, important biological functions have been uncovered for some of these molecules which appear to be mainly implicated in driving or maintaining differentiated properties in specific subpopulations of postmitotic neurons (12–14). Nevertheless, the fact that cytokine receptors are confined to limited subpopulations of differentiated CNS cells has hampered detailed functional and biochemical analysis of cytokine-elicited responses and signal transduction mechanisms activated by these molecules in mature CNS cells and their progenitors.

Cytokine effects in hematopoietic cells have been more thoroughly investigated and have led to the discovery of two families of proteins involved in cytokine signal transduction: 1) the Janus kinase (JAK) proteins, a class of four cytoplasmic tyrosine kinases (Tyk2, Jak1, Jak2, and Jak3) and 2) the signal transducers and activator of transcription (STAT) proteins, composed by six latent transcription factors (Stat1–6) that become phosphorylated on tyrosine in response to receptor activation (for reviews, see Refs. 15 and 16). To date only two reports are available describing JAK presence in the nervous system. The first, from Yang *et al.* (17), reports the cloning of Jak1 from mouse brain and provides evidence for the presence of Jak1 mRNA in retina and total brain during development, and for expression of the protein in limited groups of cells in the adult CNS. In the second, by using PCR cloning techniques Sanchez *et al.* (18) isolated tyrosine kinase sequences from embryonic hippocampal neurons. Among the amplified sequences the authors found Jak1, Jak2, and Jak3.

Prompted by these observations we first investigated whether Jak1, Jak2, or Tyk2 proteins were expressed in embryonic CNS progenitors and whether this pathway could become activated once stable expression of particular cytokine receptors was achieved. We focussed on the IL3 receptor (IL3R) as a possible activator of JAK pathways in embryonic CNS cells, particularly since *in vitro* production of IL3 by murine central nervous system neurons (10), expression of its mRNA in brain areas (9) and IL3R associated antigens (19) have been reported, indicating that endogenous IL3 might be produced locally and its corresponding receptors transiently expressed *in vivo* by certain neurons or their progenitors.

Conditionally immortalized ST14A cells previously obtained by retroviral transduction of the temperature-sensitive (*ts*A58U19) allele of the large T-antigene oncogene into pri-

* This work was supported in part by the Hereditary Disease Foundation (United States), European Science Foundation Twinning Grant 9114 (to E. C.), NATO-CRG Grant 930887 (to E. L.), and Piano Finalizzato CNR Grant 000389F99. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence should be addressed. Tel.:39-2-20488349; Fax: 39-2-29404961; E-mail: Cattaneo@ISFUNIX.FARMA.UNIMI.IT.

[1] The abbreviations used are: CNS, central nervous system; JAK, Janus kinase; STAT, signal transducers and activator of transcription; IL, interleukin; IL3R, IL3 receptor; rmILR-3, recombinant murine IL3; SFM, serum-free medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

mary striatal cells (20) were thus utilized in experiments aimed at expressing the α and β (AIC2A) subunits of the murine IL3R (for a review on the biochemistry and molecular biology of this receptor system, see Ref. 21).

In this study, we showed that Jak2 is expressed *in vivo* in the CNS at early developmental stages, as well as *in vitro* in the immortalized CNS progenitors. We found that in these cells Jak2, as well as Stat5, become phosphorylated on stimulation of the reconstituted high affinity IL3R by IL3. The triggered cells were undergoing cell division in serum free conditions. This effect was found to be strictly dependent on the presence of the cytokine.

EXPERIMENTAL PROCEDURES

Cell Culture—ST14A cell line was previously obtained from primary cells dissociated from E14 striatum primordia and immortalized using a retrovirally transduced temperature-sensitive variant (*ts*A58U19) of SV40 Large T-antigen (20).[2] ST14A cells were found to express nestin, a cytoskeletal antigen specifically present in CNS progenitors (22). The cells were propagated routinely at 33 °C in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 0.11 g/liter sodium pyruvate, 3.7 g/liter $NaHCO_3$, 0.29 g/liter glutamine, 3.9 g/liter HEPES, 100 units/ml penicillin-streptomycin (Life Technologies, Inc.), and 10% fetal calf serum (Imperial, UK).

Plasmids and Transfection Experiments—The cDNAs coding for the α (SUT-1) and β_{IL3} (AIC2A) subunits of the murine IL3 receptor were cloned into the polylinker of the pLXSP expression vector (gift from J. F. Lacoud) conferring resistance to puromycin and giving rise to the plasmids, pLXSPαIL3R and pLXSPβIL3R. Standard transfections were performed with the $CaPO_4$ kit (Stratagene, Life Technologies, Inc.). In each transfection experiment, 5 μg of each plasmid DNA were utilized. Following transfection, cells were rinsed free of the precipitate, and puromycin at 3 μg/ml was applied after a further 48 h. Resistant colonies were transferred into 24-well dishes, incubated, and cryopreserved. Of the puromycin-resistant clones, four expressed both of the IL3R subunits. These clones were named $\alpha\beta$14, $\alpha\beta$20, $\alpha\beta$28, and $\alpha\beta$29.

RNase Protection Assay—The assay was performed as described (23). Fifteen μg of total RNA were hybridized to ^{32}P-labeled antisense RNA probes at 52 °C overnight and then treated with RNase A and T1. The reaction products were loaded onto a 6% polyacrylamide sequencing gel. The βIL3R probe and the αIL3R probe were as described by Liboi *et al.* (23, 24).

[^{3}H]Thymidine Incorporation and Proliferation Assays—DNA synthesis was measured by [^{3}H]thymidine incorporation. Briefly, 2.5×10^4 cells were exposed to serum-free medium (SFM, composition: 1:1 F12: Dulbecco's modified Eagle's medium including 5 mg/ml insulin, 100 mg/ml transferrin, 20 nM progesterone, 30 nM selenium salt, 60 mM putrescine, 2 mM glutamine, 0.11 mg/ml sodium bicarbonate, 4.3 mg/ml HEPES buffer) for 16 h prior to starting the experiment, markedly reducing the ability of ST14A cells to proliferate compared to when serum was present (20). Recombinant murine IL3 (rmIL3) (Biosource International, Celbio, Italy) at 2 ng/ml was applied for the time indicated under "Results."

Tritiated thymidine, 1 μCi/ml of medium (specific activity, 2 μCi/mmol; Amersham Corp.) was added for 6 h before harvesting the cells. Rinsed cultures were solubilized in NaOH and aliquots assayed for radioactivity by addition of scintillation liquid and for protein by standard protein assays (Bradford). When this experiment was performed in cells exposed to 39 °C, [^{3}H]thymidine of higher specific activity (87 Ci/mmol; Amersham Corp.) was used. For the proliferation assay, cells from transfected clones were seeded at a density of 3×10^4 cells into each well of a six-well dish and incubated for 16 h in SFM. After 16 h (time 0), rmIL3 was added to the treated plates. The cells were then counted by Coulter Counter (model ZM from Coulter Instruments, Milan, Italy) at 24-h intervals for 7 days (168 h). Live cells were counted after trypsinization of the monolayers.

Radioiodination of IL3 and Binding Assay—Carrier-free recombinant murine IL3 was iodinated by the iodine monochloride method (25). Briefly, 5 μg of rmIL3 were incubated in the presence of 2 mCi of NaI (Amersham Corp.) and 4 mM iodine monochloride (ICl). ^{125}I-IL3 was separated from free iodine by passage through a Sephadex-G25M column. Fractions containing the radioactivity were collected, and an aliquot was examined by SDS-PAGE. The iodinated IL3 was calculated to have a specific activity of 79.7 cpm/fmol.

For the binding experiments, 3×10^5 cells were incubated with ^{125}I-IL3 for 4 h at 4° C in RPMI containing 30 mM HEPES, 1% bovine serum albumin, 0.1% NaN_3, 0.1 mM bacitracin and 5 mg/ml aprotinin. A saturation curve was obtained by incubating sets of cells with various amounts of ^{125}I-IL3 in the range of 0.5×10^3 to 20×10^4 cpm (corresponding to an estimated 0.01–20 nM of IL3). Cells were then washed several times with phosphate-buffered saline (PBS) containing bovine serum albumin and lysed. Radioactivity was measured by gamma counter and normalized over the protein content (Bradford assay). Nonspecific binding was determined in the presence of a fixed excess (100×) of unlabeled rmIL3 as competitor to the labeled species, and this value was subtracted from the total binding to yield the specific binding. All samples were tested in triplicate and used to estimate values of K_d and B_{max} by the LIGAND program.

Immunohistochemistry—Cells were plated onto 12 mm diameter glass coverslips coated with poly-D-ornithine. For the immunohistochemistry, cultures were fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) followed by two rinses in PBS. After permeabilizing the cells in 0.2% Triton X-100 in PBS, Jak2 antibodies (polyclonal Transduction Lab, DBA, Italy) were applied at 4 °C for 16 h followed by a fluorescein-conjugated secondary antibody at a 1:100 dilution (1 h at room temperature; Vector, DBA, Italy). Following removal of the secondary antibody, coverslips were mounted using Permafluor (Italscientifica, Italy).

Immunoprecipitation and Western Blot Analyses—For the immunoprecipitation experiments 1×10^7 cells from the $\alpha\beta$14 line were starved in serum-free medium for 16 h. Thirty min before treatment, 5 mM Na_3VO_4 was added to each plate. The cells were then exposed to murine recombinant IL3 for 5 or 10 min and subsequently lysed in buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM $ZnCl_2$, 10 mM NaF, 5 mg/ml aprotinin, 2 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The lysates were precleared with protein-A Sepharose (Sigma, Italy) for 30 min at 4 °C and then incubated in the presence of 4G10 anti-phosphotyrosine monoclonal antibodies (UBI, DBA-Italy) or with a rat anti-mouse βIL3 chain (anti-AIC2A) (clone 18H6–14) (26) for 16 h at 4 °C. Immune complexes were precipitated with protein A-Sepharose and washed in lyses buffer, and the eluted proteins were loaded onto 7.5–10% SDS-PAGE column. The filters were reacted with 4G10 anti-phosphotyrosine (1:1000), anti-Jak2 (1:1000; UBI, DBA, Italy), or anti-Stat5 (1:250; Transduction Lab.; DBA, Italy) followed by the respective peroxidase conjugated secondary antibodies and visualized with the ECL detection system (Amersham Corp.) as described by the manufacturer.

For Western blot analyses, the striatum primordia were dissected from E14 rat embryos as described previously (27). Membranes of total cell lysates from ST14A and $\alpha\beta$14 cells, as well as those from the embryonic striatum, were reacted with anti-Jak1 (1:1000; Transduction Lab.; DBA, Italy), anti-Tyk2 (1:1000; Transduction Lab.; DBA, Italy), and anti-Jak2 antibodies.

RESULTS

Expression of JAK Proteins in CNS Progenitors in Vivo and in Vitro—To evaluate whether members of the JAK family were expressed in CNS progenitors, Western blot analyses were performed on total lysates from the E14 striatum primordia and from parental ST14A cells. Fig. 1*A* shows the presence, in the cell line and embryonic striatal tissue, of a 130-kDa immunoreactive band in the anti-Jak2-reacted blots. As shown in the figure, in neither of the two samples could any immunoreactivity be detected for either Jak1 or Tyk2. Lysates for positive controls are shown on the *left lane* of each blot. Immunocytochemical analysis (Fig. 1*B*) showed the Jak2 signal localized in the cytoplasm of ST14A progenitor cells.

Gene Transfer and IL3R mRNA Expression—The pLXSP-derived vectors containing the α and β (AIC2A) IL3R subunits were co-transfected into nestin-positive ST14A cells (20). Simultaneous expression of the α and β IL3R chains in four puromycin-resistant clones was evaluated by RNase protection assay. As shown in Fig. 2, protected bands corresponding to the α and β IL3R subunits were present in clones $\alpha\beta$14, $\alpha\beta$20, $\alpha\beta$28, and $\alpha\beta$29. In these clones the 127-bp protected fragment corresponding to the β IL3 (AIC2A) receptor subunit (Fig. 2*B*

[2] E. Cattaneo, unpublished results.

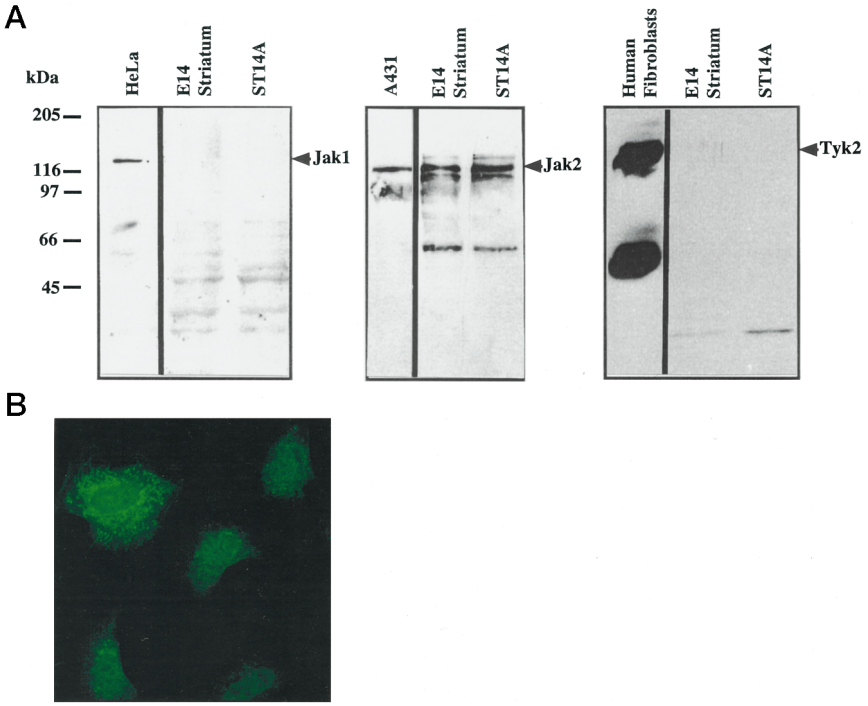

FIG. 1. **Expression of the JAK proteins in ST14A cells and E14 striatum primordia.** *A*, proteins were separated on 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Jak1 (*left panel*), anti-Jak2 (*center panel*), and anti-Tyk2 (*right panel*). In each panel, the *left column* is represented by a positive control and shows an immunoreactive signal for Jak1 in control Hela cells, for Jak2 in control A431 cells, and for Tyk2 in control human fibroblasts. A band at 130 kDa (molecular mass) is present in lysates from either ST14A cells and cells freshly dissociated from the E14 striatum primordia. *B*, immunofluorescent detection of Jak2 in ST14A cells.

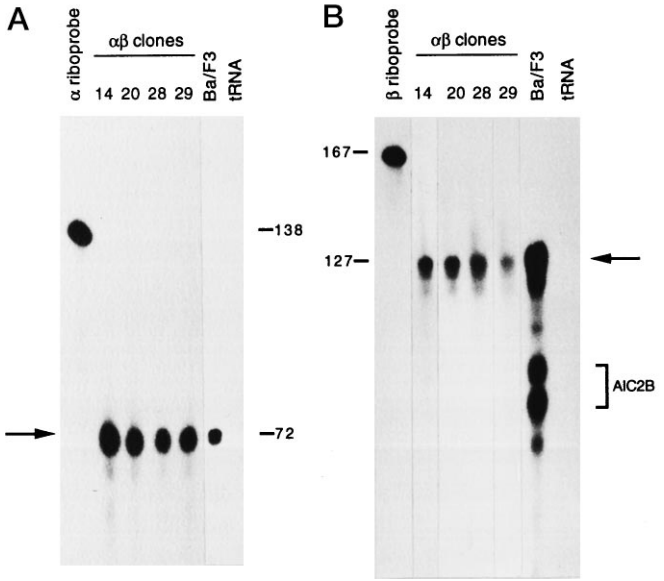

FIG. 2. **Expression of the α and β IL3R subunits in IL3R transfected CNS progenitor cells.** Total RNA from hematopoietic Ba/F3 cells (positive control) and puromycin-resistant αβ14, αβ20, αβ28, and αβ29 clones were analyzed by RNase protection assay. *A*, αIL3R riboprobe protects a 72-bp fragment in the RNAs from the four puromycin-resistant lines and in Ba/F3 cells. *Left lane*, free RNA probe. *B*, βIL3R (AIC2A) riboprobe protects fragments of 127 bp (AIC2A) in the αβ clones as well as in Ba/F3 cells. In the panel the 90- and 76-bp-protected fragments visible in control Ba/F3 cells correspond to the endogenous β_cIL3 (*AIC2B*) chain (23). Negative control tRNA lane and the DNA molecular weight marker λ (*Bst*EII digested) are shown.

comigrates with the endogenous β IL3R chain expressed in the Ba/F3 hematopoietic line. Fig. 2*A* shows the same cell clones expressing a 72-bp protected fragment that comigrates with the α IL3R chain present in Ba/F3 cells. No protected bands were seen when tRNA or RNA from parental ST14A cells were hybridized with either the α or β riboprobes.

DNA Synthesis in IL3R-transfected Lines—To determine whether activation of the transfected IL3R could stimulate DNA synthesis, [^{3}H]thymidine incorporation experiments were performed. Table I shows the data obtained for the four lines expressing both subunits of the receptor and for two lines (β11 and α9) expressing only one of the two receptor subunits. It is evident in Table I that the αβ14, αβ20, αβ28, and αβ29 lines expressing the complete receptor showed increased [^{3}H]thymidine incorporation on stimulation with IL3. Specifically, αβ14 cells not given IL3 incorporated 256 ± 91 cpm/μg of protein, while IL3-treated cultures incorporated 514 ± 13 cpm/μg of protein (100% increase over control). In β11 and α19 lines expressing only the β or the α IL3 receptor subunit, respectively, as well as in parental ST14A cells, exposure to IL3 did not elicit any effect.

Given that αβ14 cells exhibited the greatest biological effect following IL3 stimulation, this clone was chosen for further experiments. Increased thymidine incorporation was also observed in IL3-stimulated αβ14 cells exposed to 39° C. Indeed, when exposed to 39° C for 24 h and treated for 24 additional h with IL3, these cells incorporated 473 ± 60 cpm/μg of protein, while unstimulated cells incorporated significantly less thymidine (211 ± 64 cpm/μg of protein; $p < 0.001$ by Student's t test).

Binding Assays—Binding experiments were performed on αβ14 cells and in parental ST14A cells. We found saturable binding in the transfected cells. LIGAND program analysis indicated a B_{max} value of 178 fmol/mg of protein (±10%) with an apparent K_d of 212 pmol/liter for αβ14 cells. On the other hand, ST14A cells showed no specific binding. These results confirmed the presence of a functional high affinity IL3R on αβ14 CNS progenitor cells. All of the experiments reported were repeated over more than 1 year, indicating that the transgene was stably integrated.

Dose Response Curve and Antibody Blocking Experiments—We analyzed the effect of different doses of rmIL3 on the synthesis of DNA in αβ14 cells. Fig. 3 shows the linearity of the effect of IL3 in a range 0.05–2 ng/ml. Importantly, the concentration of IL3 required to obtain maximal incorporation of [^{3}H]thymidine (2 ng/ml) correlated well with the receptor K_d, both being in the picomolar range (28).

Antibody blocking experiments indicated that the effect of IL3 on DNA synthesis could be partially blocked by preincubating the cells with an antibody against the β receptor subunit

TABLE I
IL3 effect on the incorporation of [^{3}H]thymidine in IL3R-transfected ST14A cells

The experiment was performed on cultures deprived of serum for 16 h prior to treatment. [^{3}H]thymidine incorporation was measured after 24-h incubation in SFM in the absence (left columns) or presence (right columns) of 2 ng/ml recombinant murine IL3. Radioactive thymidine was added 6 h before collecting the cells. Each value is mean ± S.D.; n=6. The data were repeatedly conformed in several experiments performed over the last 2 years and on cells that were passaged several times.

Clone	−IL3	+IL3[a]
$\alpha\beta$14	256 ± 91	514 ± 13**
$\alpha\beta$20	251 ± 9	394 ± 20**
$\alpha\beta$28	181 ± 24	249 ± 18*
$\alpha\beta$29	227 ± 28	339 ± 17**
β11	182 ± 8	195 ± 21
α19	193 ± 13	191 ± 15

[a] ** $p<0.0001$ and *$p<0.001$ over the respective control cultures by Student's t test.

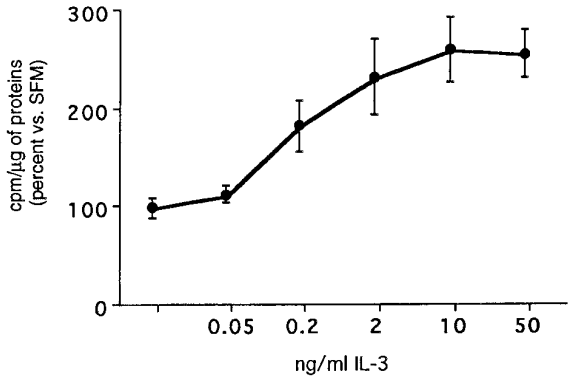

FIG. 3. **Effect of different doses of rmIL3 on $\alpha\beta$14 cells.** Triplicate samples were incubated with 0.05, 0.2, 2, 10, and 50 ng/ml of IL3 in SFM. Each value is mean ± S.D.; n = 6. Data are expressed as percentage *versus* control.

(26). Thus, cultures treated with both IL3 and the 25C9 blocking antibody (10 μg/ml) for 24 h showed a 52% reduction in thymidine incorporated compared to the cultures treated with IL3 (IL3 treated: 389 ± 40 cpm/μg of protein; IL3 plus blocking antibody: 297 ± 9 cpm/μg of protein; difference, $p < 0.001$ by Student's t test). In the presence of the antibody alone 198 ± 20 cpm/μg of protein were incorporated, while control cultures (without IL3) incorporated 177 ± 23 cpm/μg of protein. We therefore conclude that the observed effects were specifically driven by the presence of the transfected IL3R.

Growth Curve of IL3-stimulated $\alpha\beta$14 Cells—Incorporation of radioactive thymidine occurs in cells entering a new cell cycle, but it can also reflect other phenomena such as DNA repair in damaged cells. To ascertain whether the activated IL3R mediates a proliferative event in CNS progenitor cells, we performed growth curve analysis in $\alpha\beta$14 cells maintained at 33° C in SFM in the presence or absence of 2 ng/ml rmIL3. As shown in Fig. 4, a statistically significant increase in the number of cells occurred in stimulated (*broken line*) *versus* unstimulated (*solid line*) cultures 24 h following treatment. At 72 h, twice as many cells were present in IL3 treated then untreated cultures (control cultures: $0.81 \times 10^5 \pm 0.09 \times 10^5$ cells; IL3-stimulated cultures: $1.63 \times 10^5 \pm 0.2 \times 10^5$ cells). At confluency (168 h), stimulated cells did not acquire a transformed phenotype.

Immunoprecipitation Studies—In the hematopoietic system, activation of the IL3R leads to Jak2 and Stat5 phosphorylation (29, 30). We showed that Jak2 is expressed in the immortalized progenitors as well as in the embryonic material from which

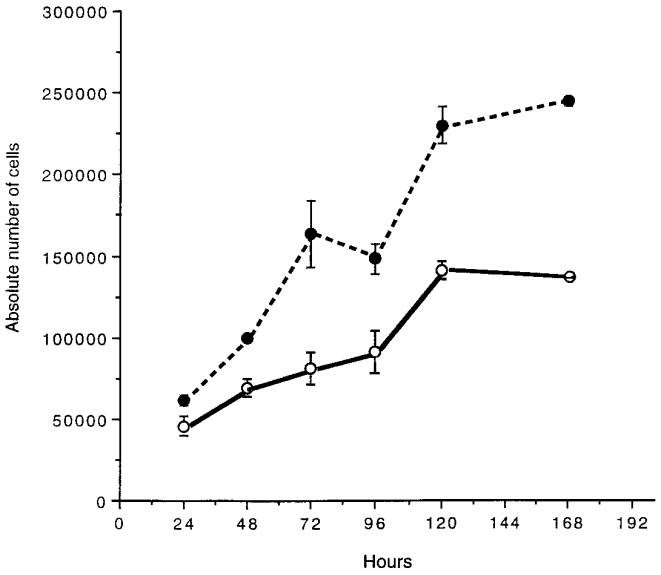

FIG. 4. **Growth curve analysis on $\alpha\beta$14 cells.** At time zero, 0.3×10^5 cells were incubated with PBS + bovine serum albumin (*open circles*) or with 2 ng/ml of rmIL3 (*closed circles*). At 24-h intervals after addition of IL3, triplicate samples were trypsinized and counted using a Coulter counter as described under "Experimental Procedures." The graphs indicate the absolute number of live cells found in the cultures in the absence (*solid line*) or presence (*broken line*) of IL3. At each time interval the number of cells found in the presence of IL3 was significantly different from that in control cultures ($p < 0.001$–0.0001 by Student's t test). Values are expressed as mean ± S.D., n = 3. Data are from one of four independent experiments giving the same growth profile.

the immortalized cells were derived (Fig. 1). We therefore wanted to determine whether the biological effect observed on stimulation of the exogenously expressed IL3R occurred through activation of Jak2 and phosphorylation of Stat5. To reveal whether Jak2 was recruited by the activated exogenous receptor, lysates from $\alpha\beta$14 cells were subjected to immunoprecipitation with the rat anti-β subunit monoclonal antibody and the membrane filter reacted with the anti-Jak2 antibody. As shown in Fig. 5*A* (*arrow*), the association of Jak2 with the exogenous β subunit is mainly prompted by the presence of the ligand. Indeed, an increased quantity of Jak2 protein could be immunoprecipitated in lysates from cells which had been stimulated with IL3 for 5 min. In a parallel filter immunodecorated with anti-β subunit antibodies, equal amounts of immunoprecipitated β chain were detected in all samples (data not shown).

When the $\alpha\beta$14 cell lysates were immunoprecipitated with the 4G10 anti-phosphotyrosine antibody and immunoblotted with anti-Jak2 (Fig. 5*B*), a tyrosine phosphorylated band of 130 kDa corresponding to Jak2 could be detected in lysates from stimulated cells (*arrow*). We also wanted to determine whether Stat5 was as well phosphorylated upon receptor stimulation. As shown in Fig. 5*C*, in lysates from stimulated $\alpha\beta$14 cells, which were immunoprecipitated with the 4G10 antibody and immunoblotted with anti-Stat5 antibodies, a 95–96-kDa reactive band could be detected.

DISCUSSION

Reports by several authors have indicated that hematopoietic growth factors play a role in the differentiation of neuronal cells. In this study we focussed on obtaining a clonal population of CNS progenitors in which one cytokine receptor (IL3R) was expressed exogenously, thus generating a tool in which signal transduction pathways and biological response activated by cytokines in CNS progenitors could be studied.

In this study we have shown that physiologically expressed

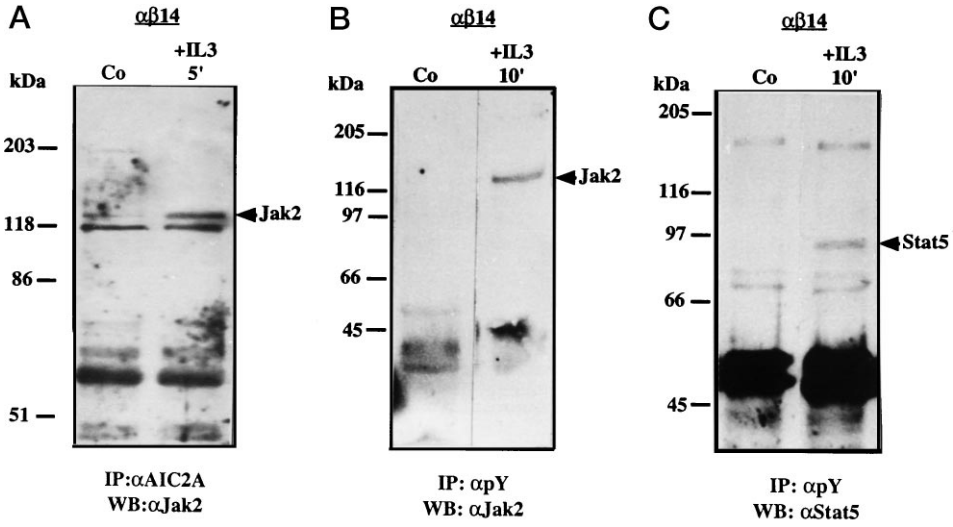

FIG. 5. **IL3-dependent Jak2 and Stat5 phosphorylation in αβ14 cells.** αβ14 cells were starved in serum-free medium for 16 h and stimulated with or without IL3 for 5 or 10 min. The lysates were immunoprecipitated with rat anti-AIC2A monoclonal antibody (*A*) or with 4G10 antiphosphotyrosine antibody (*B* and *C*). An aliquot was run on SDS-PAGE as described under "Experimental Procedures" and immunoblotted with anti-Jak2 (*A* and *B*) or anti-Stat5 antibodies (*C*).

Jak2 in embryonic CNS progenitors is phosphorylated on stimulation of the exogenous IL3R (Fig. 5, *B* and *C*), ultimately leading the stimulated cells to enter a new S phase (Fig. 4). Furthermore, in our experimental system, we found that the association of Jak2 with the exogenous β chain is largely ligand-dependent (Fig. 5*A*). Although we focussed on the IL3R, it is possible that other cytokines and cytokine receptors functioning through the Jak2 system also activate this pathway and hence importantly contribute to the differentiation of the various cell types and functions of the developing central nervous system.

It has been recently reported that, in the hematopoietic system, Jak2 activation by IL3 leads to phosphorylation of a transcription factor known as Stat5 (30). It was shown that Stat5 mRNA is expressed in the brain (30).[2] In this study we provide additional evidence for the involvement of Stat5 in CNS functions as we show that this transcription factor becomes phosphorylated on tyrosine following stimulation of the CNS cells through the reconstituted IL3R.

Entry into a new S phase was demonstrated in all the cell clones we isolated that expressed both subunits of the high affinity IL3 receptor. This result suggests that the biological effect observed is not linked to a particular integration of the transgene into the host genome, but argue in favor of a possible physiological role of the activated JAK/STAT pathway in CNS cells.

The biological effect observed was further exploited in the αβ14 clone. In these cells the K_d of the receptor-ligand complex was similar to that measured in hematopoietic cells, both being in the picomolar range (28). In addition, we demonstrated that in stimulated αβ14 cells the incorporation of [^{3}H]thymidine can be partially blocked by an antibody recognizing the transfected β receptor subunit. The absence of a complete blockade may be due to the presence of a small population of activated receptors on the cell membrane still stimulated by the agonist, perhaps as a consequence of different affinities of the agonist and the blocking antibody for the receptor complex. Growth curve analysis of stimulated αβ14 cells revealed that a single addition of IL3 was capable of sustaining cell division for several days following stimuli. Furthermore, we found that, at each time interval, the effect depended on the presence of the growth factor and conditioned medium from αβ14 cells did not elicit any change in the incorporation of [^{3}H]thymidine in parental ST14A cells (not shown), indicating that the observed effect was not due to molecules released into the culture medium following stimulation with IL3.

We have also shown that the pattern of Jak1, Jak2, and Tyk2 expression in ST14A CNS progenitors is closely similar to that found *in vivo* in the tissue from which ST14A cells were derived (Fig. 1*A*). The absence of Jak1 in both the *in vivo* material and in the immortalized progenitors seems to contrast with results of Yang et *al*. (17) who reported the presence of the mRNA for this protein in total brain from the E15 mouse. However, in that report, protein expression was confirmed primarily in sections from the developing retina or in adult tissue where immunoreactive material was found confined to a few subpopulations of cells.

We have demonstrated that CNS progenitors expressing Jak2 and devoid of intrinsic responsiveness to specific cytokines are stimulated to proliferate when a particular cytokine receptor is provided experimentally. However, it could be argued that this biological effect might not be specifically driven by the presence of an activated IL3R on the cell surface, but that the transfected receptor is activating an endogenous predetermined program of cell division. Although this possibility cannot be completely ruled out, other authors have shown that immortalized cell lines generated following the methodology we used to obtain the ST14A cells are capable of either proliferation in serum or differentiation upon stimulation of transfected neurotrophin receptors (31). Data on the capability of ST14A cells to differentiate are provided by intracerebral transplantation experiments in which ST14A cells were shown to become growth restricted (27) and to differentiate morphologically and biochemically into neurons and glial cells (11).[3] Furthermore, the IL3R-expressing clone (αβ14 cells) as well as parental ST14A cells have been found to differentiate and to express neuronal antigens when exposed *in vitro* to SFM conditions (20).[2] However, αβ14 cells continue to divide in these same conditions (absence of serum) when stimulated through the IL3R, indicating that recruitment of specific cytoplasmic transducers actively triggers a program of cell division in CNS progenitors that would otherwise differentiate.

The finding that this recently identified JAK/STAT pathway

[3] C. Lundberg, submitted for publication.

is also present in CNS cells and is susceptible to activation, indicates that the same intracellular machinery normally mediating cytokine signaling in hematopoietic cells can operate in other tissues. Through a transient and localized expression of cytokine receptors and of their ligands, activated JAK/STAT proteins can constitute the effectors involved in important biological functions elicited by cytokines during the development of the central nervous system.

Acknowledgments—We would like to thank Dr. F. Gage for helpful discussion and comments on the manuscript, Dr. R. Devos for 25C9 antibody, Dr. F. Lacoud for pLXSP vector, Celbio-Biosource, Italy, for providing rmIL3, Dr. A. Corsini for the use of the Coulter Counter, and Dr. D. McKenzie for restyling the manuscript.

REFERENCES

1. Metcalf, D. (1989) *Nature* **339,** 27–30
2. McKay, R. D. G. (1989) *Cell* **58,** 815–821
3. Gage, F., Ray, J., and Fisher, L. (1995) *Annu. Rev. Neurosci.* **18,** 159–192
4. Anderson, D. J. (1989) *Neuron* **3,** 1–12
5. Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M.-J., and Patterson, P. H. (1989) *Science* **246,** 1412–1416
6. Davis, S., Aldrich, T. H., Valenzuela, D. M., Wong, V., Furth, M. E., Squinto, S. P., and Yancopoulos, G. D. (1991) *Science* **253,** 59–63
7. Pousset, F. (1994) *Dev. Brain Res.* **81,** 143–146
8. Masuda, S., Nagao, M., Takahata, K., Konishi, Y., Gallyas, F., Jr., Tabira, T., and Sasaki, R. (1993) *J. Biol. Chem.* **268,** 11208–11216
9. Farrar, W., Vinocour, M., and Hill, J. M. (1989) *Blood* **73,** 137–140
10. Konishi, Y., Kamegai, M., Takahashi, K., Kunishita, T., and Tabira, T. (1994) *Neurosci. Lett.* **182,** 271–274
11. Lundberg, C., Martinez-Serrano, A., Cattaneo, E., McKay, R. D. G., and Bjorklund, A. (1994) *Soc. Neurosci. Abstr.* **205,** 2
12. Kamegai, M., Niijima, K., Kunishita, T., Nishizawa, M., Ogawa, M., Araki, M., Ueki, A., Konishi, Y., and Tabira, T. (1990) *Neuron* **4,** 429–436
13. Kushima, Y., Hama, T., and Hatanaka, H. (1992) *Neurosci. Res.* **13,** 267–280
14. Mehler, M. F., Rozental, R., Dougherty, M., Spray, D., and Kessler, J. (1993) *Nature* **362,** 62–65
15. Ihle, J. N., Witthuhun, B. A., Quelle, F. W., Yamamoto, K., and Silvennoinen, O. (1995) *Annu. Rev. Immunol.* **13,** 369–398
16. Schindler, C., and Darnell, J. E. (1995) *Annu. Rev. Biochem.* **64,** 621–651
17. Yang, X., Chung, D., and Cepko, C. (1993) *J. Neurosci.* **13,** 3006–3017
18. Sanchez, M. P., Tapley, P., Saini, S. S. He B., Pulido, D., and Barbacid, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1819–1823
19. Konishi, Y., Chui, D.-H., Kunishita T., Yamamura, T. Higashi, Y., and Tabira, T. (1995) *J. Neurosci. Res.* **41,** 572–582
20. Cattaneo, E., Magrassi, L., Santi, L., Butti, G., McKay, R. D. G., and Pezzotta, S. (1993) *Soc. Neurosci. Abstr.* **107,** 6
21. Miyajima, A., Mui, A., L-F., Ogorochi, T., and Sakamaki, K. (1993) *Blood* **82,** 1960–1974
22. Lendahl, U., Zimmermann, L., and McKay, R. (1990) *Cell* **60,** 585–595
23. Liboi, E., Jubinsky, P., Andrews, N. C., Nathan, D. G., and Mathey-Prevot, B. (1992) *Blood* **80,** 1183–1189
24. Liboi, E., Carroll, M., D'Andrea, A. D., and Mathey-Prevot, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11351–11355
25. Contreras, M. A., Bale, F. W., and Spar, I. L. (1983) *Methods Enzymol.* **92,** 277–309
26. Van der Heyden, J., Devos, R., Plaetinck, G., Fache, I., Fiers, W., and Tavernier, J. (1991) *J. Immunol.* **147,** 3413–3418
27. Cattaneo, E., Magrassi, L., Butti, G., Santi, L., Giavazzi, A., and Pezzotta, S. (1994) *Dev. Brain Res.* **83,** 197–208
28. Nicola, N., and Metcalf, D. (1986) *J. Cell. Physiol.* **128,** 180–188
29. Silvennoinen, O., Witthhun, B. A., Quelle, F. W., Cleveland, J. L., Yi, T., and Ihle, J. N. (1993) *Proc. Natl. Acad. Sc. U. S. A.* **90,** 8429–8433
30. Mui, A. L. F., Wakao, H., O'Farrell, A. M., Harada, N., and Miyajima, A. (1995) *EMBO J.* **14,** 1166–1175
31. Zhou, J., Holtzman, D. M., Weiner, R. I., and Mobley, W. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3824–3828