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FGF-2-Responsive Neural Stem Cell Proliferation Requires CCg, a Novel Autocrine/Paracrine Cofactor

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Summary

We have purified and characterized a factor, from the conditioned medium of neural stem cell cultures, which is required for fibroblast growth factor 2's (FGF-2) mitogenic activity on neural stem cells. This autocrine/paracrine cofactor is a glycosylated form of cystatin C (CCg), whose N-glycosylation is required for its activity. We further demonstrated that, both in vitro and in vivo, neural stem cells undergoing cell division are immunopositive for cystatin C. Finally, we showed in vivo functional activity of CCg by demonstrating that the combined delivery of FGF-2 and CCg to the adult dentate gyrus stimulated neurogenesis. We propose that the process of neurogenesis is controlled by the cooperation between trophic factors and autocrine/paracrine cofactors, of which CCg is a prototype.

Introduction

Neurogenesis, first thought to be limited to the prenatal period, occurs throughout adulthood in discrete regions of the brain, such as the subventricular zone (SVZ) (Lois and Alvarez-Buylla, 1993; Luskin, 1993) and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus of several species, including human (Altman and Das, 1965; Caviness, 1973; Eriksson et al., 1998). Stem cells are defined as undifferentiated cells that display high proliferative potential, generate a wide variety of differentiated progeny including the principal phenotypes of the tissue, possess the capacity for self-renewal, and retain their multilineage potential over time (Gage et al., 1995a). The process of neurogenesis includes proliferation, migration, survival and differentiation into postmitotic neurons of the putative neural stem cells. During brain development, an excess of neural cells is produced; the cells migrate toward their target, where a limited supply of trophic factors, produced by the target cells, regulates their survival (Oppenheim, 1991). Neurogenesis is regulated by a variety of stimuli, including steroid hormones, aging, environmental enrichment, genetic background, stresses, and running (Gould et al., 1992; Kuhn et al., 1996; Kempermann et al., 1997a, 1997b; Gould et al., 1998; Van Praag et al., 1999). However, the molecules and mechanisms controlling neural stem or progenitor cell proliferation remain only partially understood. Mitogens like epidermal growth factor (EGF) and FGF-2 influence the extent and rate of proliferation of neural stem and progenitor cell populations in vitro and in vivo (Reynolds and Weiss, 1992; Richards et al., 1992; Vescovi et al., 1993; Gage et al., 1995b; Palmer et al., 1995, 1999; Kuhn et al., 1997; Shihabuddin et al. 1997; Wagner et al., 1999; Roy et al., 2000), and studies from single cell cultures show that EGF and FGF-2 are mitogens for mouse-derived neurospheres (Reynolds and Weiss, 1996; Gritti et al., 1996) and that other unknown factors, present in conditioned medium (CM), are required to support neural stem cell proliferation (Temple, 1989; Kilpatrick and Bartlett, 1993; Davis and Temple, 1994; Qian et al., 1997; Palmer et al., 1997; Johansson et al., 1999).

We previously established long-term cultures, in the presence of FGF-2, of adult rat hippocampus-derived neural progenitor cells (AHPs) (Gage et al., 1995b) and also of adult rat neural progenitor cells derived from other areas of the adult brain (Palmer et al., 1995, 1999; Shihabuddin et al. 1997). We have previously cloned AHPs and have shown that all three lineages are generated from single genetically marked cells and that the cloned AHPs display stem cell properties, including the capacity for self-renewal and retaining their multilineage potential (Palmer et al., 1997; Takahashi et al., 1999). We have also grafted AHPs in the brain, where they integrate and differentiate into terminally differentiated neurons, appropriate for the area to where they were grafted (Gage et al., 1995b; Suhonen et al., 1996; Takahashi et al., 1998). We observed that FGF-2 requires a high cell density for culturing AHPs and also for culturing neural progenitor cells from other areas of the brain, whereas the expansion of clonal populations of AHPs, and also of neural progenitor cells from other areas of the brain, from single cells with FGF-2 was unsuccessful, unless FGF-2 was supplemented with AHP CM.

We postulated that FGF-2 requires cooperation with an unknown autocrine/paracrine cofactor(s), present in the CM, to support neural hippocampal stem cell proliferation in vitro. We further postulated that the CM-derived cofactor potentiates the activity of FGF-2 in vivo and therefore cooperates with FGF-2 to induce proliferation of endogenous neural stem cells in the adult hippocampus. Our results have lead to the discovery of a novel autocrine/paracrine factor that cooperates with FGF-2 to stimulate the proliferation of neural stem cells in vitro and neurogenesis in vivo. Furthermore, this discovery permits the formulation of the hypothesis that trophic factors can achieve activity, and therefore specificity, through required interaction with local autocrine or paracrine cofactors.

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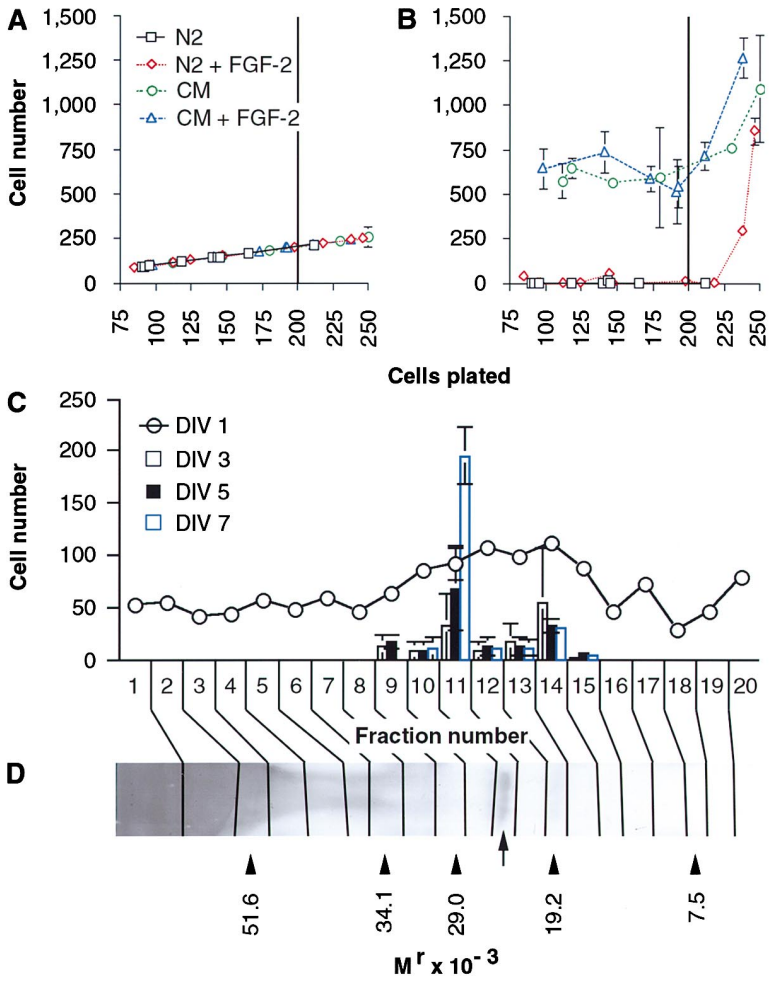

Figure 1. In Vitro Proliferation Assay and Purification of the Cofactor

AHPs, plated at different cell densities in N2, N2 + FGF-2, CM, and CM + FGF-2, were counted at DIV 1 (A) and DIV 5 (B). FGF-2 alone supports proliferation of AHPs at densities > 200 cells per well; at lower densities, CM is required. The purified 21 kDa protein ([D], arrow) exhibited most of the mitogenic activity ([C], fraction 11) at DIV 5 and DIV 7. In (C), between DIV 1 and 3 a large number of cells died, probably from the toxic compounds eluted from the membranes. Molecular weight markers (M_r) are indicated ([D], arrowhead). Data in (A) and (B) are means ± SEM of six independent experiments, and data in (C) are the means of triplicate from one of two typical independent experiments.

Results

In Vitro Proliferation Assay

To demonstrate the requirement of an autocrine/paracrine cofactor to support the mitogenic activity of FGF-2, AHPs were plated in cell culture medium (N2 medium) at densities at which the cells would die in the presence of FGF-2 alone but would proliferate with FGF-2 supplemented by AHP CM (Figure 1A). FGF-2 alone (N2 + FGF-2) supported the proliferation of AHPs when plated at densities greater than 200 cells per well (0.32 cm^2 surface area, Figure 1B). At lower densities (1–200 cells per well), cells cultured in N2 medium alone (N2) or with FGF-2 alone died by 5 days in vitro (DIV 5), whereas CM or CM containing FGF-2 (CM + FGF-2) supported their proliferation (Figure 1B). Thus, the mitogenic activity of FGF-2 is cell density dependent, with high-density cultures producing sufficient amounts of a cofactor(s) to support FGF-2's mitogenic activity, whereas low-density cultures produced an insufficient amount of cofactor to cooperate with any levels of FGF-2 to support cell proliferation. The observed mitogenic activity of the CM in the absence of FGF-2 is likely due to the continued presence of an adequate concentration of FGF-2 added for culturing. Different trophic factors/cytokines, including EGF—tested at different concentrations ranging from 10 pg/ml to 100 ng/ml and tested alone or in combinations, with or without FGF-2—failed to mimic the activity of the CM (Table 1). Heparin, a factor known to potentiate FGF-2 (Baird, 1994; Caldwell and Svendsen, 1998), was also tested at concentrations ranging from 2 ng/ml to 100 mg/ml and did not potentiate FGF-2's mitogenic activity at cell densities below 200 cells plated per well in the in vitro proliferation assay (Table 1). Statistical analysis by one-way ANOVA showed that there was no difference between the four different conditions at DIV 1 [$F(3,30) = 0.83$, $p > 0.5$]. At DIV 5, one-way ANOVA showed a significant effect due to treatments [$F(3,30) = 27.34$, $p < 0.0001$]. Specific comparisons showed significant differences between all conditions ($p < 0.0006$), except between N2 and N2 + FGF-2 or CM and CM + FGF-2. Comparisons made above and below 200 cells plated per well showed a significant difference for FGF-2 [$t(9) = 2.66$, $p < 0.026$] and CM [$t(4) = 3.17$, $p < 0.034$].

Purification and Characterization of the Cofactor

To purify the cofactor, large quantities of CM were generated from the AHPs. The cofactor was purified and analyzed by SDS-PAGE; a 21 kDa protein (Figure 1D, arrow) exhibited most of the activity in the in vitro proliferation assay (Figure 1C, fraction 11). N-terminal sequencing of the protein yielded a sequence signal of

Table 1. Effect of Neurotrophic Factors/Cytokines on AHPs Plated at Low Density

Trophic Factor/Cytokine		Cell Number			
		Number of FGF-2		+ FGF-2 (20 ng/ml)	
		DIV 1	DIV 5	DIV 1	DIV 5
FGF-2	(20 ng/ml)	15.3 ± 0	6.6 ± 3	42.1 ± 12	3.6 ± 1
FGF-2	(100 ng/ml)	41.7 ± 11	2.9 ± 1		
Heparin	(2 μg/ml)	14.6 ± 2	3.0 ± 0	10.6 ± 2	2.0 ± 2
FGF-1	(40 ng/ml)	31.6 ± 1	0.3 ± 0	34.3 ± 8	0.6 ± 0
FGF-4	(40 ng/ml)	31.6 ± 2	6.0 ± 2	25.3 ± 2	0.0 ± 0
FGF-7	(20 ng/ml)	14.6 ± 2	8.0 ± 2	14.6 ± 0	8.0 ± 2
NGF	(50 ng/ml)	35.5 ± 9	8.8 ± 5	33.3 ± 12	5.9 ± 3
BDNF	(20 ng/ml)	36.4 ± 14	5.4 ± 5	38.9 ± 12	5.6 ± 8
NT-3	(40 ng/ml)	30.6 ± 0	1.0 ± 0	28.3 ± 2	2.0 ± 1
EGF	(40 ng/ml)	39.3 ± 3	0.6 ± 0	36.6 ± 1	2.0 ± 1
CNTF	(40 ng/ml)	25.3 ± 2	0.6 ± 0	25.3 ± 0	0.3 ± 0
PDGF	(40 ng/ml)	23.3 ± 2	0.0 ± 0	17.1 ± 1	0.0 ± 0
GDNF	(20 ng/ml)	16.6 ± 2	0.0 ± 0	19.6 ± 2	0.0 ± 0
IL-3	(20 ng/ml)	32.0 ± 4	1.6 ± 0	20.6 ± 6	1.3 ± 0
IL-4	(20 ng/ml)	50.6 ± 3	2.3 ± 0	46.3 ± 3	9.0 ± 5
IL-6	(20 ng/ml)	18.0 ± 4	1.6 ± 0	18.6 ± 6	0.3 ± 0
TNF-α	(10 ng/ml)	37.9 ± 9	7.3 ± 2	37.9 ± 8	9.0 ± 4
PTN	(20 ng/ml)	20.3 ± 2	0.0 ± 0	24.6 ± 4	2.3 ± 2
Activin	(20 ng/ml)	26.3 ± 0	0.0 ± 0	37.6 ± 5	0.0 ± 0
TGF-α	(40 ng/ml)	21.3 ± 4	1.0 ± 0	17.6 ± 3	0.6 ± 0
TGF-β	(20 ng/ml)	26.0 ± 3	0.0 ± 0	36.3 ± 3	0.0 ± 0
IGF-I	(20 ng/ml)	16.0 ± 2	2.6 ± 1	17.3 ± 3	0.0 ± 0
IGF-II	(20 ng/ml)	17.0 ± 1	2.0 ± 1	21.0 ± 4	2.6 ± 0
MIP-1α	(6 ng/ml)	11.6 ± 2	0.3 ± 0	12.0 ± 0	0.3 ± 0
MIP-1β	(40 ng/ml)	13.6 ± 2	1.0 ± 0	19.3 ± 0	0.3 ± 0
Rantes	(20 ng/ml)	20.6 ± 5	0.0 ± 0	18.0 ± 2	0.0 ± 0
SCF	(20 ng/ml)	28.0 ± 4	5.1 ± 0	10.6 ± 6	0.5 ± 0

Trophic factors/cytokines were tested at different concentrations ranging from 10 pg/ml to 100 ng/ml. None of the factors tested mimicked the activity of the CM. Heparin, a factor known to potentiate FGF-2 (Baird, 1994), was also tested at concentrations ranging from 2 ng/ml to 100 μg/ml and did not potentiate FGF-2's mitogenic activity in the in vitro proliferation assay.

low intensity (GXSXXXXXLLGAXQXADA) that matched the N-terminal sequence of rat cystatin C (Esnard et al., 1990). The identity of the 21 kDa protein was further confirmed by amino acid sequencing and mass spectral analysis of the tryptic peptide fragments. All of the determined sequences (Figure 2A; 84% of the 21 kDa protein was sequenced) matched rat cystatin C (Esnard et al., 1990). Two peptides, G1752 and G1751, were identified as full-length and N-terminally truncated rat cystatin C, respectively. Peptide G1754 contained a consensus site for N-glycosylation. The discrepancy between the experimental mass and the theoretical mass calculated from the backbones for the peptides G1750, G1751, G1752, and G1754 indicated that they were indeed glycosylated (Figure 2A). Furthermore, the mass spectroscopic patterns of the peptides G1751 (Figure 2B) and G1754 (Figure 2C) were characteristic of N-glycosylated peptides, indicating that the 21 kDa protein is a glycosylated form of cystatin C (CCg). The deduced mass of the N-linked carbohydrate moiety is approximately 2.4 kDa, corresponding to 15 hexose residues. A carbohydrate moiety of 0.3 kDa is present at the O-glycosylation site (peptide G1750). The molecular weight of 21 kDa, as estimated by SDS-PAGE, was higher than the value obtained by mass spectral analysis (16 kDa, G1752, Figure 2A). This discrepancy may be due to the basic nature of the protein and the presence of covalently bound carbohydrates (Hames, 1981).

N-Glycosylation Is Essential for CCg's Activity

Cystatin C, a cysteine proteinase inhibitor (Turk and Bode, 1991), is a molecule with pleiotropic functions, with specific domains of the molecule involved in specific functions (Machleidt et al., 1989; Leung-Tack et al., 1990). Cystatin C is secreted by different cell types in vitro (Warfel et al., 1987). It is expressed in the brain (Yasuhara et al., 1993), where it is associated with cerebral pathologies such as cerebrovascular amyloidosis (Turk and Bode, 1991). Rat cystatin C is a 13–14 kDa basic protein (Turk and Bode, 1991) containing unique consensus sites for N- (Asn-79-X-Thr) and O-glycosylation (Ser-3) (Esnard et al., 1990). The existence of a glycosylated form of cystatin C has been reported in rat seminal vesicles (Esnard et al., 1988). However, the type(s) of glycosylation, N-linked and/or O-linked, and their functional relevance have not been investigated. In the in vitro proliferation assay, CCg at concentrations between 0.02 and 2 ng/ml induced cell proliferation in a dose-dependent manner and was potentiated by FGF-2 (Figure 3B). To investigate the importance of the N-linked carbohydrate moiety, CCg was treated with PNGase F, an amidase that removes N-linked oligosaccharides from glycoproteins by cleaving the N-glycosidic bond between Asn and the first hexoseamine. On SDS-PAGE, the N-deglycosylated CCg (CCg-DG) exhibited a shift in molecular weight to 19 kDa and appeared sharper than the 21 kDa band, which migrated as a diffuse band due to the presence of the carbohydrates

A Amino-acid sequences and mass

Sample (digest)	Peptide	Sequence	Experimental mass (Da, 1)	Theoretical mass (Da, 2)	Difference (1) - (2)
21 kDa	G1749	LLGAPQEADASEEGVQR	nd		
(tryptic)	G1615	(L)(L)GAPQEADASE(E)GV	1,766.6	1,769.8	
	G1748	ALDFA(V)SEYNKGNDAYH	2,242.8	2,244.0	
	G1750	(G)XSR(P)(P)(P)RLLGA(P)QEADA	2,962	2,618.3	342
	G1751	LLGAXQEADASXE(D,G)VQRALDFAV(S)(E)YNK(G)XNDA	14,494	12,480.1	2,014
	G1752	(G)X(S,C)(A,R)P(P)XRLLGAXQ(E)AD(A)	16,125	13,329.1	2,796
G1751	G1754	DVEMGR(T)XXXK(S,C)QXX(L)	4,264.4 / 5,060.3	2,483.1	1,781.3 / 2,577.2
(Asp-N)	G1755	(D)QPHL	3,878.4	3,875.9	

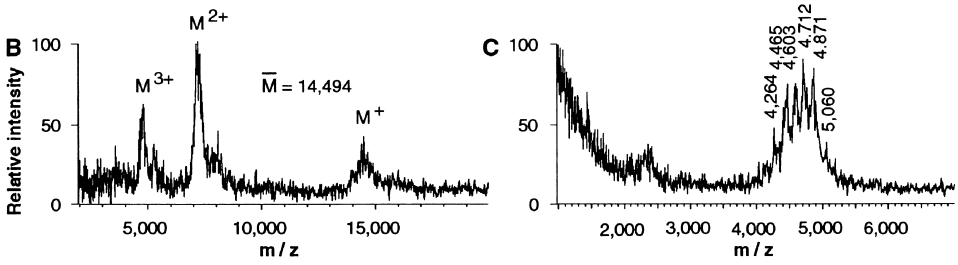

Figure 2. Sequencing and Mass Spectral Analysis of the 21 kDa Protein

The 21 kDa protein and the peptide G1751 were digested with trypsin and endoproteinase Asp-N, respectively. The peptides, separated by HPLC, were submitted to chemical sequence and mass spectral analysis. All the sequences determined (A) matched rat cystatin C. The peptide G1754 carries a N-linked carbohydrate moiety. Residues in parentheses were determined with less than 70% confidence. If two residues are listed in parentheses, no unambiguous assignment could be made. X denotes that no assignment could be made with >50% confidence. Peaks represent the different ionized forms of peptide G1751 (B) and the heterogeneity of the carbohydrate moiety of peptide G1754 (C). m/z is the mass to charge ratio, M^{n+} denotes the molecular mass of singly, doubly, and triply charged species, M is the average molecular mass calculated from these charge states (B).

(Figure 3A, lane 2 versus lane 1, arrows) (Keinanen, 1988). CCg-DG exhibited no mitogenic activity (Figure 3B). The importance of glycosylation of CCg is underscored by the fact that chicken cystatin (chCystatin), which is structurally related to rat cystatin C but lacks the N-glycosylation consensus sequence and thus is not glycosylated (Turk and Bode, 1991), had no activity (Figure 3C). In fact, it competitively inhibited the mitogenic activity of CCg (Figure 3B) and of the CM (Figure 3C). In addition, neither the purified peptide of 22 amino acid length containing the N-carbohydrate moiety (peptide G1754) nor the N-carbohydrate moiety elicited any activity in the in vitro proliferation assay. The recovery of 100% of the CM activity with CCg, together with the inhibition of the CM activity by the nonglycosylated form of cystatin, indicate that the activity in the CM may be accounted for by CCg.

To further investigate the importance of N-glycosylation versus the protease inhibitory domains for the activity of CCg, three recombinant forms of the mouse glycocystatin C were engineered (Figure 3D): (1) a wild-type form (i^+g^+); (2) a mutant form (i^-g^+), in which the protease inhibitory site was deleted by truncating the 11 N-terminal amino acids; and (3) a second mutant form where the N-glycosylation site was mutated by substituting Asn-79 for Asp (i^+g^-). Protease inhibitor-deficient forms have been shown to lose their protease inhibitory activity and exist in vitro and in vivo (Machleidt et al., 1989). On SDS-PAGE, the i^+g^+ form migrated at 16 kDa, the i^-g^+ form at 17 kDa and the i^+g^- form, as reported for mouse cystatin C (Hakansson et al., 1996), at 12 kDa (Figure 3E). The wild-type form i^+g^+, at concentrations between 2 and 200 ng/ml in the presence of 20 ng/ml FGF-2, induced cell proliferation in a dose-dependent manner (Figure 3F). While the protease inhibitor-deficient mutant (i^-g^+) retained the activity, the glycosylation-deficient form (i^+g^-) did not. The present data show that, whereas the N-linked carbohydrate moiety is necessary for the activity of CCg, the protease inhibitory domain is not directly involved. One-way ANOVA analysis of data (Figure 3B) showed that there was a significant main effect due to treatments [$F(2,6) = 54.73$, $p < 0.001$], with a significant increase in cell number with increasing concentration [$F(6,12) = 21.8$, $p < 0.0001$] as well as a significant concentration and treatment interaction [$F(12,36) = 6.23$, $p < 0.0001$]. Specific comparisons showed significant differences ($p < 0.01$) between all groups. For concentrations from 200 pg/ml and higher, there is also a significant main effect on treatments [$F(3,8) = 61.8$, $p < 0.0001$] and no significant differences between CCg-DG and CCg + chCystatin.

Cloning of Neural Stem Cells In Vitro

To investigate the ability of CCg to promote the proliferation and expansion of neural stem cells at clonal densities, single AHPs were plated in N2, N2 + FGF-2, N2 + CCg, and N2 + FGF-2 + CCg (Table 2). While N2 alone did not support cell growth, in N2 + FGF-2 and N2 + CCg, 1% and 5% of single cells, respectively, grew clones to a maximum cell number of 55 before they died. In contrast, in the presence of FGF-2 + CCg, 31% of single cells expanded to a confluent population and were passaged (Figures 4A–4D). We have investigated the presence of tubulin-β III, a neuronal marker, O4, an oligodendrocytic marker, and glial fibrillary acidic protein (GFAP), an astrocytic marker, in the culture

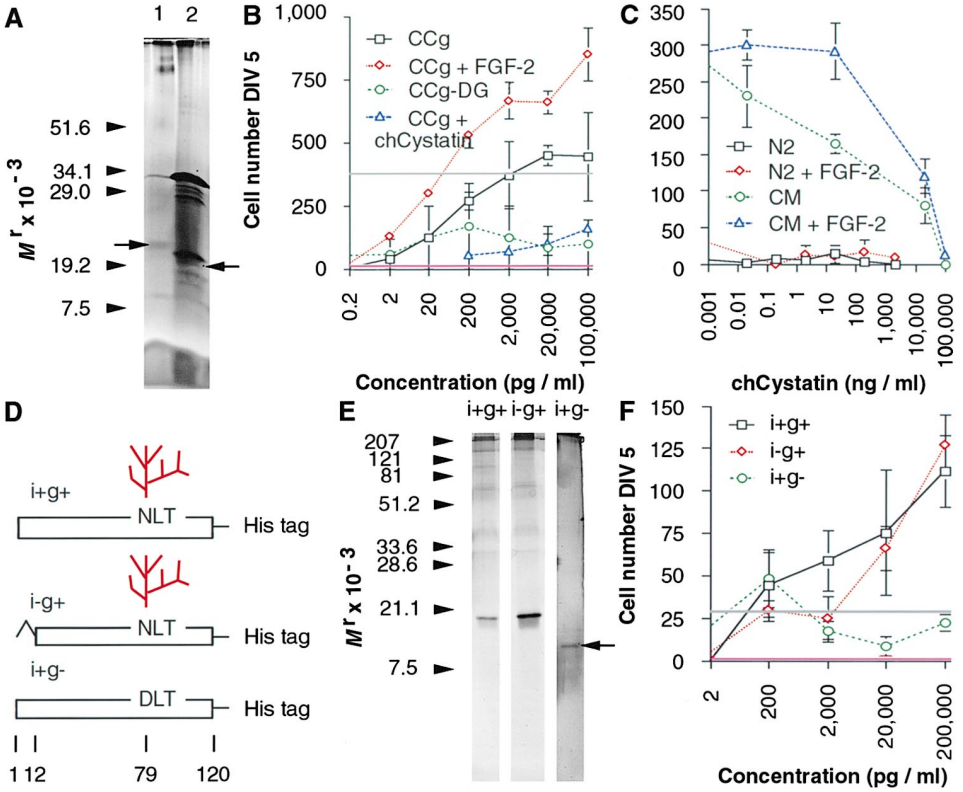

Figure 3. Structural Basis for the Activity of CCg

SDS-PAGE and silver staining of CCg untreated ([A], lane 1) or treated with PNGase F ([A], lane 2). CCg but not the N-deglycosylated form, CCg-DG, induced proliferation of AHPs plated at low density; FGF-2 (20 ng/ml) potentiated the activity of CCg, whereas chCystatin (1 mg/ml, nonglycosylated form of cystatin) inhibited it (B). ChCystatin did not induce the proliferation of AHPs in the absence or presence of FGF-2, but inhibited cell proliferation induced by CM or CM + FGF-2 (C). Three recombinant forms of the mice glycocystatin—wild type (i^+g^+), a form lacking the protease inhibitory site (i^-g^+), and a form with mutated N-glycosylation site (i^+g^-)—were engineered (D) and purified to homogeneity ([E], the form i^+g^- migrated at 12 kDa, arrow). In (D), NLT is the consensus site for N-glycosylation, DLT is the mutated site where Asn has been subsituted for an Asp; a His tag was fused in 3′ to the recombinant sequences. Only the forms i^+g^+ and i^-g^+, but not i^+g^-, induced the proliferation in the presence of FGF-2 (F). The number of cells at DIV 5 were in (B) for N2, 0; N2 + FGF-2, 5.10 ± 0.3 (purple line); CM, 354.64 ± 40.3; CM + FGF-2, 360.00 ± 33.3 (gray line); in (C) for N2, 0.33 ± 0.3; N2 + FGF-2, 0.33 ± 0.3; CM, 280.82 ± 40.5; CM + FGF-2, 306.44 ± 45.5; and in (F) for N2, 0.33 ± 0.3; N2 + FGF-2, 0.33 ± 0.3 (purple line); CM, 31.00 ± 5.0; CM + FGF-2, 30.33 ± 4.4 (gray line). The number of cells plated at DIV 1 were in (B) for N2, 92.88 ± 14.2; N2 + FGF-2, 84.88 ± 4.7; CM, 119.10 ± 9.2; CM + FGF-2, 95.76 ± 6.3; CCg, 83.96 ± 4.0; CCg + FGF-2, 87.98 ± 3.1; CCg-DG, 81.89 ± 7.8; CCg + chCystatin, 85.16 ± 6.9; in (C) for N2, 65.32 ± 15.6; N2 + FGF-2, 72.64 ± 22.3; CM, 70.82 ± 10.5; CM + FGF-2, 76.44 ± 10.5; N2 + chCystatin, 67.52 ± 5.8; N2 + FGF-2 + chCystatin, 72.12 ± 8.2; CM + chCystatin, 69.65 ± 4.2; CM + FGF-2 + chCystatin, 67.48 ± 8.3; and in (F) for N2, 24.80 ± 0.2; N2 + FGF-2, 26.40 ± 2.6; CM, 24.00 ± 1.8; CM + FGF-2, 26.00 ± 4.4; i^+g^+, 22.25 ± 1.6; i^-g^+, 20.39 ± 1.4; i^+g^-, 23.06 ± 2.6. Data in (B) and (F) are the means of triplicate from one of three typical experiments, and data in (C) are means ± SEM of three independent experiments.

generated with FGF-2 + CCg. Less than 0.01% of the cells exhibited differentiated phenotypes and were astrocytes; no oligodendrocytes and no neurons were observed (data not shown), indicating that FGF-2 + CCg treatment does not differentiate AHPs toward any lineage. It has been reported that retinoic acid (RA) differentiates neural stem and progenitor cells toward the neuronal lineage, a low concentration of FGF-2 (2 ng/ml) differentiates the cells toward the oligodendrocytic lineage, and fetal bovine serum (FBS) differentiates the cells toward the astrocytic lineage (Palmer et al., 1997; Takahashi et al., 1999). To demonstrate the multipotentiality of the cloned cells, we analyzed two passaged clones after differentiation in the presence of RA, a low concentration of FGF-2 and FBS. Both clones generated neurons, oligodendrocytes, and astrocytes (Figure 4D). Of the cells, 5% were positive for tubulin-β III, 0.50% for O4, and 8% for GFAP. Time lapse experiments were also performed on single plated AHPs in the presence of FGF-2 + CCg (Figures 4E–4P). Each single cell produced two daughter cells, both undergoing cell division. Therefore, FGF-2 in the presence of CCg induces the cloned cells to undergo symmetrical division, suggesting that the cloned cells are capable of self-renewing. The cloned AHPs were further subcloned and tested for multipotentiality. All three lineages were generated from single cells in the presence of both FGF-2 and CCg (data not shown). Taken together, these results show that (1) the cultured AHP population is heterogeneous—it contains some stem cells, but also other neural progenitor cells at different stages of maturation, as previously observed (Palmer et al., 1997); (2) FGF-2 alone is not sufficient to promote the proliferation of neural stem cells from a single AHP, although it elicits a weak mito-

Table 2. Number of Clones and Average Clone Size

Cell Type	DIV 1	DIV 3	DIV 5	DIV 10	DIV 15	>DIV 15
AHPs						
N2	98 (1)	27 (3.2 ± 1.0)	3 (2.3 ± 0.3)	0 (−)	0 (−)	0 (−)
N2 + FGF-2	80 (1)	23 (2.8 ± 0.3)	11 (6.0 ± 2.7)	3 (23.0 ± 19.5)	1 (55)	0 (−)
N2 + CCg	21 (1)	17 (2.2 ± 0.2)	16 (5.2 ± 1.4)	5 (11.6 ± 5.9)	1 (5)	0 (−)
N2 + FGF-2 + CCg	19 (1)	14 (5.5 ± 1.7)	11 (15.0 ± 5.0)	6 (67 ± 18.6)	6 (247.1 ± 52.5)	6 passaged
E17 HPC						
N2	3084 (1)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)
N2 + FGF-2	4665 (1)	103 (1)	9 (1)	1 (1)	0 (−)	0 (−)
CM	648 (1)	277 (1)	0 (−)	0 (−)	0 (−)	0 (−)
CM + FGF-2	690 (1)	342 (1)	38 (1)	14 (1.07 ± 0.07)	14 (1.46 ± 0.5)	1 expanded
N2 + CCg	685 (1)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)
N2 + FGF-2 + CCg	909 (1)	72 (1)	48 (1)	16 (1.6 ± 0.1)	9 (6.11 ± 1.0)	8 expanded
N2 + FGF-2 + chCystatin	1644 (1)	7 (1)	2 (1)	0 (0)	0 (−)	0 (−)

Quantitation of clone numbers and sizes at DIV 1, DIV 3, DIV 5, DIV 10, and DIV 15. Single cells were cultured in N2, N2 + FGF-2, CM, CM + FGF-2, N2 + CCg, N2 + FGF-2 + CCg, and N2 + FGF-2 + chCystatin. Numbers in parentheses correspond to the average sizes of the clones. FGF-2 and CCg are required to expand AHP and E15 primary hippocampal (E17 HPC) clones from a single cell. Cells were plated at a density of 1 cell per well for AHPs and 20–25 cells per well for E17 HPC cells.

genic activity on neural progenitor cells, as they did not expand farther than a few cell divisions; and (3) CCg acts in cooperation with FGF-2 to promote the proliferation of neural stem cells from a single AHP, as the cloned cells retained their self-renewal and multipotential properties.

CCg in association with FGF-2 is also required to induce proliferation of embryonic (E17) rat primary hippocampal neural progenitor cells plated at low density (Figures 4Q–4T and Table 2). In contrast to AHPs, no activity of CCg alone was observed on embryonic (E17) rat primary hippocampal neural progenitor cells plated at low density (Table 2). As opposed to embryonic (E17) rat primary hippocampal neural progenitor cells, AHPs have been cultured through several passages with FGF-2, and the residual FGF-2 bound to the surface of the cultured cells (Rogelj et al., 1988) could cooperate with CCg to promote AHP proliferation temporarily. Therefore, the cooperation of the two factors, FGF-2 and CCg, is needed for FGF-2-induced mitogenic activity on hippocampal rat neural stem cells in vitro and for their expansion from a single cell.

In Vitro and In Vivo Distribution of Cystatin C

AHPs and the DG of the adult rat hippocampus, a neurogenic region (Altman and Das, 1965; Caviness, 1973; Eriksson et al., 1998), were evaluated for the distribution of cystatin C immunopositive cells (Figure 5). No Triton X-100 was used during the staining of the AHPs to label

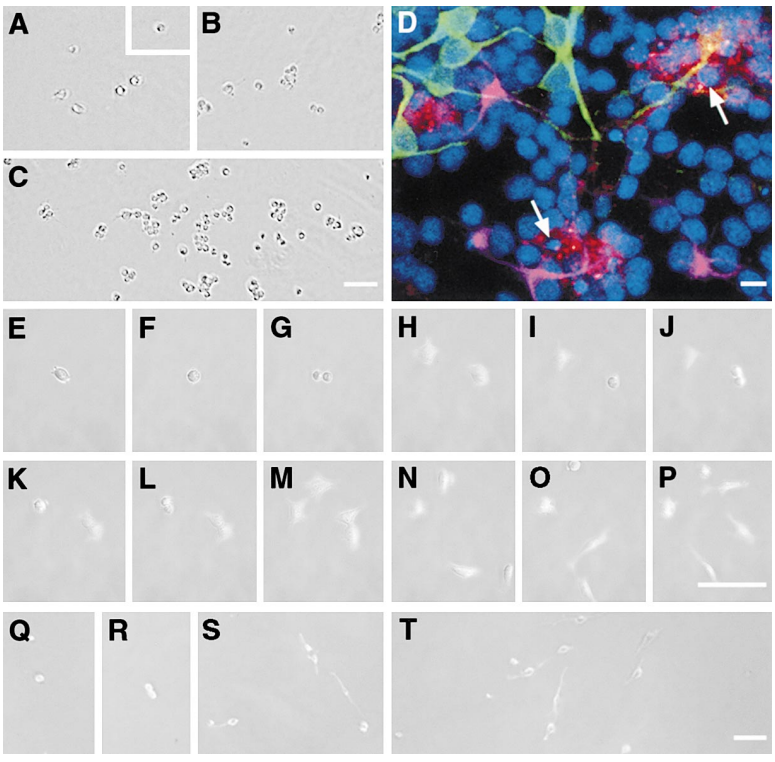

Figure 4. Cloning and Differentiation of Neural Stem Cells In Vitro

AHPs plated as single cells (A box) were cultured in the presence of N2 + FGF-2 + CCg (cells in [A]–[C] were from the same well; [A], DIV 3; [B], DIV 7; [C], DIV 15), were differentiated with RA, FGF-2, and FBS after passaging, and were examined by immunocytofluorescence (D). The three main phenotypes of the brain were detected: tubulin-β III–positive neurons (green), GFAP-positive astrocytes (purple), and O4-positive oligodendrocytes (red, arrow). Nuclei (blue) were stained with DAPI (D). Time lapse study on AHPs plated as single cells ([E], 18 hr; [F], 22 hr 30 min; [G], 23 hr; [H], 42 hr; [I], 43 hr; [J], 44 hr; [K], 50 hr 30 min; [L], 51 hr; [M], 58 hr; [N], 72 hr; [O], 81 hr 30 min; [P], 84 hr 30 min after plating). AHPs are rounding-up before dividing and the two daughter cells are dividing. Embryonic (E17) rat primary hippocampal neural progenitor cells plated at low density were cultured in the presence of N2 + FGF-2 + CCg (cells in [Q]–[T] are from the same well; [Q], DIV 6; [R], DIV 7; [S], DIV 12; [T], DIV 15). CCg in cooperation with FGF-2 could also induce proliferation of embryonic rat primary hippocampal neural progenitor cells plated at low density. Scale bars: 100 μm (A–C); 10 μm (D–P); 20 μm (Q–T).

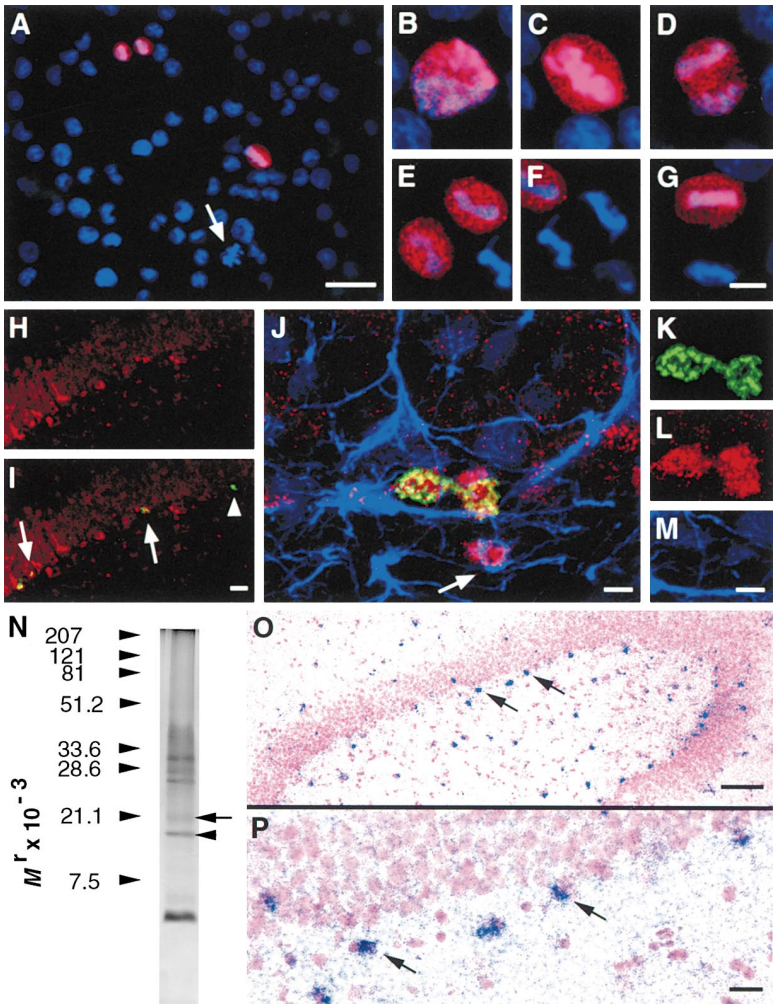

Figure 5. Immunofluorescence Labeling of Cystatin C in AHPs and in the Dentate Gyrus of the Adult Rat Hippocampus

AHPs were stained for cystatin C (red) and DAPI (blue) (A–G). Most of the AHPs immunopositive for cystatin C were in mitosis, as observed by the DAPI staining of the chromosomes ([A and B], AHP in prophase; [C], AHP in metaphase; [D], AHP in anaphase; [E], AHP in telophase). Not all the AHPs in mitosis were cystatin C–positive ([A], arrow; [F–G], AHPs in telophase). Hippocampal sections of rats injected with BrdU 2 hr before sacrifice were stained for cystatin C (red). Cystatin C–positive cells were distributed throughout the hippocampus, particularly along the SGZ of the DG (H). Colabeling of the hippocampal sections with cystatin C and BrdU (green) shows that neural progenitor cells in the SGZ undergoing cell division, as revealed by BrdU labeling, were cystatin C–positive ([I], arrows). Not all the neural progenitor cells undergoing cell division were cystatin C–positive ([I], arrowhead). By colabeling the hippocampal sections with BrdU (green, [K]), cystatin C (red, [L]), and GFAP (blue, [M]), we observed BrdU and cystatin C colabeled cells that were GFAP negative in the SGZ ([J], [J] is a merge picture of [K], [L], and [M]). Cystatin C and GFAP colabeled cells that were BrdU-negative cells were also observed ([J], arrow). The two forms of cystatin C were revealed by SDS-PAGE and silver staining from a cytosolic extract of adult rat hippocampus (N): the band at 21 kDa, corresponding to CCg (arrow), and the band at 19 kDa, corresponding to the nonglycosylated form of cystatin C (arrowhead). In situ hybridization study for cystatin C mRNA shows strong hybridization for cystatin C mRNA (arrows) in the SGZ of the adult rat DG (O and P). The brain sections were stained with nuclear fast red after performing the in situ hybridization. Scale bars: 20 μm (A, H–I, and P); 5 μm (B–G and J–M); 100 μm (O).

extracellular cystatin C. Of the AHPs in culture, 1.40% were immunopositive for cystatin C (Figure 5A). DAPI was used to identify AHPs in mitosis, based on the morphology of their DNA (Figures 5B–5G). Approximately 2.55% of the AHPs were in mitosis (Figure 5A). Strikingly, most of the AHPs immunopositive for cystatin C were in mitosis, and the AHPs immunopositive for cystatin C represented 54% of all the cells identified in mitosis (Figure 5A). We conclude that this antibody for cystatin C, in the absence of Triton X-100, labels mainly a subpopulation of the AHPs that are undergoing cell division. Immunohistology was performed on adult rat brain fixed sections (Figures 5H–5M). Rats (Fischer-344, males weighting 200–220 g) were injected with bromodeoxyuridine (BrdU, 150 mg/kg) 2 hr before sacrifice. BrdU is a thymidine analog that incorporates into the DNA of dividing cells and is used for birthdating cells and monitoring cell proliferation (Kuhn et al., 1996). Since BrdU and GFAP are intracellular antigens, the staining was performed in the presence of Triton X-100. Cystatin C–positive cells were distributed sparsely throughout the brain, particularly in the SGZ of the hippocampal DG (data not shown and Figure 5H). We observed cystatin C and GFAP colabeled cells throughout the brain (data not shown and Figure 5J), as previously observed (Yasuhara et al., 1993). In the SGZ, 50% of the cells undergoing cell division (BrdU-positive) were positive for cystatin C (Figure 5I), and none of the cells positive for BrdU and cystatin C were GFAP-positive (Figures 5J–5M). We also observed cystatin C and BrdU colabeled cells that were GFAP-negative cells throughout the SVZ (data not shown), another neurogenic region (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Doetsch et al., 1999). We conclude that in vivo as well as in vitro, this antibody for cystatin C labels a subpopulation of neural stem/progenitor cells undergoing cell division. By treatment of the CM with PNGase F and Western blot analysis, we observed that the antibody against cystatin C specifically recognizes the glycosylated cystatin, CCg, and the nonglycosylated cystatin (data not shown). Therefore, although the antibody against cystatin C did not specifically detect CCg, these results confirm the involvement of cystatin C in adult neurogenesis.

To show that CCg was present in the adult rat hippocampus, we performed a papain chromatography from

a cytosolic extract of adult rat hippocampus. The eluate was analyzed by SDS-PAGE. The two forms of cystatin C were revealed: the band at 21 kDa, corresponding to CCg, and, the band at 19 kDa, corresponding to the nonglycosylated form of cystatin C (Figure 5N). To show the presence of cells expressing cystatin C in the SGZ, we performed an in situ hybridization study of the DG of the adult rat hippocampus for cystatin C mRNA. This study revealed strong hybridization for cystatin C mRNA in the SGZ (Figures 5O and 5P) and reflected the pattern observed by immunostaining with the antibody against cystatin C (Figure 5H).

Cystatin C has been reported to be involved in cell migration and metastasis (Sloane et al., 1990). Recently, Huh et al. (1999) observed in a knock-out model of cystatin C that the reduction of metastasis was associated with a reduction in cell growth. This finding suggests that cystatin C is not only involved in metastasis but also in cell growth, as we observed. We have investigated the rate of proliferation in the DG of adult cystatin C–deficient mice, as assessed by BrdU labeling. Mice (C57BL/6J, 30- to 45-day-old female weighting 20–21 g) were injected with BrdU (50 mg/kg) three times at 12 hr intervals and were sacrificed 2 hr after the last injection. A total of 1054.00 ± 131.18 BrdU-positive cells were counted in cystatin C–deficient mice versus 2622.00 ± 295.94 BrdU-positive cells in wild-type littermate controls ($n = 6$ per group), corresponding to a decrease of approximately 60% in cell proliferation in the DG of cystatin C–deficient mice versus wild-type littermate controls. No difference in the volume of the DG was observed in cystatin C–deficient mice versus wild-type littermate controls, 0.27 ± 0.01 mm^3 versus 0.26 ± 0.01 mm^3, respectively. Although these results confirm the involvement of cystatin C in adult neurogenesis, they do not resolve the specific role of the carbohydrate moiety of CCg in cell proliferation in vivo. A knock-in mouse in which the asparagine of the N-glycosylation consensus site is substituted by an aspartic acid would be critical to investigate the importance of the N-glycosylation domain for the activity of CCg.

Stimulation of Neurogenesis In Vivo

To investigate the ability of CCg to stimulate neurogenesis, AHPs were genetically modified (Gage et al., 1995b) to coexpress a secreted form of FGF-2 (FGF-2-S), constructed by fusing the pre-pro sequence of the human nerve growth factor (NGF) 5′ to the rat FGF-2 sequence (Ray et al., 1995) and either the wild-type form of CCg (i^+g^+) or the glycosylation-deficient form (i^+g^-). AHPs prelabeled with fluorogold (FG) were grafted in adult rat hippocampus, the rats were injected with BrdU for 11 days following grafting, and the proliferation of endogenous progenitor cells was determined immunohistochemically by counting the cells positive for BrdU that were negative for FG (Figure 6A). BrdU-negative cells that were FG positive (Figure 6B) and BrdU-positive cells that were FG positive (Figure 6C) were not included as they were derived from the grafts. Cell proliferation was monitored at proximal and distal areas to the grafts, within the granular layer of the DG (Figures 6F and 6G), a neurogenic region (Altman and Das, 1965; Caviness, 1973; Eriksson et al., 1998). A 4-fold increase in BrdU-positive cells that were FG negative was observed 11 days after grafting in the DG at the proximal areas of the grafted AHPs coexpressing FGF-2-S and i^+g^+ compared to the proximal areas, where either AHPs coexpressing FGF-2-S and i^+g^- or AHPs were grafted (Figures 6F–6H). In contrast, at distal areas of the grafts, the numbers of BrdU-positive cells that were FG negative remained at basal levels in all groups (Figures 6F, 6G, and 6I).

The generation of new neurons was assessed by counting tubulin-β III and BrdU colabeled cells that were FG negative (Figure 6E) in the proximal and distal areas of the grafts (Figures 6J and 6K). A 4-fold increase in tubulin-β III and BrdU colabeled cells that were FG negative was observed in the proximal areas of the grafted AHPs coexpressing FGF-2-S and i^+g^+, compared to all other groups. Our data indicate that FGF-2 and CCg cooperate to stimulate neurogenesis in the DG of the adult rat hippocampus and that the N-glycosylation of CCg is a requirement to stimulate neurogenesis in the presence of FGF-2.

Discussion

Neural stem and progenitor cells, isolated from diverse regions of the adult brain, can be expanded in vitro in the presence of FGF-2 (Gage et al., 1995b; Palmer et al., 1995, 1999; Shihabuddin et al. 1997; Roy et al., 2000). Here we show that FGF-2 requires a high cell density to support neural stem and progenitor cell proliferation. At low cell density, FGF-2 alone does not support neural stem cell proliferation or survival, and it elicited a weak mitogenic activity on neural progenitor cells, as they did not expand farther than a few cell divisions. Supplementation with CM from neural stem and progenitor cell culture can overcome these limitations and allows neural stem and progenitor cells to proliferate. Together these findings demonstrate the existence of an autocrine/paracrine cofactor to support FGF-2's mitogenic activity on neural stem cells in vitro. Studies from single cell cultures show that EGF and FGF-2 are mitogens for mouse-derived neurospheres (Reynolds and Weiss, 1996; Gritti et al., 1996), and it has been previously reported that the cloning of neural stem cells requires other factors present in CMs, none of which has been identified (Temple, 1989; Kilpatrick and Bartlett, 1993; Davis and Temple, 1994; Qian et al., 1997; Palmer et al., 1997; Johansson et al., 1999). We have purified a CM-derived factor, CCg, that is required with FGF-2 to support the proliferation in vitro of neural stem cells from the adult rat hippocampus and for their expansion from a single cell. We conclude that neural stem or progenitor cells cultured from the adult rat hippocampus synthesize and secrete CCg, which acts as an autocrine/paracrine factor and, in cooperation with FGF-2, to support the proliferation of neural stem cells in vitro.

Furthermore, we report that the combined delivery of a secreted form of FGF-2 and CCg by genetically manipulated AHPs stimulates neurogenesis in the adult rat hippocampus. Although the intracerebral administration of FGF-2 has been shown to stimulate neurogenesis in the adult rat SVZ, FGF-2 alone in the adult rat hippocampus has a limited effect on the proliferation of neural

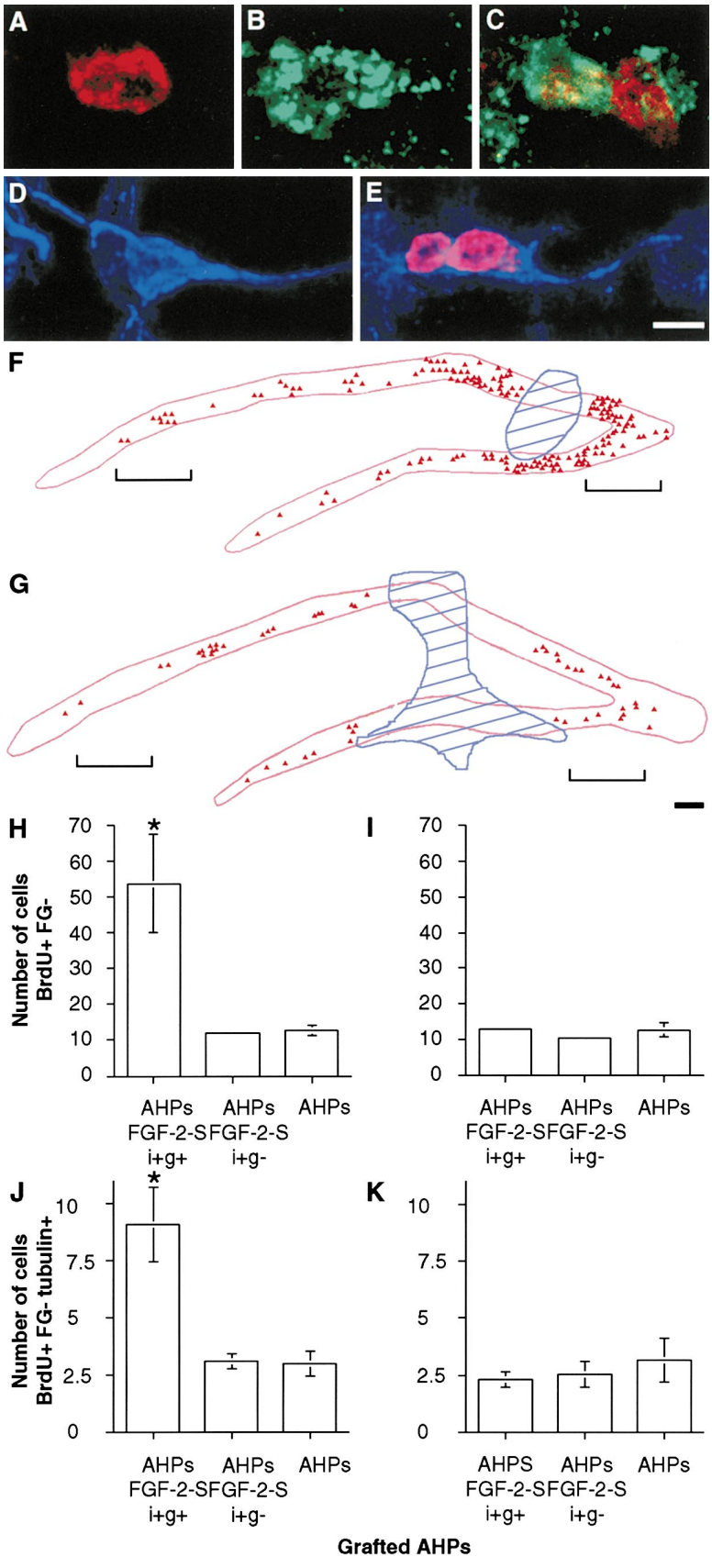

Figure 6. Stimulation of Neurogenesis in the Adult Rat DG by Grafted AHPs Coexpressing FGF-2-S and Either the Wild-Type Form of CCg or the Form of CCg with Mutated N-Glycosylation Site

AHPs prelabeled with FG were grafted in adult rat hippocampus. Newborn cells were detected by BrdU labeling (red) and grafted AHPs were detected by FG labeling (green). The proliferation of newly generated endogenous progenitor cells was determined immunohistochemically by counting the BrdU-positive cells (red) that were FG negative (A). AHPs prelabeled with FG prior to grafting were FG positive ([B], green). BrdU and FG colabeled newly generated AHPs (C) were not counted as they were derived from the grafts. Neurons were stained for tubulin-β III (blue, [D]). The generation of new neurons was determined by counting tubulin-β III and BrdU colabeled cells that were FG negative (E). (F) and (G) are camera lucida drawings of the distribution of BrdU-positive cells that were FG negative (red triangles), outside the graft area (blue line), in the DG of the hippocampus (purple line) grafted with AHPs coexpressing FGF-2-S and either the wild-type form of CCg (i^+g^+, [F]) or the form of CCg with mutated N-glycosylation site (i^+g^-, [G]). The quantification of newborn cells proximal to the grafts (H and J) was performed within the granular layer of the DG, in a 280 μm area from the edge of the graft (black segment), and the quantification of newborn cells distal to the grafts (I and K) was performed in a 280 μm area beginning at least 800 μm from the tip of the graft. An increase in tubulin-β III and BrdU colabeled cells that were FG negative was only observed in the proximal area of the DG of the rats that received grafted AHPs coexpressing FGF-2-S and i^+g^+. Control AHPs are presented. Scale bar: 5 μm (A–E); 100 μm (F–G). Data are means ± SEM for four animals in each treatment group (*$p < 0.005$ by two-way ANOVA).

stem/progenitor cells (Kuhn et al., 1997; Wagner et al., 1999). The differential effect of FGF-2 on stem/progenitor cells from the SVZ and the DG may be attributed to different populations of stem/progenitor cells; alternatively, both cell populations are responsive to FGF-2 but may also require additional factor(s) (Kuhn et al., 1997; Wagner et al., 1999). Cystatin C has been identified in cerebrospinal fluid (Lofberg and Grubb, 1979), and we also observed cystatin C and BrdU colabeled cells that were GFAP-negative cells throughout the SVZ (data not shown). Therefore, the administered FGF-2 could interact locally with CCg to support neurogenesis in the SVZ. Since it has been reported that, depending on the mode of administration, FGF-2 could be rapidly cleared from the brain (Gonzalez et al., 1994), insufficient levels of FGF-2 could prevent the stimulation of neurogenesis in the presence of CCg in the adult rat hippocampus. We demonstrate that genetically engineered AHPs, which provide a means to locally deliver a transgene for a sustained period of time (Gage et al., 1995b) by simultaneously producing FGF-2 and CCg, stimulate neurogenesis in the DG 4-fold. We conclude that in vivo neurogenesis is stimulated by the cooperation between FGF-2 and CCg. The involvement of CCg in adult neurogenesis is further confirmed by the observation of a decrease of 60% in cell proliferation in the DG of adult cystatin C–deficient mice, versus wild-type littermate controls. The decrease in cell proliferation is not associated with a decrease in the volume of the DG in cystatin C–deficient mice versus wild-type littermate controls. Several hypotheses can explain the lack of detectable change in the volume of the DG in cystatin C–deficient mice: (1) there are approximately less than 1% of the cells that are dividing over weeks period in the adult DG, (2) the rate of survival of the residual newborn cells can be extended by some other compensatory mechanisms, and (3) the cell density of the DG can also be altered. Additional experiments will be needed to tease out if there are functional consequences in the behavior or the physiology of these mice, as well as developmental consequences, that can be attributed to this decrease in cell proliferation in the DG.

Therefore, the proliferation of neural stem cells in vitro and in vivo neurogenesis are stimulated by the cooperation between FGF-2 and CCg. The postmitotic development of most neuronal populations in vertebrate animals is characterized by a period of cell death during which 40%–60% of the neurons are eliminated (Oppenheim, 1991). The degree of cell death has been shown to be controlled by the target tissues that the neurons innervate, via the restricted availability of neurotrophic factors produced by target cells. We propose that the generation of new neurons in the nervous system could be controlled by the cooperation between trophic factors and autocrine/paracrine factor(s).

Several hypotheses can be envisioned to explain how CCg and FGF-2 cooperate to stimulate neural stem cell proliferation. CCg could cooperate with FGF-2 in an autocrine/paracrine manner to promote the mitogenic activity of FGF-2 on neural stem cells. Alternatively, CCg could promote the survival, or prevent the death, of the cells that can respond to the mitogenic activity of FGF-2. Carbohydrate moieties have a broad range of activities and mechanisms of action (Varki, 1993). The observation that the N-glycosylation of CCg is necessary to support neural stem cell proliferation suggests that the N-linked carbohydrate moiety, but not the protease inhibitory domain, is directly involved in the mechanism of action of CCg. CCg could bind to FGF-2, and the resulting complex would result in a higher affinity for FGF receptor. Alternatively, CCg and FGF-2 could act synergistically in a receptor-mediated fashion to promote the proliferation of neural stem cells. This latter contention is supported by the finding that N-linked, but not O-linked, carbohydrate moieties interact with specific receptors to mediate their functions, whereas deglycosylated proteins interact with receptors but are unable to activate intracellular events, acting thereby as competitive inhibitors of protein action (Sairam, 1989), as we observed for CCg. The nonglycosylated form of cystatin C was found in larger amounts than the glycosylated form in AHP CM and in the adult rat hippocampus (Figure 5N), indicating that the activity of CCg is controlled by the synthesis and secretion of varying levels of glycosylated versus nonglycosylated forms of cystatin C. CCg is an autocrine/paracrine factor and promotes the proliferation of neural stem cells in vitro and in vivo. Therefore, the finding that the labeling of cystatin C is present on the extracellular plasma membrane of the AHPs undergoing cell division could reflect that the neural stem cells undergoing cell division bind CCg at their plasma membrane. Our data show that the protease inhibitory domain of CCg is not directly involved in neural stem cell proliferation; however, it could secondarily support cell proliferation and migration by regulating the environmental proteolytic activity (Galko and Tessier-Lavigne, 2000).

In summary, our results demonstrate that FGF-2 requires a cofactor, CCg, for its mitogenic activity on neural stem cells in vitro and to stimulate neurogenesis in vivo. CCg is a 21 kDa glycosylated form of cystatin C, whose posttranslational N-glycosylation is necessary to induce neural stem cell proliferation. Trophic factors such as FGF-2 (Baird, 1994) have a broad range of biological activities. Our findings show that their temporal and spatial activity and, therefore, specificity may be achieved by cooperation with autocrine/paracrine cofactors, of which CCg is a prototype.

Experimental Procedures

Tissue Culture, Immunofluorescence Studies, and In Situ Hybridization

AHP cultures and immunofluorescence studies were performed as described (Gage et al., 1995b). The CM was prepared by incubating 60% confluent cells (passage 17–30) for 48 hr in DMEM/F12, supplemented with 2.5 mM L-glutamine and N2 supplement (N2 medium) containing FGF-2 (20 ng/ml; recombinant human FGF-2; gift from A. Baird). The medium was replaced and conditioned for 2 more days. The CM was kept at −80°C until use. All the in vitro assays were performed in 96-well plates; cell proliferation was monitored by counting cells at DIV 1 and DIV 5 under a phase contrast microscope (× 10). In such a proliferation assay, a net increase, or decrease, in cell number is the result of both cell proliferation and cell survival. DAPI (4′,6-diamidino-2-phenylindole, Sigma) staining was performed on AHPs plated at low density, and cells in mitosis were observed, confirming that cell division occurred (data not shown). Cells were examined 4 hr after plating and wells containing clusters of >1 cell were omitted from the experiments. For differentiation experiments, cells were cultured in N2 medium containing all-*trans*

RA (1 μM, Sigma), FGF-2 (2 ng/ml), and FBS (0.50%, Hyclone) for 10 days. Time-lapse imaging was performed on a chamber mounted on an inverted microscope (Nikon TE300) with a SPOT camera. Immunofluorescence was performed with the following primary antibodies: tubulin-β III (1/1,000, Sigma), GFAP (1/500, Adv. Immuno.), O4 (gift from O. Boegler), BrdU (1/100, Accurate), cystatin C (1/1,000, Biogenesis), and FG (1/2,000, Chemicon). All secondary antibodies were from Jackson. The detection of BrdU required pretreatment of the sections in HCl 2N for 30 min at 40°C. Streptavidin-biotin amplification was used when detecting FG. Triton X-100 (0.3%) was used during the cytological and histological procedures to detect intracellular antigens, such as tubulin-β III, GFAP, BrdU, cystatin C, and FG. It was not used to reveal antigens present at the outer side of the plasma membrane, such as O4 and cystatin C. The use of Triton X-100 did not alter the quality of the immunostaining. The in situ hybridization procedure was performed as described (Lein et al., 1999). The probes used for in situ hybridization were prepared by digesting 5 μg of plasmid SPSK encoding mouse cystatin C (see below). The antisens probe was prepared by digesting the plasmid with SfiI and was amplified with T7 polymerase, whereas the control sens probe was prepared by digesting the plasmid with ClaI and was amplified with T3 polymerase.

Protein Purification

CM (10 liters) were applied to lentil-lectin Sepharose 4B (25 ml, Sigma) equilibrated with 25 mM HEPES (pH 7.0), 1 mM $MnCl_2$, and 1 mM $CaCl_2$, and the column was washed with 25 mM HEPES (pH 6.5) and 50 mM NaCl. No mitogenic activity was detected in the flow-through. The column was eluted with 250 mM α-methyl-mannoside. The eluent—with the mitogenic activity—was collected, concentrated (Biomax-5, Millipore), and applied to a strongly acidic cation exchanger column (1 ml, Hi-TrapSP, Pharmacia). The nonadsorbing effluent containing the mitogenic activity and was collected, concentrated, and resolved on a 15% SDS-PAGE under nonreducing conditions, and the proteins were transferred to an Immobilon PVDF membrane (Millipore) (Hames, 1981). For activity determination, proteins were recovered as follows: after lentil-lectin and cation exchange chromatographies, 2 ml samples were collected, dialyzed (3000 MWCO) against tissue culture medium, and filtered (0.22 μm). After blotting, the membrane was cut into 20 equal fractions, and proteins were eluted (Montalero, 1987). Since the cofactor of FGF-2 was identified as cystatin C and cystatin C binds to papain with high affinity (Turk and Bode, 1991), the 21 kDa protein was subsequently purified by a modified protocol consisting of lentil-lectin affinity chromatography followed by papain chromatography (Barret, 1981). CCg protein was purified to near homogeneity as shown by SDS-PAGE (Figure 3A, lane 1, compare with Figure 1D) and confirmed by HPLC analysis (data not shown).

Protein Sequencing and Mass Spectroscopy

The protein band at 21 kDa was excised, and 250 pmol was subjected to in situ digestion with trypsin. The digested peptides were separated by reverse phase HPLC. The resolved peaks were submitted to sequencing and mass spectral analysis. Peptide G1751 digested with endoproteinase Asp-N was separated by HPLC and analyzed by sequencing and mass spectral analysis. Sequencing was performed on Perkin-Elmer/Applied Biosystems protein sequencer (models 470A and 494) (Fischer et al., 1991). All sequences obtained were subjected to a BLAST computer homology search (Altschul et al., 1990). Major fractions were analyzed by matrix assisted laser desorption mass spectroscopy on a Bruker Reflex time-of-flight instrument.

Plasmid Construction

Three recombinant forms of CCg were engineered by PCR amplification from a plasmid encoding mouse cystatin C (ATCC 63,113): a wild-type (i^+g^+), a mutant lacking the protease inhibitory site (i^-g^+), and a mutant with a mutated N-glycosylation site (i^+g^-). To create the mutant forms, sequential PCR amplifications using primers with compatible extensions to introduce base changes were used. Sets of primers were paired appropriately to result in each construct—for i^+g^+: primer A, 5′ GAGAGAGAATTCATGGCCAGCCCGCTGCGC TCC and B, 5′ AGAGAGATCGATGGCATTTTTGCAGCTGAAATTT; for i^-g^+: primers A, B, and C, 5′ GTCCTGGGCGTGGCCTGGGCG GCCCCGGAGGAGGCAGATGCC and D, 5′ CGCCCAGGCCACGCC CAGGAC; for i^+g^-: primers A, B, and E, 5′ CGAACTACATGTAC CAAGTCCCAGACAGATTTGACTGACTGT and F, 5′ GGACTTGGTA CATGTAGTTCG. Each variant was further mutated to add an in-frame histidine (His) tag at the C terminus. As a result of the cloning procedure, two differences in the nucleotide sequence of mouse cystatin C were observed: the G in position 71 was found to be a C, and the T in position 276 was found to be a G, when compared to the published sequence (Solem et al., 1990). These nucleotide changes resulted in amino acid substitution of Gly (−5) to Ala and Phe (64) to Leu. These changes are homologous with sequences from other species (Turk and Bode, 1991). At least seven clones of each recombinant form were sequenced. The three recombinant forms were subcloned into the retroviral vector NIT for use in transient retroviral production (Pear et al., 1993). NIT (GenBank accession number AF311318) is a Moloney murine leukemia virus-based retroviral vector with an expression cassette encoding a neomycin resistance gene, an internal ribosomal entry site, and the tetracycline transactivator. The transgene is expressed from a minimal CMV promoter containing 6-tandem tetracycline operators. AHPs were resuspended in N2 medium (1 ml) containing retrovirus and polybrene (2 μg/ml, Sigma) for 30 min at 37°C. The cells were cultured and selected with G418 (400–1000 μg/ml). Recombinant proteins i^+g^+ and i^-g^+ were purified by submitting the CMs to Ni-NTA (Qiagen) and lentil-lectin affinity chromatographies. Recombinant i^+g^- was purified by submitting CM to Ni-NTA and papain affinity chromatographies. The eluents were dialyzed against tissue culture medium or 25 mM ammonium bicarbonate before lyophylisation. Starting from the same amount of CM, similar amounts of the different forms of recombinant cystatins were measured (data not shown). FGF-2-S (Ray et al., 1995) was subcloned into the retroviral vector LPCX for use in transient retroviral production. LPCX is derived from retroviral vector LNCX (Genbank accession number M28247) through substitution of the neomycin with a puromycin resistance gene. The transgene is expressed from a CMV promoter. AHPs expressing cystatin variants were infected with LPCX-FGF-2-S, cultured, and coselected with G418 and puromycin (0.8–1 μg/ml). Transgene expression was characterized in both cystatin variants and FGF-2-S coinfected AHPs by RNA blot analysis (data not shown).

Surgical Procedures

AHPs (75,000 cells in 1.5 μl) labeled for 4 hr prior to grafting with FG (0.01%, Fluorochrome) (Erickson and Goins, 1995) were stereotaxically injected into the hippocampus (anteroposterior axis, −3.5; mediolateral axis, ±3.0; dorsoventral axis, −3.9 from skull, with nose bar at 5 mm up) of anesthetized male Fischer-344 rats (200–220 g). Rats were administered a daily, intraperitoneal injection of BrdU (50 mg/kg, Sigma) for 11 days following grafting. Then animals were sacrificed and perfused (4% paraformaldehyde), and 40 μm thick brain sections were cut on a freezing microtome. To assess the relevance of the use of FG to segregate the grafted AHPs versus the endogenous cells, the following control experiments were performed. AHPs prelabeled with FG were cultured up to 11 days in the presence of FGF-2 and were also cocultured up to 11 days in the presence of FGF-2 with green fluorescent protein (GFP) transfected AHPs. FG was still detectable in over 98% of the AHPs, and none of the GFP-positive cells were labeled with FG, by immunocytochemistry (data not shown), suggesting that even after several cycles of division prelabeled AHPs remained FG immunodetectable and that there was no transfer of FG to neighboring cells in vitro. Thus, in the course of the in vivo experiment, AHPs prelabeled with FG are likely to remain FG positive. AHPs prelabeled with FG were submitted to a few cycles of freezing and thawing; the resulting dead AHPs were grafted in the hippocampus where we detected no FG-positive cells and no increase in cell proliferation in the DG. Thus, there was no transfer of FG to neighboring cells and the surgery did not have a nonspecific effect on cell proliferation in the DG (data not shown). The quantification of BrdU and tubulin-β III cells proximal to the grafts was performed within the granular layer of the DG, 280 μm area from the edge of the graft in four sections using confocal scanning laser microscopy (Zeiss Axiovert and Bio-

rad MRC1024). Similarly characterized cells distal to the grafts were counted in a 280 μm area beginning at least 800 μm from the tip of the graft (Figures 6F and 6G). The edge of the graft was delimited under UV light, since FG is autofluorescent under UV light. The trace of the edge of the graft corresponds to the limit where the UV light began to lack continuity (Figures 6F and 6G, blue lines).

Analytical Procedure and Data Analysis

Protein concentrations were determined with Coomassie protein assay reagent (Pierce). Group changes were assessed using one- and two-way ANOVA. When statistical differences were obtained at the $p < 0.05$ level between groups, post hoc comparisons were made using the Fisher least squares difference (LSD) test. Student's t test was used to make additional comparisons.

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GenBank Accession Numbers

The sequences of the wild type (i^+g^+), of the mutant lacking the protease inhibitory site (i^-g^+), and of the mutant with a mutated N-glycosylation site (i^+g^-) have been submitted to the GenBank. Their accession numbers are AF311741, AF311742, and AF311743, respectively.