Identification of a new membrane-bound heparan sulphate proteoglycan

Jorge FILMUS,*‡ Wen SHI,* Zeu Ming WONG† and Michael J. WONG*

*Division of Cancer Research, Sunnybrook Health Science Centre, †Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada

The morphological changes that occur during intestinal development have been extensively described, but the molecular basis of these changes is largely unknown. As a result of our efforts to identify molecules that play a role in intestinal morphogenesis during development, we have previously isolated a cDNA that is developmentally regulated in the intestine. This cDNA, named OCI-5, was recently shown to have 20–25% identity at the protein-sequence level with glypican and cerebroglycan, two heparan sulphate proteoglycans (HSPG) that are attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor. Here we provide experimental evidence indicating that OCI-5 is also a GPI-linked HSPG. We demonstrate this by showing that OCI-5 can be labelled with radioactive sulphate and can be digested by heparitinase, but not by chondroitinase. We also show that treatment with phosphatidylinositol-specific phospholipase C releases OCI-5 from the cell surface of COS cells transfected with an OCI-5 expression vector. The identification of OCI-5 as a GPI-linked HSPG confirms that this proteoglycan belongs to the same family of HSPGs that include glypican and cerebroglycan.

INTRODUCTION

The morphological changes that occur during intestinal development have been extensively described [1,2], but the molecular basis of these changes is largely unknown. In an effort to identify molecules involved in intestinal morphogenesis during development, we have isolated, through a subtractive hybridization technique, a cDNA corresponding to a transcript that is developmentally regulated in the intestine [3]. This transcript, which was named OCI-5, encodes a protein of 597 amino acids. At the time the cloning of OCI-5 was reported, no similarity to any of the proteins in the sequence database was found. More recently, however, the cloning of two cDNAs that are significantly similar to OCI-5 was reported [4,5]. These cDNAs, named glypican and cerebroglycan, encode the protein cores of two heparan sulphate proteoglycans (HSPG) that are attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor [4,5]. Besides the sequence similarity, the similarities between OCI-5, glypican and cerebroglycan include: the spacing between cysteines, the location of potential glycosaminoglycan (GAG) attachment sites, the presence of a leader sequence and a short C-terminal hydrophobic sequence, and the absence of a transmembrane domain [6]. Based on these similarities, it has recently been proposed that glypican, OCI-5 and cerebroglycan define a new family of membrane-bound HSPGs [6].

The pattern of expression of the three members of this family is very different. Glypican was found to be expressed in a wide range of adult and embryonic tissues [7,8]. Cerebroglycan, on the other hand, was only detected in the developing nervous system [5]. OCI-5 was detected in many fetal tissues, and in the adult lung (J. Filmus, Z. M. Wong and R. N. Buick, unpublished work).

Membrane-bound HSPGs have been shown to bind to a large variety of molecules, including growth factors, extracellular-matrix components and protease inhibitors [9–14]. Based on these interactions and on more direct experimental evidence, membrane-bound HSPGs have been implicated in important aspects of cell adhesion and proliferation [14–16]. For example, it is now well established that membrane-bound HSPGs are required for binding of basic fibroblast growth factor to its high-affinity receptor [17,18].

Although the sequence similarity between OCI-5, glypican and cerebroglycan strongly suggests that OCI-5 is a membrane-bound HSPG, until now there was no experimental evidence to support this. Here we provide data that demonstrates that OCI-5 is a GPI-linked HSPG.

MATERIALS AND METHODS

Insertion of the epitope tag

A 27 bp oligonucleotide encoding an epitope of the haemagglutinin A (HA) of the influenza virus [19] was inserted 3′ to a proline codon located 30 amino acids after the initiation codon of OCI-5 cDNA, downstream of the putative leader sequence. To do this, a *Sma*I site was created by site-directed mutagenesis, and the HA epitope was introduced by blunt-end ligation. The tagged OCI-5 cDNA was then inserted into the PECE vector under the transcriptional control of the SV40 early promoter. The inserted HA epitope is recognized by the monoclonal antibody 12CA5-I (BabCo).

Tissue culture and transfection

COS-1 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal-bovine serum. Transfections were performed by

Abbreviations used: GAG, glycosaminoglycan; GPI, glycosyl-phosphatidylinositol; HA, haemagglutinin A; HSPG, heparan sulphate proteoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C.

‡ To whom correspondence should be addressed, at: Division of Cancer Research, Reichmann Research Building, S-218, Sunnybrook Health Science Centre, 2075 Bayview Avenue, Toronto, Ontario, M4N 3M5, Canada.

the DEAE-dextran method [20]. For permanent expression, the transfection mixture also included the vector pSV2neo. At 48 h after transfection 800 μg/ml of neomycin was added to the cells. After 14 days neomycin-resistant clones were isolated by using cloning cylinders. The clones were then expanded and screened for OCI-5 expression by immunoprecipitation and Western blotting.

Immunoprecipitation and Western blots

Cells were washed with PBS, collected by scraping, and lysed with RIPA buffer (1 % Nonidet P40, 0.1 % SDS, 0.5 % sodium deoxycholate, 2 mM phenylmethanesulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin and 10 mM EDTA in PBS) at 0 °C for 30 min. After the insoluble material was discarded, portions of cell extract containing equal amounts of protein were incubated overnight at 4 °C with 2 μg of the 12CA5-I antibody, and the immune complexes were precipitated with Protein A–Sepharose. The immunoprecipitated material was then washed, mixed with loading buffer, boiled for 5 min, and run through SDS/3–8 %-polyacrylamide gradient gel under reducing conditions. The proteins were then transferred to a poly(vinylidene difluoride) membrane (Dupont). The membrane was blocked with blocking buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl; 0.1 % Tween 20, 5 % dried milk) for 1 h, and 1 μg of the 12CA5-I antibody/ml was added. After incubation for 2 h at room temperature, the binding of the antibody was detected with the ECL kit (Amersham).

Metabolic labelling and enzymic digestion of GAG chains

Cells were labelled for 24 h with 100 μCi/ml carrier-free [^{35}S]-sulphate in S-MEM medium (GIBCO BRL) containing 10 % dialysed fetal-calf serum and 100 μM $MgSO_4$. Cell lysates were then prepared as described above, and immunoprecipitation was performed with the 12CA5-I antibody by using lysate samples containing the same amount of labelled sulphate. The immunoprecipitated material was washed three times with enzyme buffer (50 mM Hepes, pH 7.0, 100 mM NaCl, 1 mM $CaCl_2$, 50 μg/ml BSA, 2 mM phenylmethanesulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin and 10 mM EDTA) and then treated in enzyme buffer with 20 m-units of chondroitinase ABC (Sigma) or with 2.5 m-units of heparitinase (Seikagaku) for 3 h at 37 °C. The digested extracts were run through a 3–8 %-polyacrylamide gradient gel. The gel was then dried and autoradiography performed.

Preparation of conditioned media

Cells were grown in serum-free medium for 6 h. Conditioned medium was collected, filtered, and OCI-5 was immunoprecipitated and analysed by Western blotting as described above.

Immunostaining

Cells growing on microscope slides were fixed with 3 % paraformaldehyde in PBS for 1 h at 4 °C. After rinsing in PBS, cells were incubated with the 12CA5 monoclonal antibody (5 μg/ml) for 2 h at 4 °C in Blotto (5 % non-fat dried milk in PBS). Cells were then washed twice with PBS and incubated for 1 h at room temperature with fluorescein-labelled goat anti-mouse IgG $F(ab')_2$ (15 μg/ml) in Blotto. After washing twice with PBS, cells were mounted and viewed under the fluorescent microscope. For phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, cells were incubated with 2.5 units of the enzyme/ml in serum-free Dulbecco's modified Eagle's medium for 1 h at 37 °C before fixation.

RESULTS

Since our initial attempts to raise antibodies with the ability to immunoprecipitate OCI-5 were not successful, we decided to insert an epitope tag in the OCI-5 cDNA. A 27 bp oligonucleotide encoding a 9-amino-acid epitope of HA was introduced at the N-terminus of the OCI-5 cDNA, immediately after the putative leader sequence (see the Materials and methods section). The tagged OCI-5 cDNA was then inserted into an expression vector, and transient expression assays were performed using COS cells. Then 48 h after transfection, cells were lysed and immunoprecipitated with a monoclonal antibody raised against the HA epitope. The immunoprecipitated material was analysed by Western blotting. Figure 1 shows that the anti-HA antibody detects a smear of high molecular mass in the COS transfected cells. Many proteoglycans are known to display this electrophoretic pattern, which is the result of heterogeneity in the length and number of GAG chains [4]. The transfected cells also display three specific bands, with apparent molecular masses of 69, 65 and 41 kDa. The 69 kDa band probably corresponds to the non-glycanated protein core, since the OCI-5 open reading frame encodes a protein of similar size. It is noteworthy in this respect that, on the other hand, the non-glycanated form of glypican has not been detected in human mammary epithelial cells and lung fibroblasts [7].

Based on these results, we decided to generate permanent COS

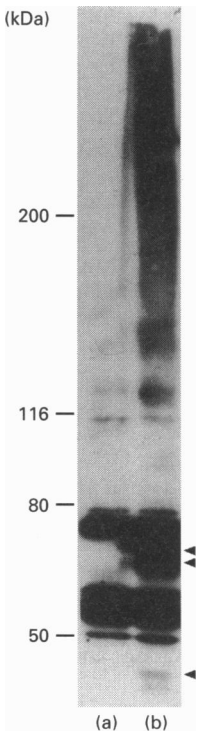

Figure 1 Analysis of OCI-5 in transiently transfected COS cells

Cells were transfected with the vector alone (lane a), or with the HA-tagged OCI-5 expression vector (lane b), and cell lysates were immunoprecipitated with the 12CA5-I antibody. The immunoprecipitate was analysed by Western blotting with the same antibody. Numbers on the left indicate the sizes of molecular-mass markers. The arrowheads point to the 69, 67 and 41 kDa bands that are recognized specifically by the 12CA5-I antibody.

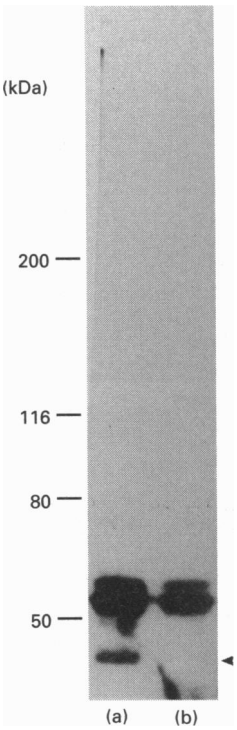

Figure 2 Detection of OCI-5 in conditioned media

Conditioned media were collected, immunoprecipitated with the 12CA5-I antibody, and analysed by Western blotting with the same antibody. Lanes: (**a**) COS cells transfected with OCI-5; (**b**) COS cells transfected with vector alone. Numbers on the left indicate the sizes of molecular-mass markers. The arrowhead points to the 41 kDa band that is specifically recognized by the 12CA5-I antibody.

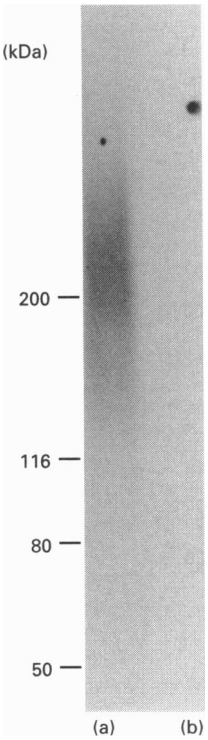

Figure 3 Immunoprecipitation of OCI-5 from transfected COS cells metabolically labelled with [^{35}S]sulphate

Cell lysates were immunoprecipitated with the 12CA5-I antibody. (**a**) Cells transfected with OCI-5; (**b**) cells transfected with vector alone. Numbers on the left indicate the sizes of molecular-mass markers.

cell lines expressing epitope-tagged OCI-5. COS 1 cells were then transfected with the tagged OCI-5 expression vector and a vector containing the gene coding for neomycin-resistance. Some 15 neomycin-resistant clones were selected and screened for the expression of OCI-5 by immunoprecipitation and Western blotting. Only two clones had detectable expression, and in one of them the expression was very low. Western-blot analysis showed that the bands detected by the anti-HA antibody in these two clones are the same as those found in the transiently transfected COS cells (results not shown). It is important to note, however, that the expression of OCI-5 in the permanent cell line was unstable, becoming undetectable after a few passages. We therefore decided to use transiently transfected cells in the rest of the experiments.

Since glypican is secreted into the medium in cultured cells [4], we decided to investigate whether OCI-5 is present in conditioned medium of transiently transfected COS cells. Figure 2 shows a Western-blot analysis of such medium. Surprisingly, only a 41 kDa band was specifically detected by the anti-HA antibody. The molecular mass and shape of this band strongly suggests that it does not contain GAG chains. Since the potential sites for the attachment of these chains in OCI-5 are located in the C-terminus and the epitope tag was inserted at the N-terminus, it is highly likely that the 41 kDa band detected in the conditioned medium is the result of proteolytic cleavage. This cleavage would separate the epitope recognized by the anti-HA antibody from the moiety containing the GAG chains.

In order to confirm that OCI-5 is in fact a sulphated proteoglycan, we incubated the transiently transfected COS cells with radioactive sulphate. Cell lysates were prepared and immunoprecipitated with the anti-HA antibody. Figure 3 shows that the autoradiography corresponding to the transiently transfected cells displays the typical smear of a proteoglycan. Next, we determined the type of GAGs present in OCI-5. Sulphate-labelled lysates of transfected cells were immunoprecipitated with the anti-HA antibody, incubated with chondroitinase or heparitinase, run through a polyacrylamide gel, and analysed by autoradiography. Figure 4 shows that the typical smear of the proteoglycan disappears almost completely after treatment with heparitinase. Chondroitinase treatment, on the other hand, does not produce any significant change.

Finally, we investigated whether OCI-5, like the homologous glypican and cerebroglycan, is anchored in the cytoplasmic membrane through a GPI anchor. When non-permeabilized, transiently transfected, COS cells are immunostained with the anti-HA monoclonal antibody, a clear membrane staining is observed in $\sim 20\%$ of the cells (Figure 5b). The percentage of positive cells probably reflects the efficiency of the transfection. The HA-specific immunostaining disappears almost completely if the transfected cells are treated with PI-PLC before adding the antibody (Figure 5c).

DISCUSSION

In this paper we have presented experimental evidence indicating that the OCI-5 cDNA encodes an HSPG that is attached to the plasma membrane through a GPI anchor. To obtain this evidence, we have inserted an epitope tag into the OCI-5 cDNA, and we have transfected the tagged OCI-5 transiently and

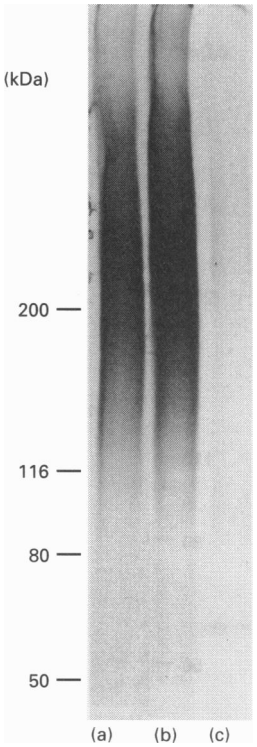

Figure 4 Analysis of the GAG chains in OCI-5

Lysates from transfected and [^{35}S]sulphate-labelled COS cells were immunoprecipitated with the 12CA5-I antibody. Immunoprecipitates were then treated with buffer alone (lane a), chondroitinase ABC (lane b), or heparitinase (lane c). Numbers on the left indicate the size of molecular-mass markers.

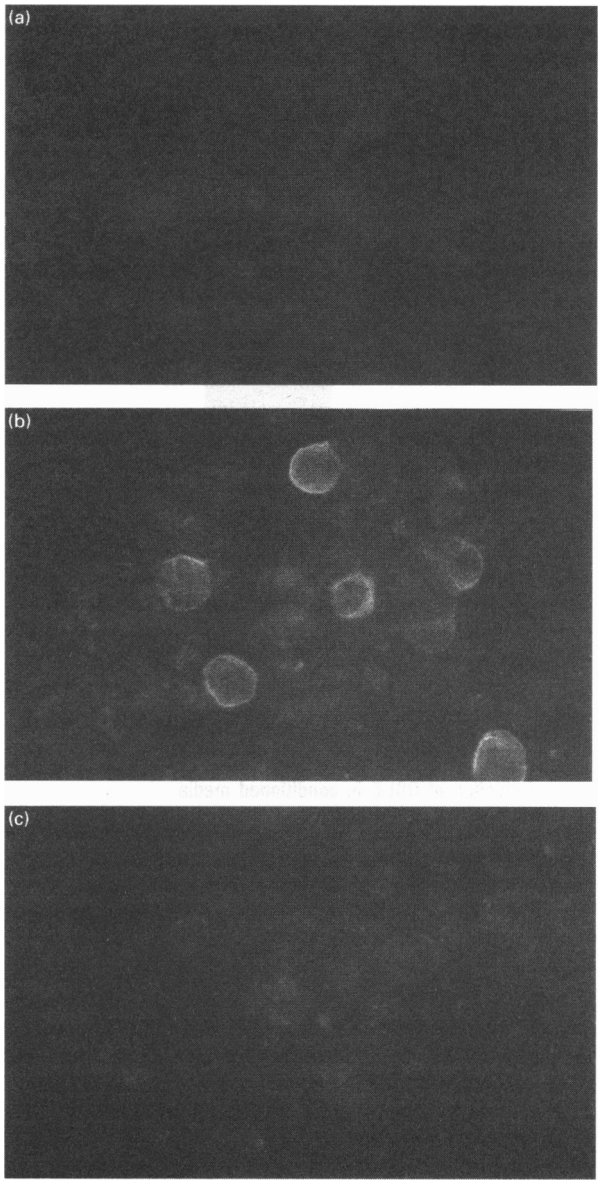

Figure 5 Detection of OCI-5 by immunofluorescence in non-permeabilized cells

Cells were immunostained with the 12CA5-I antibody. (**a**) COS cells transfected with vector alone; (**b**) COS cells transfected with OCI-5; (**c**) COS cells transfected with OCI-5 and treated with PI-PLC before immunostaining.

permanently into COS cells. In both cases we have been able to immunoprecipitate similar specific bands by using a monoclonal antibody against the epitope tag (Figure 1, and results not shown). The most abundant immunoprecipitated material behaves as an HSPG: it generates a high-molecular-mass smear when run through a polyacrylamide gel, it can be labelled with [^{35}S]sulphate, and it can be digested with heparitinase, but not with chondroitinase (Figure 4). Still, since some proteoglycans such as syndecan-1 can have either heparan sulphate or chondroitin sulphate chains, depending on the cell type [21,22], we cannot rule out the possibility of OCI-5 containing different GAG chains in other cell types.

We have also shown that OCI-5-transfected COS cells secrete into the medium a 41 kDa protein that includes the HA epitope (Figure 2). This band seems to be the result of the proteolytic cleavage of OCI-5. A band of a similar size is also immunoprecipitated from cell extracts. We could not establish whether the C-terminus, which is presumed to contain the GAG chains, is also in the conditioned media, since this moiety of the transfected OCI-5 does not contain the epitope tag.

We have demonstrated by immunostaining that PI-PLC releases membrane-bound OCI-5, confirming that OCI-5, like the homologous glypican and cerebroglycan, binds to the cytoplasmic membrane through a GPI tail (Figure 5). We have not formally proven, however, whether both the glycanated and non-glycanated OCI-5 are released by PI-PLC treatment.

The identification of OCI-5 as a membrane-bound HSPG confirms the hypothesis generated from the sequence homology between glypican, cerebroglycan and OCI-5 [6], and clearly establishes the existence of a new family of HSPGs that are attached to the cell membrane through a GPI tail. Little is known concerning the function of the members of this new family. Glypican has been found to bind laminin in Schwann cells, and it has been proposed that it is involved in the interaction between these cells and the extracellular matrix [23]. For cerebroglycan, indirect evidence suggests that this HSPG plays a role in the motility of developing neurons [5]. OCI-5 is expressed in several fetal tissues, but it is down-regulated in the adult, except in the lung ([3]; J. Filmus, Z. M. Wong and R. N. Buick, unpublished work). This suggests a role for OCI-5 during embryonal morphogenesis. A role in morphogenesis during development has also been proposed for syndecans, another family of membrane-bound HSPGs [21], and recently Nurcombe et al. [24] have

shown that HSPGs regulate the neural response to acidic and basic fibroblast growth factor during development. The observation that the expression of OCI-5 in tissue culture is regulated by cell shape is also consistent with a role for OCI-5 in embryonal morphogenesis [3].

We are currently trying to generate antibodies against OCI-5. These antibodies should allow us to perform further studies toward the understanding of the function of this novel HSPG in development.

We thank R. Buick for helpful discussions and Terrie Walker and Lynda Woodcock for assistance in the preparation of this manuscript. This work was supported by the Medical Research Council of Canada.

REFERENCES

1 Mathan, M., Moxey, P. C. & Trier, J. S. (1976) Am. J. Anat. **146**, 73–92
2 Louvard, D., Kedinger, M. & Hauri, H. P. (1993) Annu. Rev. Cell Biol. **8**, 157–195
3 Filmus, J., Church, J. & Buick, R. N. (1988) Mol. Cell Biol. **8**, 4243–4249
4 David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J. & Van Den Berghe, H. (1990) J. Cell Biol. **111**, 3165–3176
5 Stipp, C. S., Litwac, E. D. & Lander, A. D. (1994) J. Biol. Chem. **124**, 149–160
6 David, G. (1993) FASEB J. **7**, 1023–1030
7 Lories, V., Cassiman, J. J., Van Den Berghe, H. & David, G. (1992) J. Biol. Chem. **267**, 1116–1122
8 Karthikeyan, L., Maurel, P., Rauch, U., Margolis, R. K. & Margolis, R. U. (1992) Biochem. Biophys. Res. Commun. **188**, 395–401
9 Saunders, S. & Bernfield, M. (1988) J. Cell Biol. **106**, 423–430
10 Wight, T. N., Kinsella, M. G. & Qwarnstrom, E. (1992) Curr. Biol. **4**, 793–801
11 Hardingham, T. E. & Fosang, A. J. (1992) FASEB J. **6**, 861–870
12 Gallagher, J. T. (1989) Curr. Opin. Cell Biol. **1**, 1201–1218
13 Kjellen, L. & Lindahl, U. (1991) Annu. Rev. Biochem. **60**, 443–475
14 Ruoslahti, E. & Yamaguchi, Y. (1991) Cell **64**, 867–869
15 Jalkanen, M., Jalkanen, S. & Bernfield, M. (1991) in Receptors for Extracellular Matrix (McDonald, J. A. & Mecham, R. P., eds.), pp. 1–37, Adacemic Press, San Diego
16 Esko, J. D. (1991) Curr. Biol. **3**, 805–816
17 Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. & Ornitz, D. M. (1991) Cell **64**, 841–848
18 Rapraeger, A. C. (1993) Curr. Biol. **5**, 844–853
19 Field, J., Nickawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A. & Wigler, M. (1988). Mol. Cell. Biol. **8**, 2159–2165
20 Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21 Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. & Lose, E. J. (1992) Annu. Rev. Cell Biol. **8**, 365–393
22 Sanderson, R. D., Turnbull, J. E., Gallagher, J. T. & Lander, A. D. (1994) J. Biol. Chem. **269**, 13100–13106
23 Carey, D. J., Crumbling, D. M., Stahl, R. C. & Evans, D. M. (1990) J. Biol. Chem. **265**, 20627–20633
24 Nurcombe, V., Ford, M. D., Wildschut, J. A. & Barlett, P. F. (1993) Science **260**, 103–106

Received 20 April 1995; accepted 26 May 1995