Anosmin-1 is an extracellular matrix glycoprotein which underlies the X chromosome-linked form of Kallmann syndrome. This disease is characterized by hypogonadism due to GnRH deficiency, and a defective sense of smell related to the underdevelopment of the olfactory bulbs. This study reports that anosmin-1 is an adhesion molecule for a variety of neuronal and non-neuronal cell types in vitro. We show that cell adhesion to anosmin-1 is dependent on the presence of heparan sulfate and chondroitin sulfate glycosaminoglycans at the cell surface. A major cell adhesion site of anosmin-1 was identified in a 32 amino acid (32R1) sequence located within the first fibronectin-like type III repeat of the protein. The role of anosmin-1 as a substrate for neurite growth was tested on either coated culture dishes or monolayers of anosmin-1-producing CHO cells. In both experimental systems, anosmin-1 was shown to be a permissive substrate for the neurite growth of different types of neurons. Mouse P5 cerebellar neurons cultured on anosmin-1 coated wells developed long neurites; the 32R1 peptide was found to underly part of this neurite growth activity. When the cerebellar neurons were cultured on anosmin-1-producing CHO cells, neurite growth was reduced as compared to wild-type CHO cells; in contrast, no difference was observed for E18 hippocampal and P1 dorsal root ganglion neurons in the same experimental system. These results indicate that anosmin-1 can modulate neurite growth in a cell-type specific manner. Finally, anosmin-1 induced neurite fasciculation of P5 cerebellar neuron aggregates cultured on anosmin-1-producing CHO cells. The pathogenesis of the olfactory defect in the X-linked Kallmann syndrome is discussed in the light of the present results and the recent data reporting the immunohistochemical localisation of anosmin-1 during early embryonic development.

Key words: KAL-1 gene, Extracellular matrix, Cell-adhesion site, Chondroitin sulfate, Heparan sulfate, Olfactory system, Development

INTRODUCTION

During neuronal development, axons extend through various sets of extracellular environments to reach their targets. Understanding how axons grow along the proper paths to find their correct target is a major aim of developmental neurobiology. It is now clear that many adhesive, anti-adhesive, attractive and repulsive substrates are involved in this process (Goodman and Shatz, 1993; Kolodkin, 1996; see Goodman, 1996, for review), although the precise mechanisms are still not well understood. In recent years, an increasing number of proteins with such properties, as well as some of their receptors, have been identified and their function studied in vitro (Kennedy et al., 1994; Messersmith et al., 1995; Colamarino and Tessier-Lavigne, 1995; Stoeckli and Landmesser, 1995; Serafini et al., 1996; Chen et al., 1997; and see Tessier-Lavigne and Goodman, 1996, for review). The study of genetic deficiency in which axonal elongation appears to be defective is a complementary approach (Kolodziej et al., 1996; Mitchell et al., 1996; Tear et al., 1996; Leonardo et al., 1997). It has been proposed that the X chromosome-linked Kallmann syndrome offers such a model (Schwanzel-Fukuda et al., 1989). This disease is defined by the association of hypogonadism and anosmia (defective sense of smell). The hypogonadism is due to a deficiency of gonadotropin-releasing hormone (GnRH) (Naftolin et al., 1971), and the anosmia has been linked to aplasia or hypoplasia of the olfactory bulbs and tracts (De Morsier, 1954). Developmental links between the olfactory system and the GnRH neuroendocrine system have been described. Both GnRH-synthesizing neurons and olfactory receptor neurons derive from the olfactory placodes (Schwanzel-Fukuda and Pfaff, 1989). Olfactory axons contact...
the olfactory bulb primordium, and this contact is required for the development of the olfactory bulbs (Graziadei and Monti Graziadei, 1986; see Farbman, 1992, for review). The olfactory axons form an olfactory epithelium-forebrain axis of nerve fibers also comprising fibers of the vomeronasal and terminalis nerves. GnRH-synthesizing neurons ascend along this fiber axis (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Schwanzel-Fukuda et al., 1996), migrate to the medial region of the telencephalon, and ultimately reach the preoptic and hypothalamic areas.

An insight into the pathogenesis of X-linked Kallmann syndrome was provided by the study of a 19 week human fetus carrying a deletion encompassing the causative gene. In this fetus, neither the GnRH neurons, nor the axon terminals of the olfactory, terminalis and vomeronasal neurons were present in the brain. Although the GnRH neurons and the olfactory system axons had left the olfactory epithelium, they had accumulated in the upper nasal area (Schwanzel-Fukuda et al., 1989). This observation implied that the embryonic defect underlying X-linked Kallmann syndrome did not involve the initial differentiation steps of the olfactory and GnRH neurons within the olfactory placode, rather it was proposed that a defect in the subsequent elongation pathway of the olfactory axons and the associated migration of GnRH neurons is responsible for the syndrome.

The gene responsible for X-linked Kallmann syndrome (KAL-1 gene) has been cloned (Franco et al., 1991; Legouis et al., 1991). The encoded 680 amino acid protein has been named anosmin-1 in reference to the defective sense of smell which characterizes the disease. The protein sequence contains a signal peptide and no transmembrane domain or membrane anchorage domain via glycosyl phosphatidylinositol, indicating that the protein is likely to be extracellular. However, two other domains are present: (i) the N-terminal region is cysteine-rich and comprises a WAP (whey acidic protein) 4-disulfide core motif (Dandekar et al., 1982; Hennighausen and Sippel, 1982), which has been described in some proteins with serine protease inhibitory activity (Kato and Tominaga, 1979; Sippel, 1982), which has been described in some proteins with this motif is found in a wide variety of molecules with morphoregulatory roles, most of which are involved in cell adhesion (Edelman and Crossin, 1991; Engel, 1991; Edelman, 1994). Interestingly, the anosmin-1 FNIII repeats show the highest degree of homology with the FNIII repeats of the adhesion molecules L1 (Moos et al., 1988; Burgoon et al., 1991), TAG1/axonin-1 (Furley et al., 1990; Zuellig et al., 1992), and F3/F11 (Brümmedendorf et al., 1989; Gennarini et al., 1989). These molecules have been demonstrated to modulate cell adhesion, neurite fasciculation and neurite growth.

The production of anosmin-1 from the transfected CHO cell line and its purification (Soussi-Yanicostas et al., 1996), allowed us to test some properties of this protein in vitro. In the present study, we demonstrate that anosmin-1 is a cell adhesion molecule and report on the molecular basis of this property. In addition, coated anosmin-1 and anosmin-1-producing CHO cells were used to study the effect of this protein on neurite growth of cerebellar, hippocampal and dorsal root ganglion neurons. Here, we present evidence for the cell specific effects of this protein on neurite growth.

MATERIALS AND METHODS

Cells

Unless otherwise stated, culture media and additives were obtained from Gibco-BRL (Life Technologies) and fetal calf serum (FCS) from PAA (Jacques Boy, France). Cultures were grown in a 6% CO2 humidified atmosphere at 37°C. Primary cultures of hippocampal neurons from E18 mice embryos were performed as previously described (Buttiglione et al., 1996). Dissociated cerebellar neurons and cell agglomerates were obtained from 5-day old (P5) mice according to the method of Gao et al. (1995) and Buttiglione et al. (1996). Primary cultures of neurons were grown in DMEM/Ham F12 (3/1) defined medium containing 5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite and 12 mM Hepes. The diverse cell lines were maintained in their appropriate medium: the 13.S.1.24 cells (Coronas et al., 1997) in 10% FCS-supplemented DMEM; the PC12 cells (ATCC CRL-1721) (Greene and Tischler, 1976) in RPMI supplemented with 5% FCS and 8% inactivated horse serum (HyClone, without nerve growth factor; the GT1 cells (subclone 1) (Mellon et al., 1990) in glucose-enriched (25 mM) DMEM supplemented with 10% FCS and 5% inactivated horse serum; the LLLCPK cells (ATCC, CL-101) (Hull et al., 1976) in 10% FCS-supplemented, glucose-enriched (25 mM) DMEM. Parental and recombinant CHO cells (subclones I-1 and 2-3d11) (Soussi-Yanicostas et al., 1996) were cultured in 8% FCS-supplemented DMEM. The subclones isolated from the stable KAL-1-DHFR-transfected CHO cells were maintained under selective pressure (0.1 mM methotrexate, Sigma) throughout the study. Wild-type CHO-K1, mutant CHO-606 (pgsE-606) and CHO-677 (pgsd-677) cell lines (kindly provided by Dr J. Esko), and the mutant CHO-745 (pgsA-745) cell line (ATCC, CRL-2242) were maintained in 8% FCS-supplemented Ham F12.

Cell treatments

Effect of divalent cations

Cells were preincubated for 5 minutes at room temperature in Ca2+- and Mg2+-free Hanks’ balanced salt solution (CMF-HBSS) containing 5 mM EDTA, washed once with CMF-HBSS and preincubated without or with Ca2+ and/or Mg2+ in 0.1% heat-inactivated BSA-CMF-HBSS, for 1 hour at 4°C prior plating.

Inhibition by antibody

Wells were coated with anosmin-1, then saturated with 1% heat-inactivated BSA. The P34 immune serum directed against the purified anosmin-1 (see below) was thereafter added at the indicated concentrations for a 2 hour incubation period at 37°C. Cells were then applied and allowed to adhere.

Competition with synthetic peptides

Peptides (see below) (10-100 μg/ml) were directly incubated in solution with the cells for 1 hour at 4°C and were then added to anosmin-1-coated wells and incubated for a further 60 minutes at 37°C, prior to counting adherent cells.

Inhibition by heparin and chondroitin sulfate

Heparin or chondroitin sulfate were added to anosmin-1-coated wells and incubated for a further 60 minutes at 37°C, prior to counting adherent cells.

Enzyme treatment

Cells from the deficient CHO-677 and the wild-type CHO-K1 lines were preincubated with or without 0.01-1 units/ml chondroitin ABC lyase (chondroitinase ABC) (EC 4.2.2.4) (Sigma, C-2905) or heparitin-sulfate lyase (heparitinase) (EC 4.2.2.8) (Sigma, H-8891), alone or together, for 15 or 55 minutes at 37°C, then plated to anosmin-1-coated wells, and incubated for a further 1 hour.
Anosmin-1 production and peptide synthesis

Large-scale production of anosmin-1 was obtained from a stable subclone (CHO-KAL-2-3/d11) of CHO cells transfected with the full-length human KAL-1 cDNA, and the protein purified as previously described (Soussi-Yanicostas et al., 1996). A 32 amino acid peptide (32R1) derived from the first fibronectin-like type III repeat of human anosmin-1 (see Fig. 3A) was synthesised (Synt:em, Nîmes, France). Two peptides were used as controls: C17 (NH2-CSLVPITKKRRKFTGDGF-COH2), derived from the second fibronectin-like type III repeat (del Castillo et al., 1992) of human CSL VPTKKKRRKTTDGF-CONH2 (V. Kalatzis, personal communication), derived from the human EYA1 protein (Abdelhak et al., 1997).

Antibodies

The rabbit immune serum P34 raised against the purified anosmin-1 (Soussi-Yanicostas et al., 1996) and the corresponding preimmune serum were used for inhibition experiments. Immunoglobulins from preimmune and P34 immune serum were purified by affinity chromatography on Protein A-Sepharose (Pharmacia Biotech, Sweden). Fab fragments were obtained by proteolytic digestion with papain-agarose (Sigma). Undigested IgGs were eliminated by Protein A-Sepharose chromatography and purified Fab fragments were extensively dialysed against PBS.

Coating of plates

Substrate coating

The various molecules, in 0.5 ml PBS, were coated onto plastic 24-well plates (NunccloneTM) or polystyrene 96-well microtiter plates (Immulon 4, Dynatech) (overnight at 37°C). In standard experiments, purified anosmin-1 was applied at 3 μg/ml. The peptides (10 μg/ml) were pre-absorbed overnight at 37°C on polystyrene 96-well microtiter plates (Immulon 4, Dynatech), before the addition of the CHO-K1 and 13.S.1.24 cells (2×104 in a final volume of 0.15 ml). Other substrates, laminin (20 μg/ml), fibronectin (20 μg/ml) and poly-L-lysine (100 μg/ml) were used as controls. The well surfaces was washed three times with PBS, saturated with 1% heat-inactivated BSA (Sigma) for at least 1 hour at 37°C, and again washed thrice with PBS.

Adhesion assays

Assays were performed as described by Bauvois et al. (1992) with some modifications. Cells were harvested with Nozyme (JRH Biosciences), a non-proteolytic mixture; optimal duration and temperature (ambient or 37°C) for the treatment of the cells were adapted for each cell line to ensure good viability (checked by Trypan Blue staining). Dispersed cells (2–5×105 cells/0.5 ml of 0.1% heat-inactivated BSA in the appropriate serum-free medium) were added to the wells after allowing to adhere for 1 hour at 37°C. Medium was then removed and unbound cells eliminated by one wash with PBS. The adherent cells were fixed with 4% paraformaldehyde (PFA) in PBS and stained with 0.1% Crystal Violet. Quantification of the attached cells was performed using a colorimetric method (Aumailley et al., 1989) and the results expressed as the percentage of adherent cells to each substrate in reference to the poly-L-lysine control. Assays were performed in triplicate and repeated in three independent experiments.

Neurite growth

Dissociated cerebellar neurons (75×103 cells/cm2) were added to the substrate-coated 24-well plates (see above). Independently, wild-type CHO and anosmin-1-producing CHO cells (subclones 1.1 and 2.3/d11) were seeded in 8-well Labtek slides (104 cells/well) and grown for 24 hours until confluent. Thereafter, cerebellar neurons (104/well), cerebellar aggregates (approximately 50/well), hippocampal neurons (5×103/well) were laid onto these cell monolayers. Cocultures were maintained for 24 or 48 hours in the defined medium. Where indicated, preimmune or anti-anosmin-1 Fab (0.2 mg/ml) were included throughout the coculture period. Cells were fixed with PFA, permeabilised with methanol for 10 minutes at −20°C and immunostained with a polyclonal anti-GAP43 antibody (a gift from Dr Graham Wilkin, Imperial College, London), or with anti-MAP2 antiserum (a gift from Dr J. F. Leterrier, INSERM U298, Angers, France). Neurite length was quantified with a Zeiss Axioskop microscope equipped with epifluorescence and a CoolView camera (Photonic Science, France), using a Visiolab 1000 software package (Biocom, France). For cerebellar and hippocampal neurons, total neurite lengths were measured in 10 randomly selected areas (0.1 mm2) and expressed as the mean neurite length per neuron. Fasciculated and unfasciculated axons were not discriminated in the total neurite length analysis. The morphology of hippocampal neurons was analyzed. The length and branching of dendrites were determined on isolated neurons using immunofluorescence staining for MAP2. Only neurons with a polarized morphology, i.e. with an axonal length >100 μm, were analyzed (20 neurons in each experimental conditions).

RESULTS

Anosmin-1 promotes adhesion of a variety of cell types

We tested the ability of anosmin-1 to promote adhesion of different cell types. Since it has been proposed that the Kallmann syndrome results from a migration defect of olfactory axons and GnRH neurons during embryogenesis, adhesion properties of anosmin-1 were investigated using cell lines derived from rat olfactory epithelium (13.S.1.24) and mouse GnRH neurons (GT1-1). Other cell types, namely mouse P5 cerebellar neurons, as well as cell lines derived from rat pheochromocytoma (PC12), pig kidney proximal tubule (LLCPK) and chinese hamster ovary (CHO) were also used. Cell adhesion assays were performed on microtiter wells coated with anosmin-1 produced and purified from CHO cells transfected with the human KAL-1 cDNA (Soussi-Yanicostas et al., 1996). The number of adherent cells was measured after 1 hour using a colorimetric method (see Materials and Methods). The adhesion to anosmin-1 was compared to the adhesion to laminin (LN), poly-L-lysine (PLL) and bovine serum albumin (BSA). The percentage of cell adhesion observed on PLL was set to 100% and cell adhesion to the other substrates was quantified relative to this value. All the cell types tested were found to adhere to anosmin-1 after 1 hour. The percentage of adherent cells was similar for anosmin-1 and laminin, ranging from 80% to 95% (Fig. 1A), with the exception of the cerebellar neurons and LLCPK cells which were less adherent to laminin (50% and 60%, respectively) than to anosmin-1 (80%) (Fig. 1A). The percentage of cells adhering to BSA, taken as a negative control, was approximately 10% (Fig. 1A). In order to assess the specificity of cell adhesion to anosmin-1, adhesion assays were performed using anosmin-1 coated wells preincubated with increasing concentrations of the P34 immune serum directed against anosmin-1 (Soussi-Yanicostas et al., 1996). Adhesion of the 13.S.1.24 cells (Fig. 1B), PC12 cells, P5 cerebellar neurons, and CHO cells (data not shown) was prevented in a dose-dependent manner. These results establish anosmin-1 as an efficient adhesion substrate for different cell types. Whether the adhesion of PC12 and CHO cells were dependent on the presence of the Ca2+ and Mg2+ divalent cations in the medium was investigated. Ca2+ and Mg2+ ions were chelated by
incubation of the cells with EDTA for 5 minutes prior to plating on anosmin-1-coated microtiter wells (see Materials and Methods). This treatment did not modify the percentage of adherent cells (data not shown), therefore suggesting that cell adhesion to anosmin-1 is not dependent on the presence of Ca$^{2+}$ and Mg$^{2+}$.

Heparan sulfate and chondroitin sulfate glycosaminoglycans are required for cell adhesion to anosmin-1

Since anosmin-1 has been shown to bind to a heparin column (Soussi-Yanicostas et al., 1996), we tested whether cell adhesion to anosmin-1 is dependent on cell surface glycosaminoglycans. Competition experiments with exogeneous heparin or chondroitin sulfate (CS) as well as pretreatment of cells with heparitinase and/or chondroitinase ABC were performed, and several glycosaminoglycan-defective CHO cell lines were used.

In the first instance, increasing concentrations of either heparin or CS were added to microtitre wells coated with anosmin-1 prior to the addition of wild-type CHO cells (CHO-K1), and the percentage of adherent cells was determined (see Materials and Methods). Heparin at 30 $\mu$g/ml and chondroitin sulfate ABC (CS) at 15 $\mu$g/ml inhibited cell adhesion to anosmin-1 by approximately 50% (Fig. 2A,B). Similar inhibitory effects were observed with PC12 cells (data not shown). Enzymatic pretreatment of CHO-K1 cells with heparitinase (1 unit/ml) or chondroitinase ABC (1 unit/ml) reduced cell adhesion to anosmin-1 by 50% and 44%, respectively. Moreover, pretreatment with both heparitinase (1 unit/ml) and chondroitinase ABC (1 unit/ml) almost completely hindered cell adhesion to anosmin-1, leading to a residual amount of 10% of adherent cells (Fig. 2C) which is comparable to that observed with the BSA control (Fig. 1A). Consistently, the mutant CHO-745 cell line, deficient in both HS and CS glycosaminoglycans (Esko et al., 1987), showed no attachment to anosmin-1 (Fig. 2D). The CHO-677 cell line, which displays undetectable levels of HS but overexpresses CS (LeBaron et al., 1988, and Fig. 2D). Pretreatment of CHO-677 cells by chondroitinase ABC (0.01 unit/ml) resulted in an almost complete inhibition of their adhesion to anosmin-1 (Fig. 2D (c)), but did not affect their adhesion to fibronectin (data not shown). Finally, the adhesion to anosmin-1 of the mutant cell line CHO-606, which expresses normal amounts of CS and HS (although the HS is 2 to 3 times less sulfated than in wild-type CHO-K1 cells; Bame and Esko, 1989), was similar to that of CHO-K1 (Fig. 2D), suggesting that the degree of sulfatation of HS is not crucial for cell adhesion to anosmin-1. In conclusion, competition assays, enzymatic treatment, and experiments using mutant CHO cell lines all indicate that HS and CS cell surface glycosaminoglycans are required for cell adhesion to anosmin-1.

A major cell adhesion site is located in the first fibronectin-like type III repeat of anosmin-1

Sequence comparisons between the human (Franco et al.,
Anosmin-1 is an adhesion molecule (Legouis et al., 1991), chicken (Legouis et al., 1993a,b; Rugarli et al., 1993) and zebrafish (O. Ardouin and R. Legouis, personal communication) anosmin-1 highlight the strong interspecies conservation of the first fibronectin type III repeat, and in particular that of a 32 amino acid sequence which corresponds to the 6th and 7th β-strands of this repeat (del Castillo et al., 1992, and Fig. 3A). The role of this 32 amino acid sequence in cell adhesion was therefore tested. The corresponding synthetic peptide (32R1) (Fig. 3A) was used to coat microtiter wells and the adhesive property of 13.S.1.24 cells was tested. Two synthetic peptides were used as controls; a 17 amino acid peptide derived from the second fibronectin type III repeat of human anosmin-1 (17R2) (del Castillo et al., 1992) and an unrelated 16 amino acid peptide (C16V) (V. Kalatzis, personal communication). The percentage of 13.S.1.24 cells which adhered to the 32R1 peptide (80%) was not significantly different from that obtained with the entire anosmin-1 (70%) (Fig. 3B). In contrast, the cells did not adhere to either C16V (Fig. 3B) or 17R2 (data not shown). Preincubation of 13.S.1.24 cells with increasing concentrations of 32R1 prevented the adhesion of these cells to anosmin-1 in a concentration-dependent manner, whereas preincubation with the peptide 17R2 (50 μM) had no effect on cell adhesion (Fig. 3C). The adhesion of these cells to fibronectin and to laminin was not affected by preincubation with 32R1 (data not shown). Similar results were obtained with PC12 cells and CHO cells (data not shown). The 32R1 sequence therefore represents a major cell adhesion site of anosmin-1.

Anosmin-1 coated as a culture substrate promotes neurite growth from cerebellar neurons

Since cerebellar granule cells have been shown to express the KAL-1 mRNA in a human fetus at 19 weeks (Lutz et al., 1994), the effect of anosmin-1 on neurite growth was studied using mouse P5 cerebellar granule cells. Initially, neurite growth assays were performed on plastic culture dishes coated with anosmin-1. As shown in Fig. 4A and B, dissociated mouse P5 cerebellar neurons cultured on coated anosmin-1 initiated neurite growth at 24 hours and displayed long neurites at 48 hours. To determine whether the 32R1 sequence was involved in this neurite growth, cerebellar neurons were cultured in wells coated with the 32R1 peptide. As shown in Fig. 4C and D, this peptide was also a permissive substrate for neurite growth from these neurons at 24 hours and

Fig. 2. Role of heparan sulfate and chondroitin sulfate glycosaminoglycans in the adhesion of CHO cells to anosmin-1. (A,B) Competition experiments. CHO-K1 cells were added to the anosmin-1-coated wells and incubated for 2 hours at 37°C in the presence of increasing concentrations of heparin (A) or chondroitin sulfate ABC (B). (C) Enzymatic pretreatment of cells. CHO-K1 cells were incubated with heparitinase (H, 1 unit/ml), chondroitinase ABC (C, 1 unit/ml) or heparitinase + chondroitinase ABC (H+C, each at 1 unit/ml), for 55 minutes at 37°C, prior to the adhesion assays. (D) Adhesion of various mutant CHO cells to anosmin-1. No difference in adhesion to anosmin-1 is observed between wild-type cells (K1) and CHO-606 cells (606) which express undersulfated heparan sulfate. In contrast, CHO-745 cells (745), which lack both heparan sulfate and chondroitin sulfate glycosaminoglycans, do not adhere to anosmin-1; note that the adhesion of these cells to fibronectin is not modified. Finally, the percentage of CHO-677 cells (677) adhering to anosmin-1 is reduced by 70% as compared to K1 cells; the CHO-677 cells are deficient in heparan sulfate and overexpress chondroitin sulfate. Pretreatment of CHO-677 cells by chondroitinase ABC (0.01 unit/ml for 15 minutes at 37°C) results in a complete loss of adhesion to anosmin-1 (c). Results are expressed as the percentage of cells adhering to anosmin-1 relative to poly-L-lysine. Means ± s.e.m. obtained from three independent experiments.
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Fig. 3. Cell adhesion to the 32R1 peptide derived from anosmin-1 (A) Sequence conservation of the 32R1 peptide derived from anosmin-1 between human and chicken. The four fibronectin-like type III repeats (FNIII) of anosmin-1 are indicated on a schematic representation of the protein (del Castillo et al., 1992). The 32R1 peptide is derived from the first FNIII repeat of human anosmin-1. The alignment of the orthologous sequences in human (del Castillo et al., 1992) and chicken (Legouis et al., 1993b; Rugarli et al., 1993) is shown. Amino acids identical in the two species are boxed. (B) Adhesion of 13.S.1.24 cells to the 32R1 peptide. Cells were added to wells coated with anosmin-1, 32R1 peptide, C16V peptide control or BSA. No significant difference is observed in the percentage of adherent cells between anosmin-1 and 32R1. (C) Competition experiments. Cells were preincubated for 1 hour at 4°C with either the 32R1 peptide at the indicated concentrations, or with the 17R2 peptide control (50 μM) prior to addition to anosmin-1-coated wells. The adhesion of 13.S.1.24 cells to anosmin-1 is inhibited by 32R1 in a dose-dependent manner. Results are expressed as the percentage of cells adhering to anosmin-1 or 32R1 relative to the poly-L-lysine. Means ± s.e.m. obtained from three independent experiments.

48 hours. The total neurite length was measured in randomly selected areas (0.1 mm²) and expressed relative to the number of cell bodies, representing the mean total neurite length per cell body. After 24 hours of neurite growth, no significant difference was observed between anosmin-1 and 32R1 peptide (Fig. 4E). In contrast, after 48 hours the mean neurite length was significantly reduced (~50%) for the coated peptide (Fig. 4E). When competition experiments were performed on coated anosmin-1 in the presence of the soluble 32R1 peptide, the mean neurite length at 24 hours was significantly reduced (Fig. 4F) although the number of adherent neurons was not modified. Such an inhibitory effect of the soluble peptide was also observed when neurons were cultured in wells coated with anosmin-1 combined with laminin, but not when they were cultured on PLL combined with laminin (Fig. 4F).

Anosmin-1 at the surface of transfected CHO cells reduces neurite growth and induces fasciculation of neurites from cerebellar neurons

Neurite growth assays were performed using dissociated mouse P5 cerebellar neurons grown on monolayers of either wild-type or anosmin-1-expressing CHO cells. Previous immunocytofluorescence analysis of anosmin-1-expressing CHO cells indicated that anosmin-1 displays the features of an extracellular matrix component that is bound to the cell surface membrane via heparan sulfate proteoglycans (Soussi-Yanicostas et al., 1996). Such a paradigm allowed us to study the neurite growth on anosmin-1 bound to the membrane in a conformation which may reflect some of the in vivo situations.

After a 24 hour coculture period in defined medium, the majority of neurons displayed short neurites; no difference was observed between neurons growing on the two types of cell monolayers (Fig. 5A,B). After 48 hours, neurons cultured on the wild-type CHO cells displayed long and ramified neurites (Fig. 5C,E) whereas the size and branching of the neuritic network was reduced on anosmin-1-producing CHO cells (Fig. 5D,F); the mean neurite length was reduced by 40% (Fig. 5G).

To investigate whether anosmin-1 could have a general inhibitory effect on neurite growth in such experimental conditions, dissociated neurons from P1 dorsal root ganglia (data not shown) and E18 hippocampus (Fig. 6) were plated on either wild-type or anosmin-1-producing CHO cell monolayers. No significant difference in neurite length was observed after a 48 hour period (Fig. 6I), suggesting that anosmin-1 can exert a lineage-specific control of neurite elongation. In addition, the polarized morphology of the hippocampal neurons was not affected (Fig. 6G,H); the mean dendrite length per neuron (Fig. 6J) and the dendrite branching were similar on control and anosmin-1 producing CHO cells.

Subsequently, cerebellar cell aggregates from P5 mice were
Anosmin-1 is an adhesion molecule cultured on monolayers of anosmin-1-producing CHO cells from either subclone 1-1 or 2-3d11 (see Materials and Methods). Cell aggregates, obtained from dissociated P5 cerebellar neurons grown for 24 hours on plastic culture dishes (Gao et al., 1995) were transferred to the CHO cell monolayers. After 24 hours of coculture, cell aggregates grown on wild-type CHO cells had long, sinuous and unfasciculated processes (Fig. 7A,C). In contrast, cell aggregates grown on anosmin-1-expressing CHO cells displayed short, radial and fasciculated neurites (Fig. 7B,D). Both subclones, 2-3d11 (Fig. 7) and 1-1 (data not shown), exhibited the same ability to induce fasciculation and to reduce the length of the neuritic processes. To demonstrate the specificity of the effects of anosmin-1 on the fasciculation and growth of neurites, Fab fragments prepared from the P34 immune serum directed against anosmin-1 (0.2 mg/ml) were added during the entire coculture period. In the presence of anti-anosmin-1 Fab, cerebellar neurons aggregates on anosmin-1-producing CHO cells showed long and unfasciculated neurites (Fig. 7E). In contrast the Fab fragments prepared from the P34 preimmune serum had no effect on the fasciculation (Fig. 7F).

**DISCUSSION**

**Anosmin-1 is a cell adhesion molecule**
The deduced amino acid sequence of anosmin-1 suggested that this protein is an extracellular matrix (ECM) component.
Consistently, we previously reported that CHO cells transfected with the human \textit{KAL-1} cDNA secrete the corresponding protein in culture medium (Soussi-Yanicos et al., 1996). Recent immunohistochemical studies have shown that anosmin-1 is indeed an ECM component in vivo (unpublished results). In the present study, we establish that anosmin-1 is a cell adhesion molecule in vitro. A major cell adhesion site was identified in a 32 amino acid sequence, corresponding to the 6th and 7th $\beta$ strands of the first fibronectin-like type III repeat of the protein (del Castillo et al., 1992), and which is highly conserved throughout evolution between zebrafish (O. Ardouin and R. Legouis, personal communication), chicken (Legouis et al., 1993a; Rugarli et al., 1993) and human (Franco et al., 1991; Legouis et al., 1991). This peptide does not contain the integrin-recognition motif Arg-Gly-Asp (RGD) present in most extracellular matrix proteins comprising fibronectin-like type III repeats (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1986) nor does the cell adhesion to anosmin-1 appear to be Ca$^{2+}$- and Mg$^{2+}$-dependent, as seem to be all integrin-ligand interactions (Gailit and Ruoslahti, 1988; Humphries, 1996). This suggests that the putative receptor involved in cell adhesion to anosmin-1 is not a member of the integrin family.

The present results using CHO cells establish that cell-surface glycosaminoglycans are necessary for adhesion to anosmin-1. One possibility is that glycosaminoglycans are also sufficient for cell adhesion to anosmin-1, although there is at present no experimental evidence to support such a proposal. In this case, the anosmin-1-binding HS and CS glycosaminoglycans could be carried by either a single receptor molecule or by several core proteins. Alternatively,
Anosmin-1 is an adhesion molecule

These glycosaminoglycans chains would represent cofactors that could stabilize the binding of anosmin-1 to its receptor. Such a situation has been shown for the binding of many growth factors to their receptors, including bFGF (Yayon et al., 1991; see Schlessinger et al., 1995, for review). The requirement of glycosaminoglycans for cell adhesion to anosmin-1 is reminiscent of the situation for type V collagen (LeBaron et al., 1989), but distinguishes anosmin-1 from many other substrate adhesion molecules. For example, glycosaminoglycans are not necessary for short-term cell adhesion to fibronectin, although they are required for the subsequent formation of focal adhesion and stress fibers (Woods et al., 1993). Several putative HS binding consensus motifs (Cardin and Weintraub, 1989; Sobel et al., 1992; Margalit et al., 1993) are present in the deduced amino acid sequence of anosmin-1. It is noteworthy that one such motif, SKHFRS (of the XBBXBX type where B designates a basic amino acid residue and X, any other one), is present within the 32 amino acid cell adhesion site of anosmin-1.

What is the physiological significance of the cell adhesion property of anosmin-1? During development, anosmin-1 is transiently present in some basement membranes, epithelial interstitial matrices and connective tissues (unpublished results). In epithelia, anosmin-1 could be involved in the adhesion of cells to the epithelial interstitial matrices and/or anchorage of cells to the basement membrane. In connective tissues, anosmin-1 could participate in the change of the composition of the ECM which is required for tissue condensation (see Gumbiner, 1996, for review). In the context of the hypothesis that the adhesive property of anosmin-1 is not cell specific, this property would only be regulated by the temporo-spatial expression of the KAL-1 gene. Alternatively,
cell adhesion to anosmin-1 could be dependent on the presence of specific membrane proteoglycans harbouring a developmentally regulated expression (Leppä et al., 1992; Vainio et al., 1992; Salmivirta and Jalkanen, 1995; Ivins et al., 1997; Brickman et al., 1998).

**Anosmin-1 is a permissive substrate for neurite growth**

In all the experimental conditions of the present study, anosmin-1 was a permissive substrate for neurite growth. On mouse P5 cerebellar neurons, the 32R1 peptide reproduced only part of the permissive effect of anosmin-1 on neurite growth. One possible explanation is that, in addition to this site, another site(s) of the molecule is involved in the neurite growth activity of anosmin-1. It should also be kept in mind that adhesive ability of growing neurites to 32R1 could be different from that to anosmin-1, although no difference was observed in short-term adhesion assays to these two substrates. In any case, the absence of a correlation between the substrate adhesivity of neuronal cell bodies and the rate of neurite growth has been reported for several ECM components (Gunderson, 1987; Lemmon et al., 1992; see Letourneau et al., 1994, for review). For instance, laminin-1 and laminin-2 stimulate and inhibit respectively the neurite growth of a motor neuron-derived cell-line, although the adherence of these neurons to these two laminins is similar (Calof and Reichardt, 1985; Porter et al., 1995).

**Anosmin-1 can modulate neurite growth in a cell-type specific manner**

When anosmin-1 was present on the surface of transfected CHO cells an inhibitory effect on neurite growth could be detected on cerebellar granule cells, whereas no such effect was observed on hippocampal and sensory neurons. This cell-type specific effect of anosmin-1 on neurite growth suggests that the putative receptor(s) involved in the neurite growth response is expressed only in specific neuronal cells.

We also observed that anosmin-1 induces fasciculation of

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Fig. 7. Neurite fasciculation from cerebellar neuron aggregates cultured on anosmin-1-producing CHO cells. (A-F) Microphotographs of aggregates of cerebellar neurons from P5 mice cultured for 24 hours on monolayers of either wild type CHO cells (A,C) or anosmin-1-producing (B,D,E,F) CHO cells. (E) Coculture was in the presence of Fab fragments (0.2 mg/ml) from anosmin-1 P34 immune serum and in F in the presence of Fab fragments from P34 preimmune serum. Neurons were immunostained with an anti-GAP43 antibody. Anosmin-1-induced fasciculation (B,D) is prevented by the anti-anosmin-1 Fab fragments (E), whereas preimmune Fab fragments have no effect (F). Bars: 30 μm (A,B,E,F); 10 μm (C,D).
neurites from aggregates of P5 cerebellar granule cell cultures on anosmin-1-expressing CHO cells. Since this effect was concomitant with the inhibition of neurite growth, it is possible that the observed neurite fascilitation is only a consequence of the inhibitory effect on neurite growth. A balance between axon-growth cone adhesion versus substrate-growth cone adhesion (Rutishauser et al., 1988; Schubert and Klier, 1991) could account for such a proposal. Alternatively, anosmin-1 might have a primary effect on fascilitation, possibly via another ECM component which bridges anosmin-1 molecules or by inducing the expression of axonal cell surface molecules which mediate neurite fascilitation.

Implications for the pathogenesis of the olfactory defect of X-linked Kallmann syndrome

During development, olfactory axons grow from the olfactory epithelium toward the telencephalon. During their elongation, olfactory axons traverse three morphologically distinct regions: the olfactory epithelium, the frontonasal mesenchyme and the telencephalon. In each of these regions, some changes in the trajectory, the degree of fascilitation and the growth cone of the olfactory axons are observed. There have been several attempts to correlate axon behavior with the presence of definite cell adhesion molecules and/or ECM proteins (Whitesides and LaMantia, 1996; Gong and Shipley, 1996). In the olfactory system of the early human fetus, anosmin-1 was exclusively detected in the olfactory bulb presumptive region of the telencephalon (unpublished results). In this region, olfactory axons defasciculate and their growth cones become enlarged and more complex (Whitesides and LaMantia, 1996). Interestingly, preliminary results indicate that anosmin-1 is also a permissive substrate for the axon outgrowth from rat embryonic olfactory epithelium explants (data not shown). In the telencephalon, anosmin-1 may thus provide a substrate necessary for the terminal elongation of the olfactory axons. In this regard, the absence of stable connections between the olfactory axons and the olfactory bulb presumptive region of the telencephalon would explain the absence of development of the olfactory bulbs which is observed in X-linked Kallmann syndrome.

In conclusion, we have characterized some properties of anosmin-1 in vitro. This protein is a cell adhesion molecule for a variety of cell types. It is also a permissive substrate for neurite growth of several neuronal cell types and displays cell-type specific effects on neurite growth. In order to get a comprehensive view of the role of anosmin-1 in the development of the olfactory system, further investigations are required. In particular, the possible role of anosmin-1 in the defasciculation process and in the modification of the growth cone morphology that olfactory axons undergo in the terminal part of their navigation, should be investigated.

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