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Research report

Cloning, sequencing, expression and function of a cDNA encoding a receptor for the opioid growth factor, [Met^5]enkephalin [1]

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Abstract

The native opioid growth factor (OGF), [Met^5]enkephalin, is a tonic inhibitory peptide that modulates cell proliferation and tissue organization during development, cancer, cellular renewal, wound healing and angiogenesis. OGF action is mediated by a receptor mechanism. We have cloned and sequenced a 2.1-kilobase (kb) cDNA for a receptor to OGF (OGFr). The open reading frame was found to encode a protein of 580 amino acids, and eight imperfect repeats of nine amino acids each were a prominent feature. The protein encoded by this cDNA exhibited the pharmacological, temporal and spatial characteristics of the OGFr. Functional studies using antisense technology demonstrated an enhancement in cell growth. The molecular organization of the OGFr has no homology to classical opioid receptors. These results provide molecular validity for the interaction of OGF and OGFr in the regulation of growth processes.

Keywords: Antisense; Opioid growth factor receptor; ζ-Opioid receptor; Rat; Gene; cDNA; mRNA

1. Introduction

Endogenous opioid peptides, first reported by Hughes et al. [6] in 1975, have received considerable attention as potent regulators of growth [25], in addition to serving as neuromodulators [1]. One native opioid peptide, [Met^5]enkephalin, has been reported to be an inhibitory growth factor in development, cellular renewal, cancer, wound healing and angiogenesis [7,9,13,17,18,23,25,29–31]. In view of these growth properties, [Met^5]enkephalin has been termed opioid growth factor (OGF) [25]. OGF is an autocrine produced and secreted peptide that is not cell, tissue or organ specific. While OGF exhibits activity at physiologically relevant concentrations, it does not elicit physical dependence, tolerance and/or withdrawal. OGF displays a temporal and spatial distribution consistent with specific growth-related effects and is sensitive to opioid antagonist displacement. OGF has a direct, rapid, prolonged, stereospecific, receptor mediated, non-cytotoxic and reversible influence on growth both in tissue culture and in prokaryotic and eukaryotic organisms. Blockade of the interaction of endogenous opioids with opioid receptors such as naltrexone (NTX) enhances growth [11,20,21], suggesting that growth related opioid peptides such as OGF are tonically active.

The opioid-like receptor mediating OGF action is novel because of its function (i.e., growth), tissue distribution (neural and non-neural), subcellular location (nuclear-associated), transient appearance during ontogeny, ligand specificity (i.e., [Met^5]enkephalin) and competitive inhibition profile. Based on pharmacological characteristics including the binding to opioids, stereospecificity and naloxone-reversibility, this receptor was tentatively termed the zeta (ζ)-opioid receptor [24,26–28].

The molecular nature of OGF is well-documented, and this peptide is encoded by the preproenkephalin gene [5,14]. No information about the molecular features of the receptor that binds OGF is available. The present study is the first report of the molecular organization of the receptor for OGF.

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[1] The nucleotide sequence of the rat OGFr has been deposited in GenBank under accession number AF 156878.

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2. Materials and methods

2.1. Screening of the λgt11 expression library

An antibody for OGF binding protein (BO461) was produced according to published procedures [24]. In brief, two-dimensional gels of the nuclear fraction (P1) from a 6-day old rat cerebellum were transferred to nitrocellulose, and ligand blotting with [^{125}I]-[Met^5]enkephalin was performed to identify an OGF binding protein [27]. The protein was electroeluted and injected into New Zealand white rabbits to generate polyclonal antibodies. Serum was collected and purified using ammonium sulfate precipitation and DEAE Affi-blue gel filtration.

One million plaques from a λgt11 expression cDNA library (oligo-dT primed, original complexity of 1.6×10^6 pfu with insert sizes ranging from 0.6 to 4.0 kilobases (kb)), constructed from 18-day old fetal rat brain mRNA (Clontech, Palo Alto, CA), were screened using the polyclonal antibody that recognized the OGF binding protein. Immunoreactive plaques were identified by reaction with horseradish peroxidase or [^{125}I]protein A, and four clones were purified and concentrated.

2.2. Purification of cDNA inserts from λgt11

λgt11 DNA was isolated as described by Sambrook et al. [15], and cDNA inserts were released from purified phage DNA by restriction digestion with *Eco*R1. One clone (#14) was selected for further analysis, and subcloned into a pGEX vector to generate recombinant protein. A full-length cDNA sequence (clone #12) was obtained by hybridization screening of the λgt11 18-day old fetal rat brain cDNA library with the isolated 1-kb *Eco*R1 insert from clone #14. Positive clones were identified and plaque purified.

2.3. DNA sequencing

Double-stranded DNA was isolated and sequenced using the method of Sanger et al. [16] or an automated ABI Prism Model 377, Version 2.1.1. Sequence data were analyzed using the Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin Biotechnology Center). Sequence similarity was compared to the entries recorded in the Genbank database using BLAST 2.0.8 [2] in June 1999.

2.4. Rat fusion protein

Inserts from clone #14 encoding C-terminal 197 amino acids, and from clone #12 encoding all 580 amino acids, were ligated into the pGEX-3X expression vector to generate glutathione-*S*-transferase (GST) fusion proteins. When expressed in XL1-Blue strain bacteria (Stratagene, La Jolla, CA), and induced with isopropyl β-*o*-thiogalactopyranoside for 3 h, these plasmids produced GST-OGF-binding fusion proteins which were purified from crude bacterial extracts using glutathione affinity chromatography and one-dimensional PAGE gel electrophoresis.

2.5. Receptor binding assays

Fusion proteins of clone #14 (14-GST) and clone #12 (12-GST) were prepared for binding assays with [^{3}H]-[Met^5]enkephalin [26,28]. Protein (80–120 ng/tube) was incubated for 30 min with shaking at 22°C; non-specific binding was measured in the presence of unlabeled [Met^5]enkephalin. In some assays, NTX (10^{-3} M) was added to the reaction mixture to monitor opioid antagonist blockade of binding. Binding was terminated by filtering homogenates through Whatman GF/B filters. Saturation isotherms were plotted using GraphPad Prism software.

Specific competition of 12-GST fusion protein was determined in the presence of a range (10^{-10}–10^{-3} M) of ligands including [D-Ala^2,MePhe^4,Glyol^5]enkephalin (DAMGO), [D-Pen^{2,5}]enkephalin (DPDPE), dynorphin A1-8, U69,583 and morphine sulfate.

2.6. Fusion protein antibodies

Antibodies were generated by inoculating New Zealand white rabbits with 14-GST or with 12-GST fusion proteins suspended in Freund's adjuvant. The GST was not cleaved from the proteins prior to inoculating the rabbits, allowing the GST to function as a carrier protein. Rabbits were

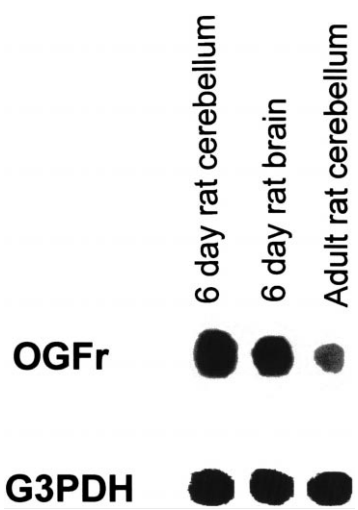

Fig. 1. Tissue distribution of mRNA detected by the cDNA clone of OGFr. Total RNA was isolated from 6-day cerebellum and brain and from adult cerebellum (25 μg/lane) and separated on a 1.2% agarose gel containing 6% formaldehyde and transferred to a nylon membrane and hybridized with [^{32}P]dCTP labeled #14. A single 2.1-kb mRNA was detected in all tissues examined. The Northern was stripped and hybridized with [^{32}P]dCTP labeled G3PDH, a constitutively expressed mRNA, to demonstrate equal loading of the RNA samples.

injected every 4 weeks for 2 months, tested for their titer, and exsanguinated 4 days after the final injection.

2.7. Western blotting

Nuclear preparations of 6-day and adult rat cerebellum, as well as GST and 14-GST fusion protein, were isolated by 12.5% SDS-PAGE, and electrotransferred to nitrocellulose. Western blotting performed with primary antibodies to either the 14-GST fusion protein [24], native OGF binding protein, or GST; [^{125}I]protein A was used for antibody detection.

2.8. Immunocytochemistry

Immunocytochemistry was performed using methodology described previously [31]. In brief, rat brains from

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-150 -TGGGCTCAGCCACGCCCCAGGGTGCCCCCAGTGGGACTAGTTCTTCATTCTGGCAGCTGCACACATCTGTCAGTGAGGGAATGTCAGGTC
 -60 -TCTCACTCTCCTCTCCTCACTATCCTTTCCGCAGAAAGCGGGTCCTCCTGCTTGTCGAGTATGGACGACCCGGACTGCGATTCCACCTGG
                                                                MetAspAspProAspCysAspSerThrTrp   10

  31 -GAGGAGGAGAGCGAGGAGGATGGCGAGGATGGCCAGGCGGATGATACGACCGATGAGGACACGGGCGACGATGACGGCGACGCGGAGGAG
      GluGluGluSerGluGluAspGlyGluAspGlyGlnAlaAspAspThrThrAspGluAspThrGlyAspAspAspGlyAspAlaGluGlu   40

 121 -GCACGGCCAAGCCTGTTCCAGTCCAGGATGACAGGGTACCGAAACTGGCGTGCTATGCAGGACATGCAAAGATACCGGCACAACTACCCG
      AlaArgProSerLeuPheGlnSerArgMetThrGlyTyrArgAsnTrpArgAlaMetGlnAspMetGlnArgTyrArgHisAsnTyrPro   70

 211 -GATTTGACAGATCAAGACTGCAATGGTGACATGTGCAACCTGAGCTTCTACAAAAATGAGATCTGCTTCCAGCCAAATGGGGCTCTCATC
      AspLeuThrAspGlnAspCysAsnGlyAspMetCysAsnLeuSerPheTyrLysAsnGluIleCysPheGlnProAsnGlyAlaLeuIle  100

 301 -GAGGACATTCTTCAGAACTGGAAAGACAACTATGACCTCCTGGAAGAGAATCACTCCTACATCCAGTGGCTGTTTCCTCTGCGGGAACCA
      GluAspIleLeuGlnAsnTrpLysAspAsnTyrAspLeuLeuGluGluAsnHisSerTyrIleGlnTrpLeuPheProLeuArgGluPro  130

 391 -GGAGTGAACTGGCACGCCAAGCCCCTCACCCTGAAGGAGGTTGAGGCATTTAAAAGCTCCAAGGAAGTCAGAGAGCGTCTTGTCCGGGCC
      GlyValAsnTrpHisAlaLysProLeuThrLeuLysGluValGluAlaPheLysSerSerLysGluValArgGluArgLeuValArgAla  160

 481 -TATGAGCTCATGCTGGGCTTCTATGGGTTCCACCTTGAGGACCGGGGCACGGGTGCTGTATGCCGTGCACAGAACTTCCAGCCGCGCTTC
      TyrGluLeuMetLeuGlyPheTyrGlyPheHisLeuGluAspArgGlyThrGlyAlaValCysArgAlaGlnAsnPheGlnProArgPhe  190

 571 -CACAATCTGAACAGCCACAGCCACAACAACCTGCGTATTACACGCATCCTCAAGTCACTGGGTGAGCTGGGCTTAGAACACTACCAGGCA
      HisAsnLeuAsnSerHisSerHisAsnAsnLeuArgIleThrArgIleLeuLysSerLeuGlyGluLeuGlyLeuGluHisTyrGlnAla  220

 661 -CCCCTGGTCCGCTTCTTCCTGGAGGAGACCCTTGTACAGCACAAACTGCCCAGCGTGCGCCAGAGTGCCCTGGACTACTTCCTGTTCGCT
      ProLeuValArgPhePheLeuGluGluThrLeuValGlnHisLysLeuProSerValArgGlnSerAlaLeuAspTyrPheLeuPheAla  250

 751 -GTGCGCTGCCGGCACCAGCGCCGGGAGCTTGTGTACTTTGCCTGGGAGCACTTCAAGCCTCGCCGAGAGTTTGTCTGGGGGCCCCGTGAC
      ValArgCysArgHisGlnArgArgGluLeuValTyrPheAlaTrpGluHisPheLysProArgArgGluPheValTrpGlyProArgAsp  280

 841 -AAGCTGCGGAGATTCAAGCCCCAGACCATACCCCAGCCACTGACGGGACCAGGGCAGGCAGATAAAGATGAGGGCTCCAGGGACCCCTCC
      LysLeuArgArgPheLysProGlnThrIleProGlnProLeuThrGlyProGlyGlnAlaAspLysAspGluGlySerArgAspProSer  310

 931 -AAGAGGCTGGCACCCAGGGTCGGACCTGTGGATCTGGAAGGGACCTGAGTGGGGACAGTGGAACAGCTGAGGATCCCTCACTGCTGAAC
      GlnGluAlaGlyThrGlnGlyArgThrCysGlySerGlyArgAspLeuSerGlyAspSerGlyThrAlaGluAspProSerLeuLeuAsn  340

1021 -ACAAAGCCCTCAGATGGGGGAACCTTGGATGGGAACCAGAGGGATGAAGCTAAGTCCCTGAGTCCCAAGGAGAGCAAGAAAAGGAAGTTG
      ThrLysProSerAspGlyGlyThrLeuAspGlyAsnGlnArgAspGluAlaLysSerLeuSerProLysGluSerLysLysArgLysLeu  370

1111 -GAGGGGAACAGGCAGGAGCAGGTCCCAGGGGAGGCAGATCCCCAGGGTGTCTCTGAGGTAGAGAAAATTGCCCTTAACCTTGAGGAGTGT
      GluGlyAsnArgGlnGluGlnValProGlyGluAlaAspProGlnGlyValSerGluValGluLysIleAlaLeuAsnLeuGluGluCys  400

1201 -GCCCTTAGCCCTATCAGCCAGGAGCCCAGGGAGGCTGAACCGCCCTGTCCTGTGGCCAGGGTGGCTAATGAGGTAAGAAAGCGGAGGAAG
      AlaLeuSerProIleSerGlnGluProArgGluAlaGluProProCysProValAlaArgValAlaAsnGluValArgLysArgArgLys  430

1291 -GTGGAGGAAGGGGCTGAGGGTGATGGAGTAGTCAGTAACACTCAAATGCAGGCCAGTGCCCTGCCTCCTACCCCTTCAGAGTGTCCTGAG
      ValGluGluGlyAlaGluGlyAspGlyValValSerAsnThrGlnMetGlnAlaSerAlaLeuProProThrProSerGluCysProGlu  460

1381 -GCCCAAAAGGATGGGAATGGGCCAGAGGACTCAAACAGCCAGGTTGGGGCAGAGGATTCCAAAAGCCAGGTGGGGCCGGAGGATCCAAAC
      AlaGlnLysAspGlyAsnGlyProGluAspSerAsnSerGlnValGlyAlaGluAspSerLysSerGlnValGlyProGluAspProAsn  490

1471 -AGCCAGGTGGGGCTGGAGGACCCAAACAGCCAGGTCGGGCCAGAGGACCCAAACAGCCAGGTCGGGCCAGAGGACCCAAACAGCCAGGTC
      SerGlnValGlyLeuGluAspProAsnSerGlnValGlyProGluAspProAsnSerGlnValGlyProGluAspProAsnSerGlnVal  520

1561 -GGGCCAGAGGACCCAAACAGCCAGGTCGGGCCAGAGGACCCAAACAGCCAGGTGGTGGGGCCAGAGCAAGCTGCCTCTAAGAGCCCTGTG
      GlyProGluAspProAsnSerGlnValGlyProGluAspProAsnSerGlnValValGlyProGluGlnAlaAlaSerLysSerProVal  550

1651 -GAGGACCCTGACTCTGACACTATGGGAACCTCAGTGGATGAGTCAGAGGAGTTGGCAAGGATTGAGGCCTCTGCTGAACCCCCAAAGCCT
      GluAspProAspSerAspThrMetGlyThrSerValAspGluSerGluGluLeuAlaArgIleGluAlaSerAlaGluProProLysPro  580

1741 -TAGAGGTGCATCTCAGTCCTACTCAGCCCACTGCAGGGGGTTTCTGAGTCCAGAGCTCTGCCGTAGGCTCTTCTTGGTGCCCCACAGTGC
1831 -TGGCCTCTCCCTAGTGGTCACTGAGGTGGCCACCAGAGGGACTGAGGCCCTGCCCTCAGGGAAGGCCAAGGCCTTCAGAACCCTCCTTAC
1921 -CTCACTGTGTCCTCCTCCACTGCCCTCTGAGCCCTGCGTTGTGATCAGACCCTAAGGGTCTAGAGGGAGGGGCCTCTTCATTAGTCTGGT
2011 -GCCAAGTGAGGCCTTTTCTGAATAAACTCTTTAGACTTTGTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide and amino acid sequences of rat OGFr. 5′- and 3′-untranslated regions are included. Repeats are denoted by single and double underlining.

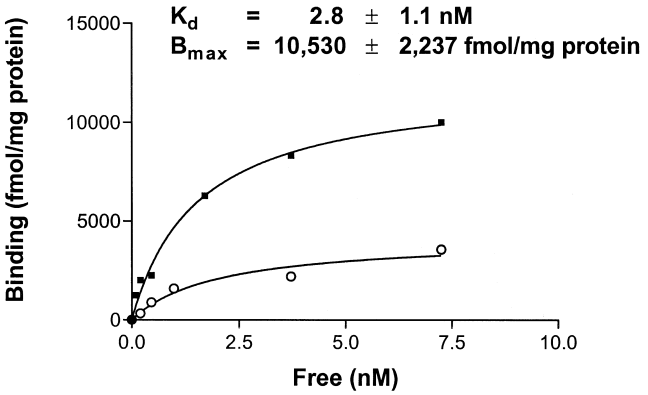

Fig. 3. Representative saturation isotherm of specific binding of [^{3}H]-[Met^5]enkephalin (■) to purified fusion protein translated in vitro from the rat OGFr cDNA clone #12. Mean ± S.E. binding affinity (K_d) for six assays was 2.8 ± 1.1 nM and binding capacity (B_{max}) was 10,530 ± 2237 fmol/mg protein. Binding was significantly reduced with the addition of 1 μM concentrations of the opioid antagonist NTX (○). Representative Scatchard plot of specific binding of radiolabeled [Met^5]enkephalin revealed a one-site model of binding (data not shown).

6-day old and adult animals were frozen and sectioned. Adjacent sections were stained with antibodies to 14-GST fusion protein or with polyclonal antibodies to the OGF binding protein. Controls included staining sections with primary antibodies preabsorbed with either 14-GST fusion protein or with OGF binding protein, as well as secondary antibodies only.

2.9. Northern blot analysis

Northern blot analysis was performed according to McLaughlin and Allar [10]. In brief, RNA was isolated from 6-day old and adult rat tissues. Membranes were hybridized at 42°C for 16–18 h in fresh prehybridization buffer containing 10^6 c.p.m./ml of random prime labeled clone #14 cDNA. Filters were subjected to final washes at 60°C with 0.1 × SSC containing 0.1% SDS for 30 min, wrapped in plastic while wet, and exposed to autoradiography film with intensifying screens for 2–4 days at −70°C. To control for differences in the amount of RNA loaded, as well as the integrity of RNA, blots were stripped and probed with [^{32}P]-labeled cDNA for G3PDH.

2.10. Functional experiments using antisense strategy

In order to study the function of the isolated cDNA with respect to growth, a 23-mer antisense S-ODN (nuclease-resistant phosphorothioate; Oligo, Etc., Bethel, ME) targeted against a sequence containing the translation initiation site of the OGF binding protein was designed and added to cultures of IEC-6 rat small intestinal epithelial cells. The sequences were 5′-GACTCAGGGACTTAGCTTCATCC-3′ (antisense) and 5′-ATAGATACTACGCCGGCTG-TCCT-3′ (scrambled).

The IEC-6 rat small intestine epithelial cell line (American Type Tissue Culture Collection, Manassas, VA) was grown in Dulbecco's medium supplemented with 10% fetal calf serum. For experiments, 10^5 cells/well in a 24-well plate were seeded and, 24 h later, 10^{-6} M concentrations of the antisense and scrambled S-ODNs were added. Some wells were exposed to 10^{-6} M NTX or an equivalent volume of sterile water. Media, S-ODNs or drugs were changed daily. After 72 h in culture, cells were trypsinized, stained with trypan blue and counted with a hemacytometer; 3 wells/treatment group were assessed.

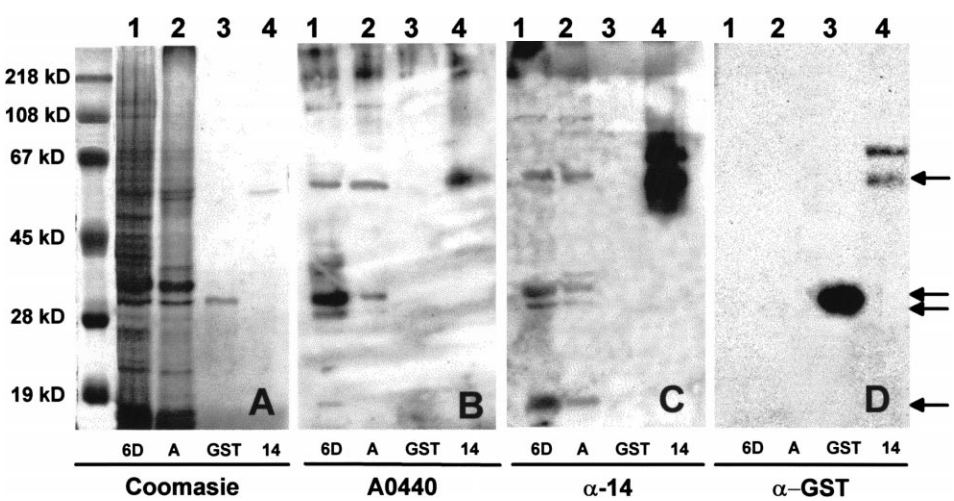

Fig. 4. Six-day old (lane 1) and adult (lane 2) rat cerebellar nuclear proteins, native GST protein (lane 3), and recombinant GST-14e protein (lane 4) were separated by SDS-PAGE and electrotransferred to nitrocellulose. (A) Coomassie blue stained gel of the electrophoresed proteins. (B–D) Western blots stained with polyclonal antibody generated against a 17-kDa OGF binding protein (B), antibody made to fusion protein 14e (C), or antibody to GST (D). Note that the staining patterns are similar in panels (B) and (C). The blot in panel (D) demonstrates the specificity of the fusion protein antibody. The control antibody to GST detected GST and GST-fusion proteins, but not native nuclear homogenate. Arrows indicate the 62, 32, 30 and 17/16 kDa OGF binding proteins.

Data from the antisense experiments were evaluated with ANOVA, and subsequent comparisons made with the Newman–Keuls' tests.

3. Results

3.1. Isolation of cDNA clone #14 by screening a fetal rat brain expression library

Out of approximately 1×10^6 recombinant plaques screened from a cDNA expression library of an 18-day old fetal rat brain, 32 reacted positively to the BO461 antiserum, of which four were purified and subjected to restriction digestion. One of these plaques, designated clone #14, was characterized further. The 1000-bp cDNA insert from clone #14 was labeled with [^{32}P]dCTP and used to probe Northern blots of total RNA isolated from 6-day old and adult rat cerebellum. As shown in Fig. 1, the cDNA probe detected a species of rat mRNA that was 2.1 kb, expressed abundantly in 6-day old cerebellum and brain, but at low levels (three-fold less than at 6 days) in adult cerebellum.

3.2. cDNA sequence analysis

Since the #14 clone was not full-length, labeled #14 cDNA was used as a hybridization probe to screen the λgt11 fetal rat brain library. Thirteen positive clones were identified and purified from the library by colony hybridization. Digestion of the purified clones with *Eco*R1 released a full size insert of 2.1 kb from clone #12. The #14 and #12 cDNAs were sequenced in both directions. Fig. 2 shows the nucleotide sequence, and the deduced amino acid sequence, of the full-length cDNA, #12; 5′- and 3′-untranslated regions have been included. The open reading frame was found to encode a protein of 580 amino acids, with eight imperfect repeat units of nine amino acids at positions 467 to 538. The molecular weight as calculated from the sequence is 58 kDa. Search of the sequences in GenBank revealed no homology to this cDNA.

3.3. Binding assays

To confirm that we have cloned the cDNA for the OGF binding protein, fusion proteins derived from clone #12 were used in receptor binding analysis with [^{3}H]-[Met^5]enkephalin (Fig. 3). Specific and saturable binding was observed, with a mean binding affinity (K_d) of 2.8 ± 1.1 nM and binding capacity (B_{max}) of $10{,}530 \pm 2237$ fmol/mg protein. Addition of NTX to the preparations significantly reduced specific and saturable binding, with reductions in B_{max} of 83% noted (Fig. 3). Representative Scatchard plot of specific binding of radiolabeled [Met^5]enkephalin revealed a one-site model of binding (data not shown).

Using a variety of ligands that recognized classical opioid receptors, no competitive binding ($> 10^{-3}$ M) for radiolabeled [Met^5]enkephalin by DAMGO or morphine sulfate (μ-receptor), DPDPE (δ-receptor), dynorphin A1–8 and U69,583 (κ-receptor) was observed (data not shown).

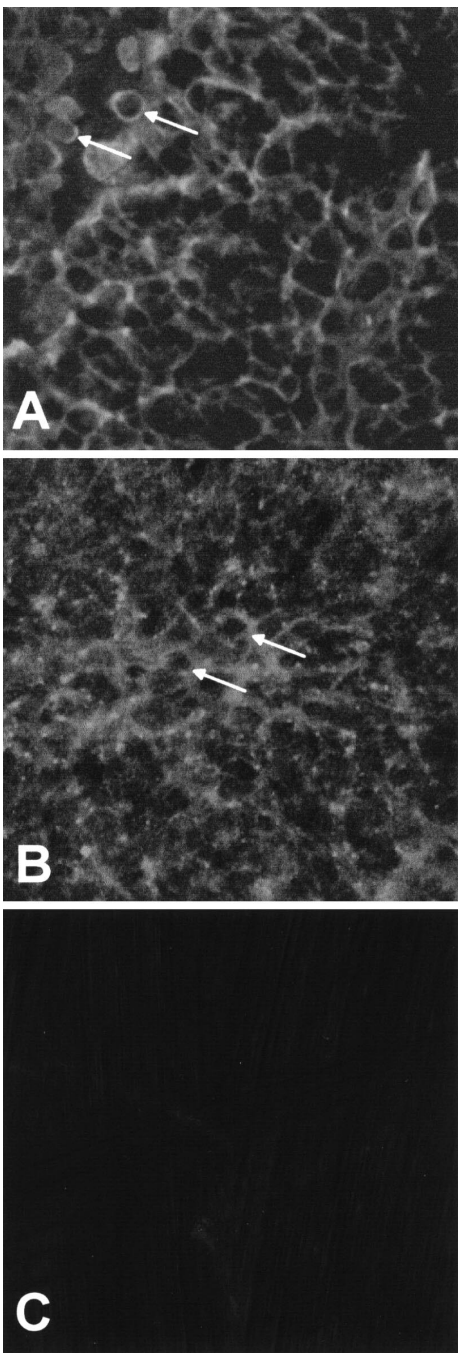

Fig. 5. Distribution of OGFr in external germinal cells in adjacent midsagittal sections of 6-day old rat cerebellum (A, B) as detected by an antibody to the fusion protein 14e (A) or an antibody to the native 17-kDa OGF binding protein (B). Internal granule cells in adult rat cerebellar sections stained with antibody to the fusion protein (14e) (C) or the 17-kDa binding subunit of the ζ-receptor) (data not shown) revealed no immunoreactivity. Arrows = immunoreactivity. Bar = 50 μm.

3.4. Western blotting and immunocytochemistry

Antibodies to the recombinant fusion protein derived from clone #14 were titered and 1:1000 dilutions detected 10 ng of fusion protein. When reacted with nuclear preparations of 6-day old cerebellum in a one-dimensional Western blot, anti-14 GST recognized five polypeptides: 62, 32, 30, 17 and 16 kDa, as well as the recombinant protein (Fig. 4). Western blots stained with antibodies generated against the native 32-kDa binding protein detected the 62, 32, 30, 17 and 16 kDa polypeptides, in addition to the recombinant protein (Fig. 4). The antibody to the recombinant fusion protein or the native 32-kDa polypeptide stained homogenates of the adult rat cerebellum, but was of a notably lesser density than in the 6-day specimen (Fig. 4).

The staining pattern in immunocytochemical preparations employing antibodies to the recombinant fusion protein was similar to that observed when using antibodies to the authentic binding protein (Fig. 5). Both antibodies revealed immunoreactivity in the 6-day old rat cerebellum, with cells of the external germinal layer exhibiting prominent staining of the cytoplasm and low reactivity of the nucleoplasm. The internal granule cells of adult rat cerebellar tissues demonstrated little specific immunoreactivity with either antibody.

3.5. Antisense studies

In order to study the function of the isolated cDNA, a 23-mer antisense S-ODN (nuclease-resistant phosphorothioate) targeted against a sequence containing the translation site of the OGF binding protein was designed and added to log-phase cultures of IEC-6 rat intestinal epithelial cells (Fig. 6). The antisense S-ODN elevated cell number by 294% from control cultures within 48 h of exposure. The cell cultures treated with the scrambled probe were similar in growth to control levels. Cultures treated with NTX, an antagonist to the opioid binding protein, also demonstrated increased cell growth from control values; the NTX group had 223% more cells than control cultures. The cells treated with scrambled message were comparable to controls in cell number.

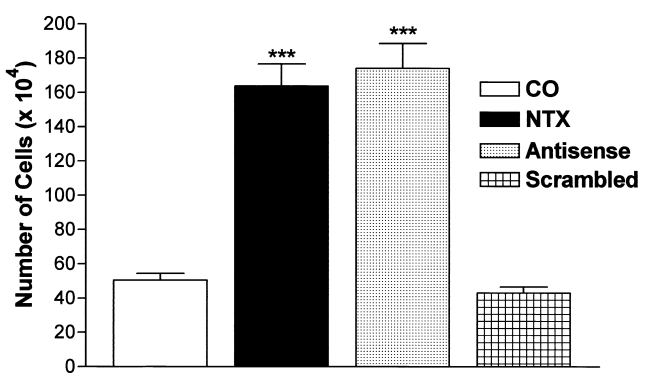

Fig. 6. Histogram of cell number in cultures of IEC-6 rat intestinal epithelial cells treated for 48 h with either sterile water (CO), 10^{-6} M NTX, 10^{-6} M 23-mer S-ODN (antisense), or scrambled oligoprobe (scrambled). Cells (10^5) were plated and compounds and fresh media added 24 and 48 h later. After 72 h in culture, cells were trypsinized, stained with trypan blue, and counted with a hemacytometer. Significantly different from CO at $p < 0.001$ (***).

4. Discussion

The role of endogenous opioid peptides as growth factors was postulated and supported by data emanating from experiments conducted with the opioid antagonist, NTX as early as 1983 [20,21]. For the last decade, the pentapeptide OGF has been identified as the native opioid involved in events related to growth [7,9,13,17,18, 22,23,25,29–31]. Pharmacological and physiological experiments indicated that OGF transduces its effects by a receptor mediated mechanism [25–27], but the receptor appeared to differ from classical opioid receptors such as μ, δ and κ [3,4,8,19]. Thus, the question has remained as to the nature of the receptor associated with OGF. For the first time, the molecular organization of this novel receptor has been elucidated. A number of pieces of evidence support the conclusion that we have cloned and sequenced the receptor for OGF (OGFr). First, pharmacological studies showed that the translated protein for the OGFr exhibited high-affinity binding that was specific, saturable, and consistent with a one-site model of binding. This binding site could be displaced by the opioid antagonist, NTX, suggesting that the binding reaction was at the level of an opioid-like receptor. Binding to an endogenous opioid peptide, OGF, was recorded but synthetic and natural ligands for classic receptors as μ, δ and κ were not competitive, indicating that OGF had special properties in recognizing the translated protein. These pharmacological characteristics of the protein translated from the cDNA identified for the OGFr are consistent with those reported in earlier studies with cells and tissues [25–27]. Second, the temporal appearance of the mRNA and the translated protein of the OGFr revealed greater expression in developing neural tissues than adult counterparts. Moreover, the temporal patterns of gene and protein expression were directly related. These findings about the prevalence of the receptor in developing rather than in postmitotic cells in adult tissue are consonant with information in earlier reports showing that OGF activity was targeted to cells that were developing, carcinogenic, renewing, or repairing [7,9,13,17,18,23,22,25,29,31]. Third, the spatial distribution of the OGFr showed that it was associated with developing neural cells but not their adult counterparts. Subcellular fractionation studies and immunocytochemical findings in cells and tissues have recorded a similar structural relationship [7,9,26–28], and the observation that

staining of the OGFr was associated with the cytoplasm of the cells indicates that the antibody is recognizing sites of synthesis as well as assembly. Fourth, antibodies to the OGFr recognized the 4 binding polypeptides/proteolytic fragments (i.e., 32, 30, 17 and 16 kDa) observed earlier [24,28], as well as a 62-kDa protein consistent with the protein resulting from the translation of the open reading frame. The 62-kDa protein was sometimes seen in preparations of native homogenates (unpublished observations), but with the elimination of β-mercaptoethanol contamination this band is now consistently visible. Whether some or all of the binding polypeptides (i.e., 32, 30, 17 and 16 kDa) are proteolytic products or the result of processing of the 62-kDa protein is presently unclear. Fifth, cell cultures treated with antisense oligonucleotides to the OGFr exhibited marked increases in the number of cells in contrast to control cultures. The magnitude of change in cell number invoked by addition of the antisense oligonucleotides was of the order occurring when endogenous opioid peptides were blocked from interacting with their receptors using NTX, suggesting that only the receptor now identified for OGF was involved with growth. Taken together these results are consistent with those in earlier reports as to the biology of OGF and its receptor [7,9,23,25–28,31], and argue in a persuasive fashion that we have identified the molecular organization of the receptor for the opioid growth peptide.

The OGFr has been tentatively identified earlier in pharmacological and physiological studies as the ζ-receptor [24,26–28]. This receptor was so named to distinguish its special characteristics such as function (i.e., growth), temporal expression (i.e., transient appearance during ontogeny in the nervous system), ligand specificity (i.e., [Met^5]enkephalin), and subcellular location (i.e., nucleus) from other classical other opioid receptors. Moreover, the ζ-receptor did share some of the pharmacological and physiological hallmarks of other opioid receptors such as reversibility by naloxone and stereospecificity. However, comparison of the molecular information in this study with the sequences of those reported for the opioid receptor family (μ, δ, κ and ORL/nociceptin/orphanin FQ) [4,8,12,19] shows no structural homology. Furthermore, although the OGFr has some pharmacological similarities with classical opioid receptors, it lacks such characteristics as a coupling through pertussis toxin-sensitive G-proteins, the similar general structure of an extracellular N-terminal region, seven transmembrane domains and intracellular C-terminal tail structure [4,8,19]. Thus, in view of such pharmacological, physiological and molecular differences we propose that the receptor tentatively identified as the ζ-receptor be termed the OGFr.

Given the extensive documentation of the function of OGFr as a mediator of OGF action in a wide variety of biological processes (e.g., development, homeostasis and cancer), the significance of alterations of OGFr with respect to disturbing biological events along with clinical repercussions should be noted. For example, if the OGFr is compromised either at the transcriptional and/or translational level(s), OGF will be restricted in function. This would be analogous to experiments performed herein with NTX to block opioid–receptor interaction, as well as with antisense oligonucleotides, whereby cells exhibit a supranormal increase in replication. Earlier studies have shown that animals with xenografts of human and animal tumors and given an opioid antagonist to block opioid peptide interfacing with endogenous opioids have an acceleration in tumorigenesis, including an increase in the incidence of cancer and a decrease in survival [20,25]. Wounded tissues exposed to NTX have an accelerated wound healing process [31], thereby demonstrating the harnessing of the endogenous opioid system to improve well-being. If OGFr is enhanced by transcriptional and/or translational events OGF function will be expected to be amplified. This may be of benefit in the case of cancer as observed in other reports [25,29], but on the other hand could be detrimental to development, angiogenesis and wound healing [25,30]. Thus, the profound influence of OGF and OGFr in biomedical science now has a molecular validity for further studies.

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