Expression and Characterization of the Extracellular Amino-Terminal Domain of the mGluR4 Subtype Metabotropic Glutamate Receptor

by

Guangming Han

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmaceutical Science
University of Toronto

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Expression and Characterization of the Extracellular Amino-Terminal Domain of the mGluR4 Subtype Metabotropic Glutamate Receptor

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Abstract

The mGluR4 subtype metabotropic glutamate receptor is a presynaptic receptor that modulates neurotransmitter release. We hypothesized that expressing the extracellular amino terminal domain (ATD) of mGluR4 would produce a soluble protein that retains the pharmacological characteristics of mGluR4.

In this study, I expressed and characterized 4 truncated versions of mGluR4. A receptor terminated 39 amino acids upstream of the first putative transmembrane domain (TMD) was secreted into the culture media of transfected human embryonic kidney cells. The rank order of potency of metabotropic ligands at this receptor was similar to that of the full-length receptor. Receptors truncated at the carboxyl-terminus of the ATD and 98 or 174 amino acids upstream from the first TMD all failed to be secreted from the cells and to bind the radioligand. The truncated mGluR4 and the equivalent mGluR8 were expressed in Escherichia coli and insect cells. Both systems produced immuno-reactive recombinant receptors that failed to display ligand binding activity.

Together, these results demonstrate that all of the LIVBP homology region and part of the cysteine-rich region are required for optimal secretion in a soluble form that retains binding activity. The successful expression of a soluble truncated mGluR4 receptor produced a useful substrate for the crystallization of the mGluR4 ligand binding domain, and may provide information for the design of subtype specific ligands for mGluRs.
Acknowledgements

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1S, 3R-ACPD</td>
<td>(1S, 3R)-aminocyclopentanedicarboxylate</td>
</tr>
<tr>
<td>1S, 3S-ACPD</td>
<td>(1S, 3S)-aminocyclopentanedicarboxylate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3 hydroxy-5-methyl-isoxazole-4-propionate</td>
</tr>
<tr>
<td>ATD</td>
<td>amino terminal domain</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>maximum number of binding sites</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaR</td>
<td>calcium-sensing receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPCCOEt</td>
<td>7-hydroxyiminocyclopropan[β]chromen-1α-carboxylic acid ethyl ester</td>
</tr>
<tr>
<td>CPPG</td>
<td>(R,S) α-cyclopropyl-4-phosphonophenylglycine</td>
</tr>
<tr>
<td>Cyclobutylene-AP5</td>
<td>(R,S) 1-amino-3-(phosphonomethylene)cyclobutane</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra acetic acid disodium salt</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglyco-bis-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt;R</td>
<td>type B γ-aminobutyric acid receptor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic glutamate receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>L-AP4</td>
<td>L-amino-4-phosphonobutyric acid</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>L-CCG-1</td>
<td>(2S, 2'S, 1'S)-2-(carboxycyclopropyl)glycine</td>
</tr>
<tr>
<td>LIVBP</td>
<td>leucine-isoleucine-valine binding protein</td>
</tr>
<tr>
<td>L-SOP</td>
<td>L-serine-O- phosphate</td>
</tr>
<tr>
<td>MAP4</td>
<td>(S)-2-amino-4-phosphonobutyric acid</td>
</tr>
<tr>
<td>MCPG</td>
<td>(R,S) α-methyl-4-carboxy-phenylglycine</td>
</tr>
<tr>
<td>M&lt;sub&gt;r&lt;/sub&gt;</td>
<td>relative molecular weight</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MPPG</td>
<td>(R,S) α-methyl-4-phosphonophenylglycine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PBP</td>
<td>periplasmic binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>S.E.M</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
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1.0 Introduction

1.1 Classification of Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) are a group of guanine nucleotide binding protein (G protein)-coupled neurotransmitter receptors (GPCRs) that are activated by the neurotransmitter L-glutamate. L-glutamate is the major excitatory neurotransmitter in mammalian central nervous system (CNS) and plays important roles in a wide variety of CNS functions. L-glutamate activates two different groups of receptors in the CNS: the ionotropic glutamate receptors (iGluRs), and the mGluRs. The family of iGluRs is comprised of the N-methyl-D-aspartate (NMDA) receptors, the α-amino-3-hydroxy-5-methyl-isoxazole-4-propioate (AMPA) receptors, and the kainate receptors. They act as ligand-gated cation channels to mediate fast excitatory synaptic transmission. The mGluRs, on the other hand, are involved in modulating different transduction pathways, including the activation of phospholipase C and the inhibition of adenyl cyclase activity.

Sequence comparisons have revealed the existence of at least three major families of GPCRs. Receptors homologous to rhodopsin receptor constitute family 1. Family 2 receptors include receptors for vasoactive intestinal peptide and glucagon. The mGluRs, the type B γ-aminobutyric acid receptors (GABA_{B}Rs), the parathyroid calcium sensing receptor (CaR), a class of pheromone receptors and a new class of taste receptors (Brown et al. 1993; Kaupmann et al., 1997; Matsunami and Buck, 1997; Hoon et al. 1999) comprise the family 3 GPCR (Bockaert and Pin, 1999). This receptor family is characterized by a large (~ 600 residues in the mGluRs) extracellular amino-terminal domain (ATD) (Figure 1). Similar to other GPCRs, the family 3 receptors contain an extracellular ATD, seven putative transmembrane domains (TMDs) and an
Figure 1  Schematic representation of the proposed structure of an mGluR protein. The region rich in cysteine residues is indicated with black circles. The segment in the second intracellular loop that is important for G-protein coupling specificity is indicated in black.
Cysteine rich 7-TMD G-protein coupling

LIVBP-like region

Cysteine rich region

7-TMD G-protein coupling

C-terminal Tail
intracellular carboxyl-terminal domain (Figure 1). However, they do not share sequence homology with receptors from the other families. The overall sequence identity between mGluRs, CaRs and GABA<sub>B</sub>Rs is about 20% (Figure 2). The mGluRs and the CaRs also share a highly conserved cysteine-rich region in the ATD, whereas the GABA<sub>B</sub>Rs lack this region.

The cDNA of the first member of mGluR family, the mGluR1, was cloned independently by two groups in 1991 (Houamed et al., 1991; Masu et al., 1991). To date, eight members of the mGluR family have been cloned. They have been classified into three subgroups according to their sequence homology, pharmacological profiles, and signal transduction mechanisms (Conn and Pin, 1997). The group I mGluRs include mGluR1 and mGluR5, the group II mGluRs include mGluR2 and mGluR3, and the group III mGluRs include mGluR4, mGluR6, mGluR7 and mGluR8. These mGluRs share roughly 45% sequence homology among the subgroups, whereas the degree of sequence identity increases to about 70% when comparing mGluRs within the same subgroup (Figure 2).

The group I mGluRs are coupled to the activation of phospholipase C, resulting in an increase in phosphoinositide turnover and the release of Ca<sup>2+</sup> from internal stores (Conn et al., 1995). A recent study suggested that in addition to acting through transduction cascades involving G-proteins, mGluR1 is also able to mediate G-protein independent signal pathway through a Src-family protein tyrosine kinase (Heuss et al., 1999). The splice variants of group I mGluRs include mGluR1a, 1b, 1c, 1d, 1f, and mGluR5a and 5b. Recently, a novel splice variant of mGluR1 (mGluR1E55) was cloned from the mouse CNS. The predicted protein product of mGluR1E55 contains only the extracellular domain of the receptor and may be secreted (Zhu et al., 1999). The function of this receptor remains unknown as it contains no TMD or carboxyl-terminal domain, and cannot couple to G-protein.
Figure 2  Dendrogram and classification of the members of the mGluR family, together with the parathyroid Ca\(^{2+}\)-sensing receptor (PCaR1) and type B GABA receptor (GABA\(_B\)R1a).
The group II mGluRs are negatively coupled to adenylyl cyclase and the production of cyclic adenosine monophosphate (cAMP) through a pertussis toxin-sensitive G-protein (G\(_i/G_o\)) when heterologously expressed in cell lines (Thomsen et al., 1997; Flor et al., 1997; Corti, et al. 1998). There is no evidence for the existence of splice variants of mGluR2 and mGluR3 to date. The group III mGluRs and their splice variants (mGluR4a, 4b, mGluR6, mGluR7a, 7b, mGluR8a and 8b) are also negatively coupled to adenylyl cyclase and the formation of cAMP through a G\(_i/G_o\) protein. One exception is the newly cloned human mGluR8c (Malherbe et al., 1999). The mGluR8c cDNA contains a 74-bp out-of-frame insertion which results in a unique 49 amino acid carboxyl terminus inserted at amino acid 452 in the ATD. Similar to mouse mGluR1E55, human mGluR8c appears to be a truncated receptor that lacks the seven putative TMDs and the carboxyl-terminal domain, and may exist as a soluble form of the receptor.

1.2 Pharmacology of Group III mGluRs

The three groups of mGluRs differ considerably in their ligand selectivity. Group I receptors are selectively activated by agonists quisqualate and 3,5-dihydroxyphenylglycine. Group II receptors show high affinity for the agonists (2S, 1'R, 2'R, 3'R)-2-(2,3-dicarboxycyclopropyl)glycine, (2S, 2'S, 1'S)-2-(carboxycyclopropyl)glycine (L-CCG-1), and 1'S, 3S aminocyclopentanedicarboxylate (1S, 3S-ACPD) (Pin et al., 1999).

The group III mGluRs are characterized pharmacologically by their high affinity towards the agonists L-amino-4-phosphonobutyric acid (L-AP4) and L-serine-O-phosphate (L-SOP) (Tanabe et al., 1993; Brabet et al., 1998). Both L-AP4 and L-SOP are structurally similar to L-glutamate, with the \(\gamma\)-carboxyl group of glutamate being substituted for a phosphonate group (Figure 3). Both L-AP4 and L-SOP are very specific for group III mGluRs, as they are inactive at other
Figure 3  Chemical structures of group III mGluR agonists and antagonists used in this study.
Agonists for Group III mGluRs

- L-Glutamic Acid
- L-AP4
- L-SOP
- Cyclobutane AP5
- PPG

Antagonists for Group III mGluRs

- MAP4
- CPPG
- MPPG
mGluRs. A number of other group III mGluR ligands appear to be active at group I and/or II mGluRs as well. For example, although α-methyl-L-amino-4-phosphonobutyrate (MAP4) is a more selective mGluR4 antagonist (Figure 3, Table 1), it is also antagonist with low potency on mGluR2 (Johansen and Robinson, 1995; Gomeza et al., 1996). Most mGluR1 and mGluR2 antagonists are inactive on group III mGluRs, except (R, S) α-methyl-4-phosphonophenylglycine (MPPG), which appears to be slightly more potent to mGluR4 than to mGluR2 (Gomeza et al., 1996A).

The pharmacological profiles of the mGluRs have been established using functional assays that measure the accumulation or inhibition of second messenger molecules. Inhibition of cAMP formation is the most commonly used assay for the characterization of the group II and III mGluRs. An alternative method involves co-transfection of receptors with chimeric G proteins and the measurement of phosphoinositides or intracellular calcium levels (Gomeza et al., 1996A; Hampson et al., 1999). Studies using the functional assays have established a rank order of potency for agonists at the group III mGluRs of L-AP4 > L-SOP > L-glutamate > L-CCG-I > (1S, 3S)-ACPD >> (1S, 3R)-ACPD. Interestingly, functional assays measuring the inhibition of cAMP revealed that the agonist potencies of mGluR7 were almost 1,000 times lower than the other group III mGluRs (Okamoto et al., 1994; Saugstad et al., 1994), despite the 70% sequence homology shared by the group III receptors. This suggests that mGluR7 may either bind ligand with a much lower affinity or transduce its activation signal with greatly reduced efficacy as compared with the other group III mGluRs. Sequence comparison between mGluR7 and the other group III mGluRs may therefore reveal key amino acid residues involved in the ligand binding or signal transduction process of the group III mGluRs.
Although L-AP4 and L-SOP are specific for group III receptors, high potency and subtype-specific ligands for individual group III receptors have yet to be developed. An analogue of AMPA, homo-AMPA has been shown to be specific for mGluR6. However, the potency of this agonist is 4-fold lower than that of L-glutamate (Bräuner-Osborne et al., 1996). Therefore, the search for subtype-selective, high affinity ligand for group III mGluRs is still an ongoing endeavor. A number of new group III mGluR agonists and antagonists are now available for testing, including the selective and potent agonist (R, S)-4-phosphonophenylglycine (PPG) (Gasparini et al., 1999) and the antagonist (R, S)-α-cyclopropyl-4-phosphonophenylglycine (CPPG).

1.3 Distributions of Group III mGluRs

The group III mGluRs are widely distributed throughout the CNS, with the exception of mGluR6, which is expressed exclusively in the retina. Immunocytochemical studies have shown that the mGluR4a protein is expressed in discrete neuronal populations in the rat and mouse CNS, with the highest level found in the cerebellum (Kinoshita et al., 1996; Mateos et al., 1998). A study on mGluR4 knock-out (KO) mice using $[^3]$H-L-AP4 autoradiography also confirmed the presence of high levels of mGluR4 protein expression in the cerebellum (Thomsen and Hampson, 1999). Mice lacking mGluR4 displayed a significant decrease in $[^3]$H-L-AP4 binding compared with the wild-type mice in the molecular layer of the cerebellum. mGluR4 is also expressed in the hippocampus, cerebral cortex, olfactory bulb, striatum, piriform cortex/amygdala, and thalamus (Risso Bradley et al., 1996; Phillips et al., 1997; Risso Bradley et al., 1999). Within the hippocampus, moderate mGluR4a expression is present in the molecular layer of the dentate gyrus, while in the cerebral cortex mGluR4a expression is distributed evenly throughout
the cortex at the interface of cortical layers IV and V (Shigemoto et al., 1997; Phillips et al., 1997). No study on mGluR4b protein expression has been reported to date.

The expression of mGluR6 protein is restricted to a post-synaptic site on rod bipolar cells of the inner nuclear layer of the retina. It is suggested that this specific localization of mGluR6 is mediated by a 9.5 kilobase sequence located upstream from the translational initiation site on the mGluR6 gene (Ueda et al., 1997). This is based on experiments using transgenic mice with a β-galactosidase reporter gene fused to this 9.5 kilobase fragment. In these mice, expression of the enzyme was found restricted to the retinal bipolar cells.

The mGluR7a is the most abundant group III mGluR in the brain. It is widely distributed throughout the CNS, with the highest levels in the olfactory bulb, hippocampus, and cerebral cortex of the adult rat. The mGluR7a is the principal member of the group III mGluRs expressed in the hippocampus and is labeled with high intensity in the CA1, CA3 and dentate gyrus regions (Shigemoto et al., 1997; Kinoshita et al., 1998). Moderate levels of mGluR7a are found in the amygdala, basal ganglia, superior colliculus an spinal cord and lower levels in the thalamus and hypothalamus (Ohishi et al., 1995; Kinoshita et al., 1998). The mGluR7b protein is expressed in a limited fashion and is almost always co-localized with the mGluR7a (Kinoshita et al., 1998). The functional significance of the co-localization is unclear, and no evidence for the direct interaction between mGluR7a and mGluR7b has been reported to date.

mGluR8 protein is expressed most prominently in the olfactory bulb, pontine grey, and the lateral reticular nucleus of the thalamus (Duvoisin et al., 1995; Saugstad et al., 1997). Lower levels of mGluR8 expression were found in the rat cerebral cortex, cerebellum, hippocampus, and mammillary body (Saugstad et al., 1997). In the hippocampus, mGluR8a shows prominent expression in the molecular layer of the dentate gyrus and the terminal zone of the lateral
perforant path in the CA3 region (Shigemoto et al., 1997). Detailed immunohistochemistry studies on mGluR8b have yet to be conducted.

Except for mGluR6, which is expressed post-synaptically in the retina, immunohistochemical studies have shown that mGluR4, mGluR7, and mGluR8 are primarily located presynaptically at asymmetric glutamatergic synapses (Shigemoto et al., 1997). In addition, the presynaptic localization of mGluR4, mGluR7 and mGluR8 is also observed at the inhibitory GABAergic synapses in various regions in the brain (Salt et al., 1996; Risso Bradley et al., 1996; Kinoshita et al., 1998).

1.4 Possible Physiological Function of Group III mGluRs

Activation of group III mGluRs has been shown to inhibit adenylyl cyclase activity and cAMP formation in neuronal cultures (Prézeau et al., 1994; Bruno et al., 1995). The negative coupling to cAMP formation, together with their presynaptic localization at glutamatergic synapses, suggests a role for mGluR4, 7 and 8 as autoreceptors in regulating glutamate release from nerve terminals. Furthermore, agonists at group III mGluRs have been shown to inhibit voltage-gated Ca$^{2+}$ channels in cultured olfactory bulb neurons, cultured hippocampal cells, and in isolated pyramidal neurons (Sahara and Westbrook, 1993; Trombley and Westbrook, 1992; Stefani et al., 1998). Evidence indicates that the inhibition of Ca$^{2+}$ currents is likely due to interactions between the G-proteins and the Ca$^{2+}$ channels, and is not dependent on protein kinase A (Herrero et al., 1996). Since voltage-gated Ca$^{2+}$ currents are known to play an important regulatory role in the modulation of central synaptic transmission, these observations fit well with the hypothesis that group III mGluRs function as presynaptic autoreceptors. Indeed,
group III mGluR agonists have been shown to cause a reduction in synaptic transmission at glutamatergic synapses (Attwell et al., 1995; Dietrich et al., 1997).

Studies on KO mice lacking mGluR4 have provided more direct insights on the biological roles played by the receptor. These mice showed normal gross motor performance, but were deficient on the rotating rod motor-learning test, suggesting that mGluR4 KO mice may have an impaired ability to learn complex motor tasks (Pekhletske et al., 1996). An analysis of presynaptic short-term synaptic plasticity at the parallel fiber-Purkinje cell synapse revealed that paired-pulse facilitation and post-tetanic potentiation were impaired in the mutant mice, whereas long-term depression was not impaired (Pekhletske et al., 1996). Taken together, these results suggested an important function of mGluR4 in maintaining synaptic efficacy during repetitive activation. A plausible mechanism is that mGluR4 may act to conserve the synaptic stores of glutamate for release upon repetitive stimulation; in mice lacking the receptor, the stored glutamate would be rapidly depleted during repetitive firing (Pekhletske et al., 1996). The mGluR7 KO mice have been shown to have reduced fear response and a deficit in conditioned taste aversion (Masugi et al., 1999). Since the amygdala is the most likely region being involved in both of the phenotypes, it has been suggested that mGluR7 is critical in amygdala-dependent aversion learning (Masugi et al., 1999).

1.5 Potential Clinical Use of the Group III mGluR Agonists

One of the potential beneficial therapeutic effects of group III mGluR agonists is reduction of neuronal damage that occurs after stroke, traumatic brain injury, or in certain neurodegenerative disorders. Marked increases in the extracellular levels of glutamate are observed following brain or spinal cord trauma and are correlated to injury severity (Faden et al., 1989; Panter et al.,
It is well accepted that the excessive release of glutamate causes neurotoxicity. Activation of iGluRs including the NMDA and AMPA receptors induces large increases in the concentration of neuronal cytosolic free Ca\textsuperscript{2+}, due to Ca\textsuperscript{2+} influx through NMDA receptors and/or secondary activation of voltage-gated Ca\textsuperscript{2+} channels. This will eventually result in neuronal death, via a pathophysiological process commonly known as excitotoxicity (Choi, 1988; Faden et al., 1989; Wrathall et al., 1992).

It has been shown that mGluR4, but not group I or group II mGluR has an increased expression in vulnerable brain areas following global ischemia in rats (Iversen et al., 1994). In addition, reduced level of mGluR4 expression was observed in cerebellar granule neurons undergone apoptotic neuronal death (Borodezt et al., 1998). Both observations suggest the possible involvement of mGluR4 in regulating the downstream signaling events that are elicited following neuronal damage. Consequently, agonist activation of group III mGluRs may inhibit the release of glutamate, and impart neuroprotective properties to these receptors. A number of studies support this hypothesis. Maiese et al. have shown that administration of L-AP4 before nitric oxide exposure significantly increased survival of rat hippocampal neurons in culture (Maiese et al., 1996). Selective group III agonist also have been shown to protect cortical cells or cerebellar granule cells in culture against β-amyloid peptide induced apoptosis (Copani et al., 1995), or cortical neurons exposed to a toxic pulse of NMDA (Bruno et al., 1995). Moreover, treatment of L-AP4 or L-SOP to cortical neuronal/glial cultures subjected to mechanical injury resulted in dose-dependent neuroprotection (Faden et al., 1997).

In addition to their neuroprotective properties in neuronal injury, agonists of the group III mGluRs may also have anti-convulsant function. In support of a regulatory role for the group III mGluRs in epilepsy, an increase in mGluR4 mRNA levels has been reported in rats exhibiting
status epilepticus (Aronica et al., 1997). A recent study indicated that the expression of mGluR4 in hippocampal neurons from patients with chronic mesial temporal lobe epilepsy was upregulated at both mRNA and protein levels (Lie et al., 2000). The upregulation of mGluR4 would likely counteract excessive calcium influx and glutamate release, and subsequently reduce synaptic transmission. Moreover, the sensitivity of the group III mGluRs towards L-AP4 was found to be enhanced in rat with amygdala-kindled seizures (Neugebauer et al., 1997). L-AP4 produced depressions of lateral amygdala evoked monosynaptic excitatory postsynaptic currents (EPSC) in kindled neurons with a EC₅₀ value of 10.8 nM, which was 30 times lower than that of control neurons (297 nM). This observation suggests that during a seizure, the group III mGluRs become more sensitive to stimulation with a given level of glutamate, either due to the changes in receptor affinities or the alterations in second messenger systems. Together, these findings suggest that the group III mGluRs can be useful drug targets in the treatment of epilepsies. In support for this notion, administration of group III mGluR agonists has been shown to protect against both chemically and electrically induced seizures in several rodent models of epilepsy (Abdul-Ghai et al., 1997; Thomsen and Daldy, 1998; Gasparini et al., 1999).

1.6 The Three Structural Domains of mGluRs

The mGluRs are large molecules ranging in size from 95 kDa for mGluR6 to 133 kDa for mGluR1a. The mGluRs have a large extracellular ATD, followed by a hydrophobic region containing seven putative TMDs and an intracellular carboxyl-terminal domain with variable lengths. The ATDs of the mGluRs are highly conserved. There are 17 conserved cysteine residues within the predicted ATD of the mGluRs, some of which likely to form disulfide bonds and play a role in the protein folding and/or signal transduction (Conn and Pin, 1997). One
significant difference between mGluRs and the family 1 and family 2 GPCRs is the location of their ligand binding sites. While the ligand binding regions of most GPCRs reside within their TMDs, ligand binding sites of the mGluRs and the other members of the family 3 GPCRs are contained within the ATD region.

In most conventional GPCRs (family 1), the third intracellular loop plays a critical role in the G-protein coupling specificity. Interestingly, in mGluRs, the second intracellular loop likely plays a role equivalent to that of the third intracellular loop of the other GPCRs. A study on chimeric receptors between the phospholipase C-coupled mGluR1 and the adenylyl cyclase-coupled mGluR3 has shown that the second intracellular loop of mGluRs plays a critical role in G-protein coupling specificity (Gomez et al., 1996B) (Figure 2). Studies also indicated that all intracellular segments are likely involved in the coupling and activation of the G-protein (Gomez et al., 1996B, Pin et al., 1994). The amino acid sequence of the first and third intracellular loops is highly conserved among all the mGluRs and PCaR1, suggesting that these domains play important roles in G-protein activation. Indeed, a mutation in the third intracellular loop of the human CaR1 that prevents the receptor from activating phospholipase C has been found in familial hypocalciuric hypercalcemia patients (Pollak et al., 1993).

In a recent study, 7-hydroxyimino-cyclopropan[b]chromen-1α-carboxylic acid ethyl ester (CPCCOEt) has been shown to act as a noncompetitive mGluR1 antagonist which inhibits receptor signaling without affecting glutamate binding (Litschig et al., 1999). This compound selectively inhibited glutamate-induced increase in intracellular calcium at human mGluR1b with an IC₅₀ of 6.5 μM while having no agonist or antagonist activity at human mGluR2, 4, 5, 7 and 8. It has been suggested that CPCCOEt acts in a noncompetitive manner by decreasing the efficacy of glutamate-stimulated phosphoinositide hydrolysis without affecting the EC₅₀ value of
glutamate. Chimeric and mutagenesis studies have shown that Thr815 and Ala818 of human mGluR1b, located at the extracellular surface of the seventh TMD, are critical in the binding of CPCCOEt. It has been therefore proposed that the interaction of CPCCOEt with Thr815 and Ala818 of mGluR1 disrupts receptor activation by inhibiting an intramolecular interaction between the agonist-bound extracellular ATD and the TMDs and very likely the subsequent intramolecular transduction (Litschig et al., 1999).

The carboxyl-terminal domain of group III mGluRs has been shown to interact with G protein βγ subunits (Gabellini et al., 1993; Prézeau et al., 1996). Moreover, it has been shown that a subdomain in the carboxyl-terminal tail of group III mGluRs binds calmodulin and G protein βγ subunits in a mutually exclusive manner (O'Connor et al., 1999). This result suggests a novel mechanism of presynaptic modulation in which Ca2+-calmodulin is required to release G protein βγ subunits from the carboxyl-terminal domain of group III mGluRs in order to mediate glutamatergic autoinhibition. However, the carboxyl-terminal domain is likely to have other functions. For example, chimeric and deletion studies revealed that axon exclusion of mGluR2 versus axon targeting of mGluR7 is mediated by their carboxyl-terminal domains (Stowell and Craig, 1999). In addition, this domain may also be the target for a number of protein kinases.

1.7 Oligomerization of mGluRs

A unique structural feature shared by all eight mGluRs is the presence of 21 conserved cysteine residues. Seventeen of these are in the ATD and two are in the extracellular loops. Among the seventeen cysteines in the ATD, nine are at the C-terminal portion of the ATD (Figure 2). Although the function of these cysteines is currently unclear, the strict conservation of position implies that their function is shared and important for this family of receptors.
One possible function for these cysteines is mediating dimerization of mGluRs. Under non-reducing condition, mGluR5 expressed in human embryonic kidney (HEK) cells was shown to migrate on polyacrylamide gel as a homodimer but not as a mGluR1/mGluR5 heterodimer (Romano et al., 1996). The existence of dimer under non-reduced condition was also observed in our study on truncated mGluR4 (Han and Hampson, 1999). Co-immunoprecipitation experiments have shown that a mutant mGluR5 receptor truncated after the first TMD was able to retain the ability to form dimer with either itself or the full-length mGluR5, suggesting that the locus for disulfide-mediated dimerization is within the extracellular domain of the receptor. Furthermore, using proteolytic removal of all or part of the extracellular domain by trypsin revealed that the cysteine(s) responsible for disulfide bond formation are in the amino-terminal 17 kDa of mGluR5. In a more recent study, the dimerization of mGluR1a was reported in membranes isolated from both rat brain and transfected BHK cells (Robbins et al., 1999). The dimerization was suggested to form in the endoplasmic reticulum.

Interestingly, these 17 cysteines are also present in the ATD of CaR at equivalent positions. A series of studies have shown that intermolecular interactions between dimeric calcium-sensing receptor monomers are important for its normal function (Bai et al., 1998; Bai et al., 1999;). Moreover, mutagenesis studies indicated that several cysteine residues within the ATD of CaR were critical in the dimerization of the receptor (Pace et al., 1999; Ray et al., 1999). The more distantly related GABA\(_B\)Rs lack the cysteine rich region altogether, and form heterodimer by interactions between their carboxyl-terminal tails (Jones et al., 1998; Kaupmann et al., 1998).

1.8 The ATD and the Ligand Binding Domain of mGluRs
In 1993, O’Hara and colleagues reported low homology between the large ATD of the mGluRs and a group of bacterial proteins called the periplasmic binding proteins (PBPs); the most homologous of the PBPs was the leucine-isolucine-valine binding protein (LIVBP) (O’Hara et al., 1993). A three-dimensional compatibility search that identifies sequences folding into a known structure (Bowie et al., 1991) showed that mGluR1 scored in the top 0.1% of all database sequences queried with a LIVBP profile. A comparison of mGluR1-5 with LIVBP and a number of other PBPs in a multiple alignment indicated that the observed similarity might be biologically meaningful (O’Hara, et al., 1993).

The PBPs are a family of proteins that act as transporters to traffic amino acids and sugars from the periplasmic space across the bacterial inner membrane and into the cells. These proteins have an affinity of approximately 1 μM for their ligands, and function to concentrate important growth substrates inside the cell (Kellerman and Szmelcman, 1974; Ames 1988). The crystal structure of the LIVBP has been resolved. The LIVBP is composed of two globular lobes connected by three short stretches of peptide (the hinge region, Sack et al., 1989). This bi-lobed structure exists in an open and a closed form, and acts through the so-called Venus flytrap mechanism. Upon binding of substrate in the open configuration, the PBP adopts a closed conformation and interacts with a unique set of membrane-bound proteins that catalyzes the actual membrane translocation of the substrate (Adams and Oxender, 1989).

Based on the homology, a three-dimensional structure of mGluR1 ATD sequences that aligned with the available PBPs was modeled. The model has the characteristics of the bacterial PBPs: two large, globular lobes consisting of β sheets flanked by α helices, linked by a hinge region consisting of three interdomain crossover segments. The ligand is thought to interact with a binding site on one of the lobes (O’Hara et al., 1993). The hinge region has been implicated in
the transduction of binding signals. Mutations in close proximity to the hinge region of the parathyroid calcium sensing receptor results in patients exhibiting genetic disease that affected calcium homeostasis, which may due to the loss of the function of the receptor (Pollak et al., 1993, Pollak et al., 1994).

In the PBPs, substrate binding occurs in a cleft and is stabilized by hydrogen bonds. Two ligand-bound forms of PBPs have been observed: an open form with ligand initially bound to one domain, and a closed form in which ligand is bound to both domains and enclosed within the cleft (Sack et al., 1989). In the open form of LIVBP, Ser79 and Thr102 are involved in hydrogen bonding to LIVBP ligands. Arg116, Phe276 and Asp323 are involved in the LIVBP-binding pocket structure (Sack et al., 1989), part of which is a salt bridge between Arg116 and Asp323. Interestingly, these residues align with identical residues in mGluRs.

A number of experiments have been carried out over the last few years providing informative clues about the localization of the L-glutamate binding domain in mGluRs. Takahashi et al. have produced a series of chimeric receptors by exchanging various parts of the ATD of rat mGluR1 to the corresponding segments of mGluR2 (Takahashi et al., 1993). The subsequent pharmacological characterization of these chimeric receptors allowed them to conclude that substitution of up to half of the mGluR2 ATD in the homologous region of mGluR1 ATD is sufficient to convert the pharmacological profile of mGluR1 into that of mGluR2. Data obtained from experiments on chimeric constructs of the ATD of human mGluR4 with the TMDs and carboxyl-terminal regions of mGluR1b also indicated that pharmacological selectivity is conferred by residues located in the ATDs of mGluRs (Tones et al., 1995). In another approach, polyclonal antibodies against two different portions of the ATD of rat mGluR1 was shown to
inhibit the action of L-glutamate on the stimulation of phosphatidylinositol hydrolysis when tested on CHO cells stably expressing mGluR1 (Shigemoto et al., 1994).

In the study conducted by O'Hara et al. (1993), site-directed mutagenesis was used to probe the mGluR1 binding pocket for residues that are essential for ligand binding. As mentioned previously, the amino acid residue Ser79 and Thr102 side chain atoms in the LIVBP are known to form a hydrogen bond with the \(\alpha\)-ammonium and \(\alpha\)-carboxylate atoms of the glutamate ligand (Sack et al. 1989). Alignment of the LIVBP with mGluR1 shows that Ser165 and Thr188 residues of mGluR1 correspond to Ser79 and Thr102 in the LIVBP. Single point mutations changing either Ser165 or Thr188 in mGluR1 to alanine resulted in significant decrease in affinity of mGluR1 for its ligand. No functional response was observed with the T188A mutant, and the affinity of mGluR1 for both glutamate and quisqualate was reduced by over 10,000-fold for the S165A/T188A double mutant (O'Hara et al., 1993).

In a more recent study, Hampson et al. (1999) used a molecular model of the tertiary structure of rat mGluR4 ATD as guiding tool to probe the ligand-binding pocket of mGluR4. In mGluR4, Ser159 aligns with Ser79 in the LIVBP and Ser165 in mGluR1. Thr182 aligns with Thr102 in the LIVBP and Thr188 in mGluR1. Mutation of Ser159 to alanine in mGluR4 resulted in a 95% reduction in \([^{3}H]L-AP4\) binding compared with the wild-type mGluR4, whereas the mutation of Thr182 to alanine produced a 96% decrease in \([^{3}H]L-AP4\) binding, despite that the expression levels of the mutant receptors were comparable to that of the wild-type mGluR4. These observations are in agreement with the loss of activity seen in the analogous mutation in mGluR1. Arg78, which is conserved in all mGluRs, was identified as the third residue critical in ligand-binding to mGluR4. Mutation of R78A in mGluR4 resulted in a nearly complete loss of \([^{3}H]L-AP4\) binding. It has been suggested that the \(\gamma\)-carboxy group on L-glutamate or the \(\gamma\)-
phosphonate group on L-AP4 and L-SOP forms an ion pair with the positive charge on the side chain of Arg78 as one of the essential components of the ligand-binding pocket of mGluRs (Hampson et al., 1999).

The most direct evidence showing that the ATD alone is sufficient for ligand binding and selectivity came from the study on soluble truncated mGluRs. The production of the entire rat mGluR1 ATD as an individual protein in insect sf9 cells revealed that ligand binding in mGluR1 can be dissociated from the membrane domain (Okamoto et al., 1998). The truncated mGluR1 was a 74 kDa soluble protein that was secreted into the cell culture medium. The pharmacological profile of this soluble receptor was found similar to that of the full-length membrane bound mGluR1: the rank order of inhibition was quisqualate >> L-glutamate ≈ ibotenate ≥ (1S, 3R)-ACPD for full-length mGluR1a expressed in sf9 cells while the rank order of inhibition for truncated soluble mGluR1 expressed in sf9 cells was quisqualate >> L-glutamate ≈ (1S, 3R)-ACPD ≥ ibotenate. Our study (initiated in January of 1998 as part of this thesis work) on mGluR4 indicated that a truncated soluble mGluR4 lacking the last 39 amino acids of the ATD was secreted from HEK cells into the culture medium. This soluble receptor maintained the ligand selectivity, and displayed similar pharmacological characteristics as that of the full-length mGluR4a (Han and Hampson, 1999; for details please refer to later sections).

1.9 Objectives and Rational

Given their regulatory effects on glutamatergic synaptic transmission, and their influences in the process of neuronal degeneration, group III mGluRs are considered as promising targets for the development of therapeutic agents for the treatment of epilepsy and other neurological disorders. Moreover, because of the wide diversity and heterogeneous distribution of mGluR
subtypes, the opportunity exists for developing highly selective drugs that affect a limited number of CNS functions. More detailed analyses on the ligand binding mechanism of these receptors are therefore required in order to design subtype specific drugs.

Since it is usually difficult to conduct structural analysis such as X-ray crystallography on membrane-bound proteins, a soluble segment of the ATD of mGluR4 that retains similar pharmacological profile as the wild-type receptor would be desirable for detailed structural studies. O'Hara et al.'s model of mGluR ATD suggested the possibility of isolating such a segment. A similar attempt in the study of the structure of iGluR ligand-binding core was successful. The iGluRs appear to have an LIVBP-like domain, followed by a distinct glutamate-binding protein-like region, which is split into two by the first three of the four putative transmembrane regions (the S1 and S2 regions). By directly connecting the two DNA stretches that encode the S1 and S2 segments of iGluR4, Keinänen and his colleagues elegantly created a soluble mini-receptor that lacked the putative transmembrane domains. This soluble protein displayed a ligand-binding profile typical of the AMPA-subtype receptors (Kuusinen et al., 1995). In a similar study on the NMDA receptor NR1, it has been shown that the S1-S2 fragment is responsible for the binding of Glycine (Ivanovic et al., 1998). Using this S1-S2 fragment, the crystal structure of GluR2 ligand-binding region in a complex with kainate was finally solved (Armstrong et al., 1998).

Based on the homology between the extracellular ATD of the mGluR4 receptor and the LIVBP protein, and the evidence from previous studies, we hypothesized that a truncated receptor protein containing all or part of the mGluR4 ATD would be soluble, yet retain the ligand binding activity of the full-length mGluR4. Such a receptor protein could be a substrate for protein crystallization. One objective of my study was to produce a truncated mGluR4
receptor with only the ATD or part of the ATD. Pharmacological and biochemical characterization of this truncated receptor would provide valuable answers to a series of questions regarding the ligand binding to mGluR4, including the impact of glycosylation, the possibility of receptor dimerization, and most importantly, the role of ATD in ligand selectivity. A radioligand binding assay using the agonist [³H]L-AP4 provides a means to assess the pharmacological profile of the truncated mGluR4, which can be compared to the wild-type receptor. A second objective was to define the boundary for amino acid residues required in ligand binding to mGluR4. The goal was to identify the shortest segment of the ATD of mGluR4 that can be expressed as a soluble protein and retain pharmacological activity. This study would produce a soluble receptor protein containing only regions necessary and sufficient for ligand binding, and would eliminate regions of the mGluR4 ATD not involved in ligand binding but could potentially complicate the expression of the protein in other host systems such as bacteria. This study would also explore the possible existence of other ATD regions outside the LIVBP homology region that may be important in maintaining the pharmacological characteristics of the receptor.

The third goal of this study was to express the truncated forms of mGluR4 and the closely related mGluR8 in different expression hosts such as E. coli and insect cells for high-level protein expression. The purification and production of crystals of truncated mGluRs are prerequisites for conducting structural studies such as X-ray crystallography, which will in turn provide direct information on the structure of the receptor binding pocket and the interaction between ligands and the binding site. mGluR4 and mGluR8 share high sequence homology (over 70% identity). They display similar affinities for most of the ligands including L-AP4, L-SOP and L-glutamate (Pin et al., 1999), but different affinities for ligands such as PPG, which
appears to have higher potency at mGluR8 than at mGluR4 (Gasparini et al., 1999). Comparison between the ligand binding domains of these two receptors would provide useful insight for the design of subtype-specific ligands.
2.0 Materials and Methods

2.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Canada and BDH Inc., unless otherwise indicated.

2.2 Standard Molecular Biology Procedures

2.2.1 Bacterial Transformation

All solutions and plates used for *Escherichia coli* (*E. coli*) competent cell preparation and transformation were prepared using Millipore water and were autoclaved. Bacterial cultures were grown in Luria-Bertani (LB) medium ((w/v): 1.0% bacto-tryptone, 0.5% yeast extract, 1.0% NaCl) and transformed bacteria were plated on LB-agar plates prepared by supplementing LB medium with 1.5% bacto-agar. Appropriate antibiotics were added to the plates for selection of transformed bacteria. All incubation steps were carried out at 37°C unless otherwise specified, and all shaking steps were carried out at 225 rpm on a New Brunswick orbital shaker.

For the preparation of competent *E. coli*, a single colony of DH5α (Gibco BRL) *E. coli* was inoculated in 2 ml of LB medium and incubated at 37°C with shaking for 16 hours. An aliquot of 15 μl of this overnight culture was added to 25 ml of fresh LB and incubated at 37°C with shaking and aeration until reaching an OD₆₀₀ of 0.3. Competent cells were then prepared using the calcium chloride (CaCl₂) method outlined in Sambrook, Fritsch and Maniatis (1989). After the preparation of the competent DH5α cells, 10 ng of supercoiled plasmid DNA or 50 ng of ligated relaxed plasmid DNA was added to 200 μl of competent cells and incubated on ice for 40 minutes. The cells were then heat-shocked for 45 to 60 seconds at 42°C and immediately
chilled on ice for 2 minutes. The cells were then incubated in 0.8 ml of fresh LB with shaking at 37°C for 1 hour. The transformed bacteria were then spread on LB agar plates containing 100 μg/ml ampicillin (Boehringer Mannheim). The plates were incubated at 37°C for 16 hours to allow for selection of ampicillin-resistant colonies.

2.2.2 Plasmid Preparation

Ampicillin-resistant colonies were inoculated in 3 ml of LB medium containing 100 μg/ml ampicillin, and incubated for 16 hours at 37°C with aeration. Overnight cultures were harvested by centrifugation in 1.5 ml microfuge tubes at 3000 x g for 4 minutes. Plasmid DNA was then isolated from the cells using the QIAspin Miniprep Kit (Qiagen) according to the manufacturer's protocol. Plasmid DNA concentration and purity were verified by spectrophotometric analysis at 280 nm and 260 nm. A small amount of the plasmid DNA product (approximately 0.2 μg) was subjected to restriction digest and gel electrophoresis to confirm the presence of the correct plasmid and orientation of the inserted DNA.

2.2.3 Restriction Enzyme Digestions and Agarose Gel Electrophoresis

Restriction enzyme digestions were used for subcloning purposes and to screen transformants for the appropriate size and orientation of DNA inserts in plasmids constructed. Digestions were performed in reaction volumes vary from 10 to 100 μl. The digestion reactions typically contained 1-2 μg DNA, deionized sterile water, specific 10X reaction buffer supplied by the manufacturer, and 5-10 units of specific restriction enzyme (New England Biolabs, Gibco BRL). All reactions, unless specified were incubated at 37°C for 2 hours to ensure complete digestion of the DNA.
Agarose gel electrophoresis was used to visually analyze restriction digests and quantify purified plasmid DNA or DNA fragments. Unless specified, electrophoresis was performed using 0.8% agarose gels dissolved in 0.5 x Tris-acetate/EDTA buffer (20 mM Tris-acetate, 0.5 mM EDTA) supplemented with 0.5 μg/ml ethidium bromide. DNA-gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% (w/v) glycerol in water) was added to each sample at 1/6 of the total volume prior to loading on the gel. Samples were run on a Mupid-21 Mini-Gel apparatus (Helixx Technologies) at a setting of 100 volts until adequate separation of digested bands had been accomplished. Fluorescent bands were visualized using an ultraviolet transilluminator (UVP Incorporated). Gels were also loaded with a 1-kilobase or 100 bp ladder (Gibco BRL) for determination of DNA fragment lengths, and DNA was quantified by comparison with a known amount of Hind III digested λ DNA (Gibco BRL).

2.2.4 Blunt-end Reactions, DNA Dephosphorylation and DNA Ligation

Treatment with T4 polymerase was used to create blunt ends in DNA fragments for subcloning purposes. In a typical reaction, approximate 1 μg of DNA in a volume of 25 μl was mixed with 5 μl of 10X reaction buffer, 2.5 μl of bovine serum albumin (BSA, 1 mg/ml), 4 μl of dNTPs (1.25 mM), 2 μl of T4 polymerase (3000 u/ml, Pharmacia) and 11.5 μl H2O. The reaction was carried out at 12°C for 20 minutes.

To prevent self-ligation in vectors with blunt ends, these vector were dephosphorylated by calf intestinal alkaline phosphatase prior to ligation reactions. The typical reaction mixture contained 1 μg of DNA in 35 μl dH2O, 5 μl of 10X reaction buffer and 10 μl diluted enzyme, which was made from 0.5 μl calf intestinal alkaline phosphatase (30 units/ml, Gibco BRL) and
28 µl dilution buffer. The reaction was carried out at 37°C for 1 hour. The dephosphorylated product was precipitated as described above.

In DNA ligation reaction, DNA fragments with competitive ends were joined by T4 ligase. Typically, a 20 µl reaction mixture contained approximate 30 ng of insert (50 ng for blunt-ended ligation) and 10 ng of vector in Tris-HCl (pH8.0), 1 µl of T4 DNA ligase (400,000 units/ml, New England Biolabs), 2 µl 10X reaction buffer, 2 µl of 10 mM ATP and H2O to make the final volume of 20 µl. The reaction was done at 16°C for 16 hours.

2.3 Plasmids for Expressing Truncated mGluR4 Receptors in Mammalian System

2.3.1 C-myc tagged full-length mGluR4a in pcDNA3

Plasmid expressing wild type mGluR4a (mGluR4a-pcDNA3) was constructed by inserting Bgl II-EcoR I fragment of rat mGluR4a cDNA in the pBluescript SK phagemid (provided by Dr. S. Nakanishi) into the pcDNA3 mammalian expression vector (Invitrogen Corp.) at the BamHI I site. For the construction of c-myc-tagged mGluR4a, the mGluR4a-pcDNA3 plasmid was cut with Xho I, and the larger fragment containing the pcDNA3 backbone was ligated to itself (the 5'-mGluR4a-pcDNA3 plasmid). The oligonucleotides encoding the c-myc epitope 5'-GTCACGAAAAGGCTTATTTCTGAAGAAGACTTGATCCAG-3' (f-BstE II-myc) and 5'-GTGACCTGGATCCAGTTTTCTTCTTCTTCAGAAATAAGCTTTTGTC-3' (rev-BstE II-myc) were phosphorylated, annealed to each other, and inserted into the 5'-mGluR4a-pcDNA3 plasmid at the BstE II site to produce 5'-mGluR4a-pcDNA3. The 931-bp Nde I-Xho I fragment from 5'mGluR4a-myc-pcDNA3 and a 3335-bp Xho I-Not I fragment of mGluR4a-pcDNA3 were subcloned into pcDNA3 at the Nde I-Not I sites using a three-piece ligation to produce myc-
mGluR4-pcDNA3. Plasmids mGluR4a-pcDNA3 and mGluR4a-myc-pcDNA3 were constructed by Dr. Roman Pekhletske.

2.3.2 Truncated mGluR4 receptors m4Tr-P586 and m4Tr-Y548

The DNA fragment encoding the ATD of mGluR4 from amino acid 1 to 586 was generated by polymerase chain reaction (PCR). The forward primer 5'TTTCCGAAATGTCGGGAAGG3' (m4-8 olig) included the start codon, and annealed upstream to the signal peptide sequence in mGluR4a-myc-pcDNA3. The reverse primer 5'CTTACGGCGAGTCCCACCTCCA3' (mR4-trunc-Rev) created a stop codon after Pro586, the second last amino acid before the beginning of the first TMD. In order to produce PCR product with phosphorylated ends, the two primers were treated with T4 kinase prior to the PCR reaction. 300 pmol of each primer together with 1μl of 10X T4 kinase buffer (New England Biolabs), 1μl of T4 kinase (10,000 units/ml, New England Biolabs), and 1μl of 1mM ATP were incubated in a 10μl reaction mixture at 37°C for 18 minutes. The PCR reaction consisted of a 100μl mixture containing: 2.5 units of Taq polymerase (Pharmacia), 10 μl of 10X Taq reaction buffer (Pharmacia), 2 μl of the T4 kinase reaction mixture containing 60 pmol of each primer (added immediately after the 18 minutes phosphorylation reaction described above), 500 ng of mGluR4a-myc-pcDNA3 as the template, and 125 n mole of each of the four dNTPs (Pharmacia). The PCR products were purified with QIAquick PCR purification kit (Qiagen). Procedures were carried out according to manufacturer’s instruction. The purified PCR fragment was subjected to blunt-end treatment, and isolated using QIAquick gel extraction kit (Qiagen) according to the manufacturer’s protocol.
The pcDNA3 vector was digested with EcoR V (Pharmacia), and extracted with equal volume of phenol-chloroform-isooamyl alcohol (25:24:1 v/v, Gibco BRL). To precipitate the DNA from the digestion mix, 1/10 volume of 3M sodium acetate (pH 5.2) and 3 volumes of anhydrous ethanol were added. The mixture was left at -20°C for 1 hour, and centrifuged at 14,000 X g for 10 minutes. The pellet containing the vector DNA was washed with 70% ethanol (v/v), and air-dried. The DNA was then dephosphorylated.

To prepare the insert for construct m4Tr-Y548-pcDNA3, a 1686 bp fragment was isolated from the mGluR4a-myc-pcDNA3 by digesting with Kpn I (Gibco BRL). This fragment encoded mGluR4 signal peptide, myc epitope, and the ATD truncated after Tyr548, which was 39 amino acids from the beginning of the first TMD.

The ligation reaction for the PCR fragment and the EcoR V digested pcDNA3 vector had an approximate 5:1 insert to vector molar ratio. A ratio of 3:1 was used for the ligation reaction for the Kpn I fragment from mGluR4a-myc-pcDNA3 and the Kpn I digested pcDNA3 vector. Ligation products were transformed into E. coli XL-1 DH5α. Plasmid was isolated from the ampicillin resistant colonies, and the orientation of the cDNA inserts was checked by restriction enzyme digestion.

2.3.3 Plasmids Expressing m4Tr-Y489 and m4Tr-V413

cDNA fragments encoding m4Tr-Y489 and m4Tr-V413 were generated by PCR with Vent DNA polymerase (New England Biolabs). The PCR primers were synthesized by Gibco BRL. Both reactions used the same forward primer, which had the sequence of 5'-CCGAGGTTCATGGGTCTCTAGATCTT-3' (GM-F1). Restriction sites for EcoR I and Bgl II were incorporated for convenient subcloning. The reverse primers for m4Tr-Y489
(GCACGAGCGGGGTACCTAGTACTC, GM-R-Y489) and m4Tr-V413 (5'-GCGCGGTACCCATGCCCTACAG-3', GM-R-V413) had stop codons after the Y489 and V413, respectively, followed by a restriction site for Kpn I in both primers.

The PCR products encoding m4Tr-Y489 and m4Tr-V413 were digested with EcoR I and Kpn I and inserted into the pcDNA3 vector digested with the same enzymes. In the second subcloning step to construct m4Tr-Y489-pcDNA3 and m4Tr-V413-pcDNA3, EcoR I fragments containing cDNA sequences were isolated and blunt-ended. The blunt-ended fragments were inserted into pcDNA3 at the EcoR V site. The orientations of the inserts were examined by restriction enzyme digest.

2.4 Construction of Plasmids for E. coli Expression

2.4.1 pBAD/Myc-His System for Intracellular Expression in E. coli

The DNA fragment encoding amino acids 1 to 549 of mGluR4 was subcloned into pBAD/Myc-His (version B, Invitrogen), and coding sequence was in-frame with the vector at both 5' and 3' junctions. First the mammalian expression plasmid m4Tr-Q549-pcDNA3 was constructed. A PCR reaction was carried out using the c-myc-mGluR4-pcDNA3 as the template and the GM-F1 primer as the forward primer. In the reverse primer for m4Tr-Q549 (5'-CAGGTCAATTCTTACTGGTACCC-3', GM-R-Q549), a stop codon was incorporated after the codon for Q549, and an EcoR I site was downstream from the stop codon. The PCR product for m4Tr-Q549 was subjected to restriction digestion by EcoR I (Gibco BRL), and ligated with EcoR I digested pcDNA3 vector. The insert for constructing bacteria mGluR4 expression plasmid was prepared by first digest m4Tr-Q549-pcDNA3 plasmid with EcoR I. The digestion product was blunt-ended and gel-purified. The linear, blunt-end DNA was digested with Kpn I
and the 1714 bp fragment was isolated by gel-extraction. The vector was digested with Bgl II and blunt-ended. The product was purified by gel-extraction and subjected to Kpn I digestion. The final double-digested vector was purified by gel-extraction. To express the amino acids 1 to 543 of rat mGluR8 with the pBAD/Myc-His vector, the insert was isolated as an EcoR I fragment from the plasmid m8Tr-pBlueHis2, and inserted into vector pBAD/Myc-His version B at the EcoR I site.

The ligation products were transformed into E. coli strain TOP10 (Invitrogen) following the transformation procedure described previously. Transformed bacteria was selected by ampicillin supplemented LB agar plate and the orientation of the inserts was checked by restriction digestion.

2.4.2 pET-22b System for Periplasmic Expression in E. coli

pET-22b vector was purchased from Novagen. It allows the fusion of the protein in interest with an E. coli signal peptide, therefore, the production of a recombinant protein secreted into the periplasmic space. It also provides 6X His tag to the carboxyl-terminus of the protein.

To insert the DNA fragment encoding the amino acids 1 to 549 of rat mGluR4 into pET-22b, a Hind III-Kpn I fragment containing the cDNA sequence was isolated from m4Tr-pBlueBac4.5. Both ends of this fragment were blunted using T4 DNA polymerase. This fragment was then ligated with pET22b(+) vector, which was digested with EcoR I and Nat I, blunt-ended, and dephosphorylated. The orientation of the insertion was verified by restriction enzyme digestion. The reading frames at both 5' and 3' ends of the insertion were confirmed by DNA sequencing.

To insert the DNA fragment encoding the amino acids 1 to 543 of rat mGluR8 into pET22b, an EcoR I-Xba I fragment was isolated from T40-pcDNA3.1/Myc-His (a mammalian expression
plasmid producing a soluble truncated rat mGluR8, constructed by Dr. Vanya Peltekova), followed by blunt-end reaction. The vector was prepared in the same way as for m4Tr-pET22b.

2.5 Baculovirus Transfer Vectors for Protein Expression in Insect Cells

2.5.1 Truncated mGluR4 and mGluR8 with Original Mammalian Signal Peptides

To construct m4Tr-pBlueBac4.5 plasmid, a 1707 bp Bgl II-EcoR I fragment was isolated from m4Tr-Q549-pcDNA3. The baculovirus transfer vector pBlueBac4.5 (Invitrogen) was also digested with Bgl II and EcoR I. In constructing the transfer vector expressing truncated mGluR8, a plasmid m8Tr-pBlueHis2 was first made. To prepare the insert for m8Tr-pBlueHis2, T40-pcDNA3.1/Myc-His was digested with Xba I (Gibco BRL) and blunt-ended. The DNA was then digested by Kpn I, and the 1739 bp fragment was isolated. The pBlueHis2 vector (version B, Invitrogen) was digested with EcoR I and blunt-ended. The DNA was precipitated as described in previous section, and subjected to Kpn I digestion and dephosphorylation. The ligation, transformation and plasmid isolation procedures were carried out as described previously. An EcoR I fragment containing the cDNA sequence was isolated from m8Tr-pBlueHis, and was inserted into EcoR I digested and dephosphorylated pBlueBac4.5. The plasmids, m4Tr-pBlueBac4.5 and m8Tr-pBlueBac4.5, were transformed into DH5α E. coli strain. The correct orientations of the inserts were verified by restriction enzyme digest.

2.5.2 Truncated mGluR4 and mGluR8 with a Baculoviral Signal Peptide

To replace the rat signal peptides of the truncated rat mGluR4a and mGluR8a with a baculoviral signal peptide that is specific for secretion in insect cells, cDNA fragments of mGluR4 and mGluR8 were generated via PCR and inserted into pFastBac vector derivatives.
(pK509-8 and pK503-19) containing a viral signal sequence (provided by Dr. K. Keinänen). A PCR product was generated using mGluR4a-myc-pcDNA3 as the template. The primer sequences were: 5'-CCGAGGTTTCATGGGTCTCTAGATCTTT-3' (Trm4-pK509-F) for the forward primer and 5' -TAGCGGTCCACTCTAGACTGGTAC-3' (Trm4-pK509-R) for the reverse primer. The reverse primer contained an Xba I site, which allowed the cDNA fragment to be in-frame with the His-tag sequence in the vector. PCR reaction using Vent polymerase (New England Biolab) was carried out as described previously. The PCR product was digested with Xba I and ligated with the vector, which was prepared by digesting pK509-8 with Not I, followed by blunt-end reaction and finally digestion with Xba I. After the construct was obtained and verified, it was subject to restriction digestion with BamH I. A 1578 bp fragment was isolated and blunt-ended. This fragment was inserted into pK503-19 that was digested with EcoR I-BamH I and blunt-ended. DNA sequencing confirmed that the PCR fragment was error-free, and the insertion in m4Tr-pK503-19 was in-frame with both 5' viral signal peptide and FLAG epitope tag and the 3' His-tag.

A 287 bp PCR product was generated with the forward primer 5'-GATCCTCACGCGGCGCAAAGAACTC-3' (Trm8-pK509-F) and reverse primer 5'-CTGGAACACGTGTCAAGGA-3', using the plasmid T40-pcDNA3.1-Myc/His as the template. This fragment incorporated a Not I restriction site at its 5' end. It was digested with Acc III (Stretagen) at 60°C for 3 hours and used as the insert in the first subcloning step. To prepare the vector, T40-pcDNA3.1-Myc/His was transformed into and then isolated from E. coli strain SCS110 (Stretagen) so that the DNA was not methylated. The isolated plasmid was digested with EcoR I, followed by blunt-end reaction and then digestion with Acc III. Ligation and transformation procedures were described above. A 1558 bp fragment was then isolated from
this construct by digesting with Not I-Xba I, and inserted into pK509-8 treated with the same enzymes. The product of the second subcloning step was digested with Not I and blunt-ended, followed by a subsequent ligation of the plasmid itself. The final construct was confirmed by DNA sequencing to be PCR error-free and in-frame with the signal peptide, FLAG epitope and His tag.

2.6 Recombinant Protein Expression in Mammalian Cells

2.6.1 Transfection

Transient transfection of HEK 293 TSA-201 cells was performed using the calcium-phosphate precipitation method described by Gorman et al (1990). For plasmid DNA to be used for transfection, a 280/260 nm absorbence ratio ≥ 1.8 was required. HEK cells were cultured in 100 mm sterile dishes (Nunclon) to a confluency of 80%. These cell cultures were maintained in minimal essential medium (Gibco BRL) supplemented with 6% fetal bovine serum (Gibco, BRL) and an antibiotic solution containing penicillin and streptomycin (Gibco, BRL). Cells were grown at 37°C and 5% CO₂.

Three hours prior to transfection, 10 ml of fresh supplemented medium were added to each plate. Transfections were performed using 20 μg plasmid DNA per 100 mm plate. The plasmid DNA was combined with 450 μl 1/10 TE (1 mM Tris-Cl, 0.1 mM EDTA, pH 8.0) and 50 μl of 2.5 M CaCl₂. This solution was thoroughly mixed and added to 500 μl 2X HEPES Buffered Saline (HBS: 0.28 M NaCl, 50 mM HEPES, 3 mM Na₂HPO₄) to form an opaque precipitate. This final solution was mixed and added dropwise to each plate. The cells were incubated at 37°C for 4 hours before exposure to a glycerol shock. The medium was aspirated from each dish and 6 ml of 15% glycerol in phosphate-buffered saline (PBS: 10 mM Na₂HPO₄, 3
mM KH$_2$PO$_4$, 0.12 M NaCl, pH 7.2) was added. Cells were exposed to the glycerol solution for 30 seconds. The glycerol solution was aspirated and the cells were washed with 6 ml of PBS. The PBS was aspirated, 10 ml of fresh supplemented medium was added to the cells and they were placed at 37°C incubator. 24 hours post-transfection, each transfected plate was subcultured into 2 separate dishes and placed at 37°C.

2.6.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used in conjunction with immunoblotting to confirm expression of all receptor constructs. Equivalent amounts of protein were loaded for each sample and the proteins were separated by electrophoresis on 8% or 10% gels using an SE 250 SDS-PAGE apparatus (Hoeffer Scientific Instruments). Electrophoresis was carried out followed by placing the gel in a transblotting cassette for transfer of the proteins to nitrocellulose membrane (0.45 μm pore size, Schleicher and Schuell). Proteins were transferred for 2 hours at 4°C, under a constant current of 225 mA and were then placed in blocking solution (10 mM Tris-Cl, 150 mM NaCl, 0.2% polyoxyethylene-sorbitan monolaurate, 5% powdered milk, pH 7.6) overnight. The membranes were washed 3 x 15 minutes with wash buffer (10 mM Tris-HCl, 150 mM NaCl, 0.2% polyoxyethylene-sorbitan monolaurate, pH 7.6), and then incubated for 2 hours with gentle rocking with the appropriate primary antibody diluted in wash buffer. Following incubation with the primary antibody, blots were washed 3 x 15 minutes with wash buffer. Blots were then incubated for 2 hours with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Amersham) diluted at a ratio of 1:10,000 in wash buffer. Blots were washed for a final 3 x 15 minutes and were then exposed to enhanced chemiluminescence
(Amersham) reagents for two minutes. Immunoreactive bands on the nitrocellulose blots were visualized on enhanced chemiluminescence hyperfilm.

2.6.3 Immunocytochemistry

HEK cells transiently transfected with the two constructs were examined by immunostaining. Twenty-four hours after the transfection, transfected HEK cells were subcultured and grown in 35mm plates with a glass cover slip attached at the center. The cells were first placed with 200 µl of OptiMEM medium on the central glass cover slip that was pre-incubated with 0.01% poly-L-ornithine at 37°C for 14 hours. After growing the cells at 37°C for 4 hours, another 800 µl of OptiMEM medium was added to each plate. The following day, cells were washed for two times, 2 minutes each time with PBS. After the removal of PBS, methanol was added onto the glass cover slip of each plate, and the plates were placed at -20°C for 20 minutes. Methanol was then removed and the plates were air-dried for 10 minutes. The fixed cells were washed with PBS for two 5 minutes washing periods, and blocked in 10% BSA for 1 hour. The cells were then incubated with mouse monoclonal anti-c-myc IgG (200 µg/ml, Santa Cruz Biotechnology, 1:1400 dilution in 1% BSA) for 1.5 hours. After three 5 minutes washes in PBS, anti-mouse Ig biotin conjugate (Sigma, 1:1500 dilution in 1% BSA) was used to incubate the cells for 1 hour. The cells were washed with PBS for three 5 minute periods, and then incubated in ExtrAvidin FITC conjugate (Sigma, 1:600 dilution in 1% BSA) for 1 hour. After three 10 minutes washes in PBS, cells were mounted with 50% glycerol in PBS (v/v). The depositions of the proteins were visualized as FITC fluorescent under a Zeiss Axiovert 135TV fluorescent microscope.

2.7 [3H] L-AP4 Radioligand Binding Assay
2.7.1 Crude Membrane Preparation from Transfected HEK cells

The membrane preparation of transfected HEK cells was carried out according to the methods used by Eriksen and Thomsen, 1995. Transfected cells were harvested 48 hours post-transfection by gently pipetting them off of the plates. The cells were then centrifuged at 1,380 x g (3,000 rpm for JA-14 rotor, Beckman) for 10 minutes and then resuspended in 20 ml lysis buffer (30 mM HEPES, 1 mM EDTA, 5 mM MgCl₂, pH 7.4) and polytroned (Polytron, Inc) for 5 seconds at level 5. The cells were then centrifuged at 48,400 x g (20,000 rpm for JA-20 rotor, Beckman) for 20 minutes. The pellet was resuspended in 15 ml lysis buffer supplemented with 0.08% Triton X-100 and incubated at 37°C for 10 minutes. An additional 12 ml of non-supplemented lysis buffer was added, and the cell lysate was centrifuged again at 48,400 x g. The pellet was resuspended in 15 ml binding assay buffer (30 mM HEPES, 0.1 M NaCl, 1.2 mM MgCl₂·6H₂O, 5 mM KCl, 2.5 mM CaCl₂, pH 8.0) and centrifuged as before. For all membrane preparations, both assay buffer and lysis buffer were supplemented with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 0.1 mM. Finally, the pellet was resuspended in 1-5 ml assay buffer (according to desired concentration of final membrane fraction) and homogenized with a 5 ml glass homogenizer. The homogenized membranes were aliquoted into 1 ml fractions and frozen immediately at -70°C. A small aliquot of membrane was also removed for protein concentration measurement. Determination of the protein concentration was done according to the method of Bradford (1976) using BSA as a protein standard.

2.7.2 Soluble Sample Preparation

At 24 hours post-transfection, medium was changed to OptiMEM serum reduced medium (Gibco BRL). Each plate of transfected cells was subcultured into two plates with 7 ml of
OptiMEM medium in each plate. At 48 hours post-transfection, media and cells were collected separately. The medium was centrifuged at 48,400 x g for 30 minutes at 4°C, and the supernatant was subjected to dialysis to remove the glutamate in the medium. Dialysis was carried out in 2,400 ml of dialysis buffer (same as assay buffer above) supplemented with 0.1 mM PMSF and 1 mM EGTA. Samples were dialyzed at 4°C with gentle stir. Dialysis buffer was changed 3 times over a 24-hour period. After the dialysis, the samples were pooled, transferred to microfuge tubes as 1 ml aliquots, and stored at -70°C.

2.7.3 Membrane Binding Assay

[^H]-L-AP4 and other mGluR ligands used in the radioligand binding assays were purchased from Tocris Cookson. The[^H]-L-AP4 binding assay was performed on ice using a constant concentration of[^H]-L-AP4 (30 nM) and a range of concentrations for the cold ligands (10 nM - 1 mM). The concentration of 30 nM of[^H]-L-AP4 was employed, as this represented the lowest concentration of ligand that could specifically label the group III mGluRs. The assay for membrane-bound receptors was carried out as described by Enksen and Thomsen (1993) and Thomsen et al., (1997) except that 300 μM L-SOP was used for blanks. Each assay point was performed in triplicate with each tube in the assay containing 25 μl assay buffer/competing drug, 200 μl diluted membranes with a total protein concentration of 0.625 mg/ml, and 25 μl[^H]-L-AP4 (300 nM), for a final volume of 250 μl. All drugs used in the binding assays were dissolved in assay buffer (+PMSF) and were prepared as 10X stocks. L-SOP (300 μM) was used as the 'blank' in the assays to determine the amount of non-specific binding, as at such a high concentration, it effectively saturates all receptor binding sites. Reagents for the assay were added in the following order: 25 μl of assay buffer/L-SOP/competing drug, followed by 200 μl
of diluted membranes, and at last 25 μl of [3H]-L-AP4. After the addition of all reagents, the tubes were vortexed and incubated on ice, with shaking (154 rpm) for 30 minutes. After the incubation period, the mixtures were centrifuged at 13,000 x g for 2 minutes. The supernatant was aspirated and the pellets washed once with 1 ml of binding assay buffer without PMSF. Pellets were dissolved in 500 μl 1M NaOH for 16 hours at 22°C. Samples were then transferred to Pony liquid scintillation vials (Packard) and supplemented with 5 ml of Ultima Gold scintillation cocktail (Packard). Samples were left for 3 hours to allow for equilibration of the samples with the scintillation fluid, and radioactivity was then measured by liquid scintillation spectrometry. Triplicate sets of binding counts were summed and the mean values calculated. The mean of the non-specific or ‘blank’ binding was subtracted from the mean binding counts for each of the assay points to calculate each point’s specific binding. This number was divided by the total specific counts (from samples without competing drug) to yield a numerical value representing each point’s percentage of total binding. All radioligand binding data analyses were performed using the GraphPad Prism software program. Percent total binding values were fitted into a one-site competition model for ligand binding to determine an IC₅₀ value for each curve. Kᵢ values were also calculated using GraphPad Prism program. The calculations were based on the IC₅₀ value of each competition assay and a Kᵦ value of 470 nM for L-AP4 at the full-length mGluR4a receptor. Competition curves for each drug were performed in triplicate and mean IC₅₀ and Kᵢ values were determined.

2.7.4 Soluble Binding Assay

Each sample of the soluble [3H]L-AP4 binding assay was in a total volume of 250 μl. The addition of each reagent was in following order: for each tube, first add 25 μl 10X competing
drug, 25 μl of 3 mM L-SOP (for blank) or 25 μl of assay buffer (for total binding), then 100 μl assay buffer, followed by addition of 100 μl dialyzed sample, and finally 25 μl of 300 nM [3H]L-AP4 to give a 30 nM final concentration of [3H]L-AP4 (except for autocompetition experiments with L-AP4 in which 10 nM final concentration was used). The solutions were mixed, then incubated on ice (shake at 154 rpm) for 40 minutes. After incubation, 20 μl of 17 mg/ml γ-globulin (425 μg) was added to each tube, followed by the addition of 200 μl 30% polyethyleneglycol (w/v). The mixture was vortexed, and incubated on ice for 2-4 minutes. It was then centrifuged at 14,000 x g for 2 minutes. The supernatant was aspirated. The pellet was washed with cold 15% polyethyleneglycol, then dissolved in 500μl 1M NaOH. The rest followed the same procedure for binding assay with membrane sample, as described above. An IC50 value for L-AP4 at the soluble receptor was generated from autocompetition curve obtained with GraphPad Prism software, and the KD value for L-AP4 at the soluble receptor was calculated using the equation IC50 = KD (1 + [P]/Kp) where [P] is the concentration of the labeled probe ([3H]L-AP4, 10 nM), and Kp is the KD of the labeled probe. Unpaired t test was conducted using Prism software for statistic analysis.

2.8 Deglycosylation and Lectin Binding Test

For each 23 μl dialyzed sample, 3 μl 10X G7 Buffer (New England BioLabs) and 3 μl 10% NP-40 were added. 1 μl PNGase F (500,000U/ml, New England BioLabs) was added to each mixture, and the reactions were incubated at 0°C, room temperature, and 37°C for 1 hour, respectively. After incubation, 4X SDS sample buffer was added to the reaction mixtures, and the mixtures were subjected to immunoblotting analysis with anti-c-myc antibody.
Culture medium samples containing m4Tr-Y548 protein were dialyzed in buffer A (50 mM Tris-Cl, 2 mM CaCl₂ and 100 mM NaCl, pH 7.5) for 16 hours in cold room. Wheat germ agglutinin-sepharose was washed with 1 ml of buffer A, and centrifuged at 6,000 x g for 4 minutes at 4°C. After the addition of 1 ml of the dialyzed sample, the sample-lectin mixture was incubated for 2 hours at 4°C with shaking. The mixture was then centrifuged at 10,000 x g for 1 minute. An aliquot of the supernatant was saved for immunoblotting analysis. The pellet was washed with 1 ml of buffer A. The elution was carried out by incubating the pellet with 1 ml of buffer A containing 300 mM N-acetyl-D-galactosamine for 2 hours at 4°C with shaking. The mixture was centrifuged at 10,000 x g for 1 minute and the supernatant was saved for immunoblotting analysis.

2.9 Recombinant Protein Expressions in E. coli

2.9.1 L-Arabinose Induced Expression of m4Tr-pBAD/Myc-His and m8Tr-pBAD/Myc-His

In the pilot expression test, 2 ml of LB containing 50 μg/ml of ampicillin was inoculated with a single colony from each transformant. The cultures grew at 37°C with shaking for 16 hours. For each transformant, 5 tubes each containing 2 ml of fresh LB supplemented with 50 μg/ml of ampicillin were inoculated with 15 μl of the overnight culture. The cells were incubated at 37°C with shaking to an OD₆₀₀ = 0.5. An aliquot of 1 ml cell culture of each transformant was removed and centrifuged at 14,000 x g for 30 seconds in a 1.5 ml microcentrifuge tube. The supernatant was aspirated and the cell pellet was frozen at −20°C to serve as the zero time point samples in later analysis. Five L-arabinose solutions were made, at concentrations of 20%, 2%, 0.2%, 0.02% and 0.002% (w/v). For each of the 5 tubes from the same transformant, 20 μl of each L-arabinose stock solution was added to. The cells were grown for 4 hours at 37°C with
Cells were collected as described above at the end of the incubation and kept at -20°C for further analysis.

After all the samples had been collected, the pellets were resuspended in 100 µl of bacterial lysis buffer (3 mM KH₂PO₄, 47 mM K₂HPO₄, 400 mM NaCl, 100 mM KCl, 10% glycerol (v/v), 0.5% Triton X-100 (v/v), 10 mM imidazole, pH 7.8). The samples were placed on ice and sonicated for 2 x 10 seconds. To separate soluble proteins from the insoluble ones, the sonicated samples were centrifuged at 100,000 x g for 45 minutes in an Airfuge™ air-driven ultracentrifuge (Beckman). The supernatants and pellets were subjected to SDS-PAGE and immunoblotting experiments separately.

For radioligand binding analysis on the soluble and insoluble samples from the transformed E. coli, the expression experiment was scaled up accordingly. The soluble fractions were dialyzed against binding assay buffer supplemented with PMSF and EGTA. The [³H]L-AP4 binding assay was carried out as the same procedure for mammalian soluble samples.

2.9.2 IPTG Induced Expression of m4Tr-pET22b and m8Tr-pET22b

Plasmids were transformed into E. coli strain BL21(DE3) (Novagen) following the transformation procedure described previously. For pilot test, single colonies were inoculated in 3 ml of fresh LB supplemented with 100 µg/ml ampicillin. The growth of the bacteria was monitored every hour until the density of the culture reached OD₆₀₀ = 0.4. Each tube of bacterial culture was divided into two tubes with equal volume. IPTG was added to one of the tube at a final concentration of 0.4 mM, whereas the other tube served as the negative control. The incubation was then continued for another 3.5 hours, and samples were collected at the end of the incubation time.
Each 1.5 ml of bacterial culture was transferred to a microcentrifuge tube and subjected to centrifugation at 14,000 x g for 2 minutes. The cell pellet was resuspended in 1 ml of PBS by gentle vortex. The centrifugation step was repeated and the pellet was resuspended in 150 μl of cold 20% (w/v) sucrose in 10mM Tris-HCl (pH7.5). After vortex, 25 μl of 0.1M EDTA (pH8) was added to the cell suspension. A 50 μl aliquot was saved as the whole cell fraction for SDS-PAGE analysis. The remainder was incubated on ice of 10 minutes. The samples were centrifuged for 5 minutes at 14,000 x g, and the cell pellet was resuspended in 100 μl of 2.5 mM MgCl₂. The samples were again incubated on ice for 10 minutes, and the centrifugation step was repeated. The supernatant fractions were collected as the periplasmic extracts for SDS-PAGE analysis. The pellets were resuspended in 100 μl of cold H₂O and collected as the cytoplasmic/membrane fractions. Samples for SDS-PAGE were prepared by mixing with equal volume of 2X SDS sample buffer. To prepare periplasmic extracts for radiolabeled [³H]L-AP4 binding assay, the bacterial culture was 30 ml in volume and the entire procedure was scaled up accordingly. The periplasmic extracts were dialyzed against binding assay buffer supplemented with PMSF and EGTA, as described previously. The [³H]L-AP4 binding assay was carried out as the same procedure for mammalian soluble samples.

2.10 Recombinant Protein Expressions in Insect Cells

2.10.1 Insect Cell Cultures

EX-CELL 400™ serum free insect medium (EX-400) was purchased from JRH Biosciences. Fetal bovine serum and cell culture media supplements were purchased from Gibco BRL. Cell culture flasks, dishes and pipettes were from Corning, Falcon Becton-Dickenson Labware, Nunc and Sarstedt.
Insect Sf-9 cells (*Spodoptera frugiperda*) were obtained from Invitrogen Corporation. The cells were cultured and maintained in EX-400 media supplemented with 2% fetal bovine serum, 1% fungizone and 0.1% gentamicin at 27°C. To establish a culture, a vial (1.0 ml) of frozen cells (2 X 10⁷ cells) in frozen media consisting of 10% dimethylsulfoxide, 10% fetal bovine serum and 80% culture media (v/v) was taken out of liquid nitrogen storage and thawed rapidly with gentle agitation in a 37°C water bath. The cells were then transferred to 6 ml of culture media in a 25 cm² tissue culture flask and incubated at 27°C for 1-5 days with media changes every 3 days. Once the cells reached 80-90% confluence, they were suspended in culture media by agitation. The cell viability and density was determined using a hemocytometer and staining with 0.045 (v/v) trypan blue. The cells were replated either at 1 x 10⁶ cells per 100 mm culture plate as a monolayer culture, or in a 100 ml spinner flask with 85-90 ml of completed media. The spinner flask was kept at 27°C with constant stirring at 80 rpm.

2.10.2 Generation of Recombinant Baculoviruses

2.10.2.1 Co-transfection for pBlueBac4.5 constructs

Sf9 cells were seeded at 2 x 10⁶ cells per 60 mm tissue culture plates with completed EX-400 media. After the plates were rocked gently to evenly distribute the cells, the cells were left undisturbed for 15 minute to attach to the bottom of the plates. Meanwhile, in a 1.5 ml microcentrifuge tube, 10 µl (0.5 µg) of the Bac-N-BlueTM DNA (Invitrogen), 4 µl of recombinant transfer plasmid (1 µg/µl), 1 ml of FBS-free EX-400 media and 20 ml of Insectin-Plus™ Liposomes (Invitrogen) were added and mixed by vortexing vigorously for 10 seconds. The transfection mixture was incubated at 22°C for 15 minutes. While the mixture was incubating, the medium was removed from the cells without disrupting the monolayer and the
cells were washed by 2 ml of fresh FBS-free EX-400 media. After the media was again removed, the entire transfection mix was added into the dish dropwise. The dish was then incubated at 22°C for 4 hours on a side-to-side, rocking platform. Following the 4-hour incubation period, 1 ml of complete EX-400 medium was added to each 60 mm dish. The plates were placed in a sealed plastic bag with paper tower wetted with 5 mM EDTA inside and were incubated at 27°C for 72 hours. EDTA was used to prevent the growth of mildew and bacteria on the paper towels.

2.10.2.2 Transposition and Transfection using Bac-To-Bac Baculovirus System

The vectors used for the construction of m4Tr-pK503-19 and m8Tr-pK509-8 are derived from pFastBac donor plasmid, which is a component of the Bac-To-Bac baculovirus expression system (Gibco BRL). The generation of viral DNA using this system involves transposition of insert from donor plasmid to the bacmid within E. coli cells. For transposition, 1 ng of each recombinant donor plasmid was transformed into MAX EFFICIENCY DH10BAC competent cells (Gibco BRL). E. coli containing recombinant bacmid DNA was selected on LB plate containing 50 μg/ml kanamycin, 7 μg/ml gentamicine, 10 μg/ml tetracycline, 100 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 40 μg/ml IPTG. After 40 hours incubation at 37°C, white colonies were selected from the plates and replated on another fresh plate for reconfirmation. The recombinant bacmid DNA was isolated from the white colonies using Plasmid Mini Kit (Qiagen), following manufacturer’s instruction for large size DNA isolation.

Transfection of Sf9 cells with recombinant bacmid DNA was carried according to manufacturer’s instruction. CELLFECTIN was purchased from Gibco BRL. Cells were seeded at
9 x 10^5 cells per 35-mm well in 2 ml of serum free medium with 50 units/ml of penicillin and 50 μg/ml of streptomycin. The cells were washed with medium without antibiotics, and the wash medium was aspirated. For each transfection, 0.5 μg of DNA was diluted into 100 μl of serum free medium without antibiotics, whereas 6 μl of CELLFECTIN was also diluted into 100 μl of serum free medium without antibiotics. The two solutions were combined and incubated for 30 minutes at 20°C. Each 200 μl of the mixture was further diluted with 800 μl of medium, and the diluted lipid-DNA complexes were overlaid onto the cells. The cells were incubated at 27°C for 5 hours, and the transfection mixtures were removed, and replaced by 2 ml of serum free medium with antibiotics. The cells were incubated for another 72 hours at 27°C. The transfection procedure was done by Ms. Xi-Ping Huang.

2.10.2.3 Purifying Recombinant Baculovirus by Plaque Assay and Titering of Viral Stocks

At 72 hours post-transfection, the media from the transfection dishes, or transfection viral stock, was collected by centrifugation. The transfection viral stock was vigorously vortexed before used to prepare 10-fold serial dilutions in complete EX-400 media. For each dilution, the final volume was 1 ml. The dilutions made were 10^-2, 10^-3 and 10^-4 dilutions. Cells were carefully seeded at a density of 5 x 10^6 cells/100 mm plate in 5 ml complete EX-400 medium to give a 50% confluence. Three plates were prepared for each viral dilution. The plates were rock at a speed of 8 side-to-side motions per minutes for 10 minute at 22°C to form an evenly distributed cell monolayer. The plates were left standing at a level position for 15 minutes the rocker, and underwent another 30 minutes incubation at 22°C to allow the cells to attach the bottom of the plates.
Once the cells had firmly attached to the plates, 3 ml of medium was removed from each plate. One ml of viral dilution was added dropwise to each plate. The infected plates were then incubated at 22°C on a slowly rocking platform for 1 hour. The medium was then aspirated from each plate, and 5 ml of agarose-medium mix was withdrawn using a sterile 10-ml plastic pipette. The mix was prepared during the incubation time by combining 50 ml of 2.5% baculovirus agarose with 50 ml of complete EX-400 medium supplemented with 150 μg/ml of X-Gal, and kept in 47°C water bath. The agarose was slowly streamed against the side edge of the plate so that it flew down the side and across the plate with minimum disturbance of the cell monolayer. After the agarose was set, the plates were sealed in a plastic bag with paper towels slightly dampened with 5 mM EDTA to prevent the plates from drying out. The sealed plates were incubated at 27°C for 5-6 days, or until plaques were well formed.

To isolate viral DNA from the recombinant blue plaques, a sterile pipette tip was used to penetrate the agarose and remove the cell monolayer containing the plaque. The agarose plug was transferred to one of the wells on a 12-well microtiter plate in which 5 x 10⁵ Sf9 cells were seeded with 2 ml of complete EX-400 medium. The microtiter plate was then placed in a sealed plastic bag and incubated at 27°C for 72 hours. The entire procedure was repeated using the viral stock purified from the first plaque assay to further improve the purity of the stock.

High titer viral stocks were generated by infecting 100 ml (1x10⁶ cells/ml) of Sf-9 cell culture in a 100-ml spinner flask and incubating for 7 days at 27°C with constant stirring at 80 rpm. At 7 days post-infection, the culture medium was collected by centrifugation at 1,500 x g for 10 minutes at 22°C. The supernatant, denoted the P3 stock, was transferred to new tubes and stored at 4°C, while an aliquot for the purpose of long-term storage was taken and kept at -70°C.
Once the P3 stock was generated, the titer of the virus was determined by an end-point-dilution assay. The procedures for plaque assay and titration of baculovirus stocks were carried out by Ms. Xi-Ping Huang.

2.10.3 Expression Analysis of Truncated mGluR4 and mGluR8 in Insect Cells

Both the infected Sf-9 cells and the culture media were collected 48 hour post-infection, and subjected to SDS-PAGE and immunoblotting analysis. To examine the solubility of the recombinant proteins, P3 stocks were used to infect Sf-9, MG-1 and High 5 cells. For each type of cells, four 100-mm plates of cells were infected with m4Tr-549-pBlueBac4.5 P3 stock. Cells were harvested at 48 hours post-infection. One ml of the harvested cell-medium mix was removed from each culture, and centrifuged at 14,000 x g in a 1.5 ml microcentrifuge tube. The cell pellets were resuspended in 100 μl of PBS and subjected to sonication for two 10-second periods at level 14 (Heat System, Ultrasonics Inc.). The samples were then centrifuged at 100,000 x g for 45 minutes in an Airfuge™ air-driven ultracentrifuge (Beckman). The pellets and the supernatants were analyzed by immunoblotting separately.

The rest of the cell cultures were subjected to centrifugation at 1,380 x g, and crude membrane preparations were obtained as described in previous section. To test the ligand-binding ability of the truncated mGluR4 expressed in insect cells, these membrane samples were subjected to single point [³H]L-AP4 binding assay using 30nM [³H] L-AP4. Same procedures were repeated for sf9 cells infected with m8Tr-pBlueBac4.5 P3 stock.

Sf9 cells infected with m4Tr-pK503-19 and m8Tr-pK509-8 were subjected to immunoblotting experiment to examine the protein expression. Samples were collected 72 hours post-transfection by washing the cells off the plate with culture media. Whole cell fractions were
separated from the culture media by centrifuging at 1,380 x g for 5 minute at 22°C. The supernatant was subject to centrifugation at 100,000 x g for 45 minutes before undergoing dialysis. For sample preparation for [3H]L-AP4 binding assay, soluble samples were dialyzed against the binding assay buffer supplemented with 0.1 mM PMSF and 1 mM EGTA. The dialyzed samples were subjected to soluble binding assay as described previously. The binding test was done in two repeats.
3.0 Results

3.1 Expression of Truncated Variants of mGluR4 in HEK Cells

A series of plasmids were constructed to produce segments of varying length at the ATD of the mGluR4 receptor. The purpose was to find suitable constructs that were soluble and retained the pharmacological characteristics of the full-length receptor. All the constructs were made using the pcDNA3 mammalian expression vector. This vector contains the transcription enhancer and promoter elements from the immediate-early gene of human cytomegalovirus for high-level, constitutive expression. By using PCR and restriction endonuclease digestion, five constructs were made from the original full-length rat mGluR4a cDNA construct tagged with a myc epitope immediately downstream from the receptor signal sequence. A schematic diagram of the full-length and truncated mGluR4 receptors analyzed in this study is shown in Figure 4.

m4Tr-P586 encodes a receptor that is truncated one amino acid upstream of the junction of ATD and the first TMD. It contains the entire LIVBP homology region and the cysteine-rich region. m4Tr-Y548 terminates 39 upstream of the first TMD. This region includes the entire LIVBP homology region and part of the cysteine-rich region. Both m4Tr-P586 and m4Tr-Y548 contain 115 bp of the 5' untranslated region (UTR) from the original rat cDNA. m4Tr-Y489, which has only 24 bp of the 5' UTR, terminates at the 3' end of LIVBP homology region. The shortest truncated construct, m4Tr-V413, lacks the last 98 amino acids of the ATD, and terminates 76 amino acids upstream of the 3' end of LIVBP homology region.

Since all the truncated constructs retained the original signal peptide from the rat mGluR4a receptor, and none of the constructs contained any putative TMDs, we initially predicted that all the proteins produced from the constructs would be secreted from HEK cells into the cell culture
Figure 4  Schematic diagram of mGluR4 mammalian expression constructs

The truncated mGluR4 receptors are named according to the last amino acid they encode (numbering includes amino acids in the putative signal peptide regions). The dashed lines represent 5' UTR. Open boxes represent the rat signal peptide. Gray boxes represent the seven putative TMDs. Black bars indicate the position of the c-myc epitope.
medium. Furthermore, since m4Tr-P586, m4Tr-Y548 and m4Tr-Y489 all include the entire LIVBP homology region, they were likely to retain ligand-binding properties. Accordingly, we also predicted that m4Tr-V413, in which the LIVBP homology region was truncated, would not have intact binding activity.

The expression plasmids were transiently expressed in HEK cells using calcium phosphate method. Two days after the transfection, both the whole cells and culture media portions of the transfected cultures were collected and examined by immunoblotting. The results of immunoblotting experiments are shown in Figure 5. Immuno-reactive bands were detected in the cellular samples from cells transfected with the expression plasmids, but not the sample from cells subjected to mock transfection. The immuno-reactive proteins appeared to be either very close to or slightly larger than their predicted molecular masses (Figure 5). Glycosylation most likely accounts for the differences between the calculated molecular masses of these proteins and the apparent molecular masses in immunoblot. However, in contrast to the prediction, while immuno-reactive proteins were detected in cellular portions from all the transfected cultures, m4Tr-Y548 was the only truncated receptor that was secreted into the culture medium. Immunoreactive receptor protein was detected in the medium sample from cells expressing m4Tr-Y548, but not in the medium samples from cells expressing full-length mGluR4a, m4Tr-P586, m4Tr-Y489 or m4Tr-V413.

3.2 Analysis of the Oligomeric Structure of m4Tr-Y548

The effects of the reducing agent DTT on the truncated receptor m4Tr-Y548 were examined using SDS-PAGE and immunoblotting. In the presence of high concentrations of DTT (1 – 100 mM), the receptor migrated as a monomer with a $M_r = 71,000$ (Figure 6). Lowering the
Figure 5  Immunoblotting analysis of full-length and truncated mGluR4 receptors expressed in HEK cells. Total cellular proteins (panel A) and media-secreted proteins (panel B) were isolated from transiently transfected HEK cells. Samples containing 12 μg of total cellular protein or 2 μg of total media protein were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with an anti-c-myc mouse monoclonal antibody. The standard molecular weights are indicated in kilodaltons. For both panel A and B: lane 1: mock transfected HEK cells; lane 2: full-length c-myc-mGluR4a; lane 3: m4Tr-P586; lane 4: m4Tr-Y548; lane 5: m4Tr-Y489; lane 6: m4Tr-V413.
concentration of DTT from 1 mM to 0.1 mM produced an abrupt shift in the pattern of the immunoreactive bands; the band corresponding to the monomer disappeared, and two higher immunoreactive bands appeared. The lower molecular weight band migrated at $M_r = 145,000$, whereas the upper band migrated with an estimated $M_r = 202,000$; these likely correspond to dimers and trimers of the truncated receptor. The pattern of bands observed in the presence of 0.01 mM DTT was identical to that seen with 0.1 mM DTT. These results indicate that oligomers of the truncated receptors are formed by intermolecular disulfide bonds.

3.3 Immunocytochemical Analysis on Transfected HEK cells

Immunocytochemistry was carried out using anti-c-myc antibody to further examine the localization of the expressed truncated receptors in HEK cells. Transfected cells were fixed with methanol, and labeled with an anti-c-myc antibody. In cells expressing the full-length mGluR4a, intense fluorescent labeling was seen around the periphery of the cell, indicating cell surface expression. In cells expressing the m4Tr-P586, the immunolabeling was detected in the intracellular compartment. The intracellular labeling was also present in cells expressing m4Tr-Q549, m4Tr-Y548 and m4Tr-Y489 (data not shown).

3.4 Ligand Binding Properties of Truncated Variants of mGluR4

High affinity binding of L-AP4 and L-SOP are unique pharmacological features of group III mGluRs. The binding of group III mGluR specific agonist L-AP4 to the truncated receptors was accessed in a $[^3H]$ L-AP4 binding assay. Binding was examined in both the soluble fractions and the membrane fractions. In the soluble fractions, no specific binding of $[^3H]$ L-AP4 was detected in samples from full-length mGluR4a, m4Tr-P586, m4Tr-Y489 and m4Tr-V413. However, in
Figure 6 The effect of the reducing agent DTT on the soluble truncated m4Tr-Y548. The electrophoresis samples were treated with various concentrations of DTT as indicated, separated on a 10% polyacrylamide gel, and transferred to nitrocellulose. The concentration of DTT in each sample is indicated in the figure. The immunoblot was probed with an anti-c-myc mouse monoclonal antibody.
<table>
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the soluble fraction of the cells expressing m4Tr-Y548, a high level of specific binding was consistently observed (Figure 7, panel A). In the membrane fractions, the amount of specific binding for all the truncated mGluR4 receptors was very low compared with the full-length membrane bound mGluR4a (Figure 7, panel B).

To examine the ligand selectivity of m4Tr-Y548 as a group III mGluR, the binding properties of the soluble truncated mGluR4 were compared with the membrane-bound full-length receptor using glutamate receptor ligands including the nonselective mGluR agonist (1S, 3R)-1-amino cyclopentane-1,3-dicarboxylic acid (1S, 3R ACPD), the group I mGluR antagonist (R, S) a-methyl-4-carboxyphenylglycine (MCPG), and the ionotropic glutamate receptor agonists kainic acid and NMDA (Figure 8). Each drug was tested at a concentration of 100 μM. At the truncated receptor m4Tr-Y548, kainic acid, NMDA and MCPG produced less than 10% inhibition of [3H] L-AP4 binding; a similar low level of inhibition was observed with the full-length mGluR4a. The very low potency of MCPG for mGluR4 has also been reported for experiments on human mGluR4 expressed in CHO cells (Flor et al, 1995). (1S, 3R) ACPD showed a greater degree of inhibition at m4Tr-Y548 (27% of control binding) compared with the full-length mGluR4a (61% of control binding), indicating that this agonist may be more potent at the truncated receptor. This difference in degrees of inhibition was statistically very significant, with a p value = 0.005. The relatively low potency of (1S, 3R) ACPD compared with group III mGluR ligands such as L-AP4 and L-SOP is consistent with previous binding data from mGluR4 expressed in Sf9 insect cells (Thomsen et al., 1997).

The inhibitory potency of the agonists L-AP4, L-SOP, L-glutamate, and cyclobutylene AP5 and the group III mGluR antagonists MAP4, CPPG, and MPPG for the soluble m4Tr-Y548 were compared with the full-length mGluR4a. The rank order of potency of the agonists at m4Tr-
Figure 7 Relative level of $[^3\text{H}]$ L-AP4 binding to the full-length mGluR4a and its truncated variants. All binding assays were carried out using 30 nM $[^3\text{H}]$ L-AP4. Panel A: the soluble, media-secreted fractions containing 7 μg protein from the HEK cell culture transfected with truncated mGluR4. The data are expressed as percentages relative to the soluble m4Tr-Y548. No specific binding was detected in the soluble fraction of the m4Tr-Y489 and m4Tr-V413. Panel B: membrane fractions were prepared from HEK cells transfected with full-length or truncated variants of mGluR4a, containing 125 μg protein in each sample. The relative amounts of binding of the truncated variants are presented as percentage relative to the full-length e-myc-mGluR4a. Each column depicts the mean ± SEM of 3 determinations conducted in triplicate.
A. 

![Graph A: Percent Normalized $[^3H]L$-AP4 Binding vs. Culture Medium Fractions](image)

B. 

![Graph B: Percent Normalized $[^3H]L$-AP4 Binding vs. Cell Membrane Fractions](image)
Figure 8 Inhibition of $[^3H]L$-AP4 binding to the full-length mGluR4a and m4Tr-Y548 by ionotropic and metabotropic glutamate receptor ligands. The specific binding of $[^3H]L$-AP4 to the full-length c-myc-mGluR4a is represented by the open bar, and the specific binding of $[^3H]L$-AP4 to m4Tr-Y548 is represented by the solid bar. Each drug was tested at a concentration of 100 μM. Each column is the mean ± SEM of three experiments.
Y548 was identical to that of the full-length receptor. The affinities of all four agonists were higher for the truncated receptor compared with the full-length receptor. This was also consistent with observation that (1S, 3R) ACPD had higher inhibition of $[^3$H]L-AP4 binding at the truncated receptor. The affinity for the endogenous ligand L-glutamate at m4Tr-Y548 was about 2-fold higher than that at the membrane-bound full-length mGluR4a (Figure 9; Table 1). Except for CPPG, which had a 7-fold decrease in affinity for m4Tr-Y548, the antagonists displayed similar affinities for m4Tr-Y548 compared with the membrane-bound full-length mGluR4a. The rank order of the antagonists at m4Tr-Y548 (MAP4 > MPPG > CPPG) was similar to that at the full-length mGluR4a (CPPG = MAP4 > MPPG) except for CPPG, which was the most potent of the three compounds for the full-length receptor but was the least potent at the soluble receptor (Table 1; Figure 9). The unpaired $t$ test indicated that the differences in affinities of agonists at the truncated and full-length receptors were statistically significant or very significant (the $p$ values are 0.003 for L-AP4, 0.012 for L-SOP, 0.016 for L-glutamate and 0.016 for cyclobutylene-AP5). While the difference in affinity of CPPG at the truncated and full-length receptors was statistically very significant ($p = 0.007$), the differences for the other two antagonists were not statistically significant ($p = 0.139$ for MAP4 and $p = 0.670$ for MPPG).

3.5 Deglycosylation of m4Tr-Y548

The soluble truncated receptor migrated on SDS-PAGE with a $M_r = 71,000$; this is approximately $M_r = 8,000$ larger than the predicted molecular weight based on the amino acid sequence, suggesting that the truncated receptor was glycosylated. Treatment of the soluble fraction containing m4Tr-Y548 with Endo H, which cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from asparagine-linked glycoproteins, produced a small shift
Figure 9  Competition curves for the inhibition of $[^3]H$ L-AP4 binding to m4Tr-Y548 by the agonists L-glutamate, L-AP4, L-SOP and cyclobutylene-AP5 (A) and the antagonists MAP4, MPPG, and CPPG (B). The soluble binding assay was conducted in triplicate using 30 nM $[^3]H$ L-AP4 (except for L-AP4, for which 10 nM $[^3]H$ L-AP4 was used). Each point represents the mean ± SEM of three determinations on samples obtained from two to three different preparations.
A

% of Control vs. Log [Agonist] (M)

L-AP4
L-SOP
Glutamate
Cyclobutylene-AP5

B

% of Control vs. Log [Antagonist] (M)

MPPG
MAP-4
CPPG
Table 1. Binding Constants for Agonists and Antagonists at the Full-Length mGluR4a and m4Tr-548

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<th>m4Tr-548</th>
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<tr>
<td><strong>Agonists</strong></td>
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<tr>
<td>L-AP4</td>
<td>0.12±0.02</td>
<td>0.47±0.05</td>
</tr>
<tr>
<td>L-SOP</td>
<td>0.23±0.03</td>
<td>2.4±0.5</td>
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<td>L-glutamate</td>
<td>2.2±0.6</td>
<td>5.3±0.5</td>
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<tr>
<td>Cyclobutylene-AP5</td>
<td>3.2±0.9</td>
<td>12±2</td>
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<tr>
<td><strong>Antagonists</strong></td>
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<tr>
<td>CPPG</td>
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</tr>
<tr>
<td>MAP4</td>
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<td>25±2</td>
</tr>
<tr>
<td>MPPG</td>
<td>74±28</td>
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All values represent the mean ± SEM of 3 independent measurements. The $K_D$ and $B_{max}$ values for the truncated receptor were 120 ± 10 nM and 33 ± 4 pmol/mg protein, while the $K_D$ and $B_{max}$ values for the full-length receptor were 470 ± 30 nM and 9 ± 0.4 pmol/mg protein.
in the immuno-reactive band from $M_r = 71,000$ to $M_r = 69,000$ (data not shown). However, treatment with PNGase F, which cleaves all asparagine-linked carbohydrates, produced a larger shift to $M_r = 61,500$ (Figure 10, panel A). This molecular weight estimate was slightly smaller than the predicted molecular mass of the nonglycosylated protein (62,700 daltons), but close to that of the nonglycosylated protein after the cleavage of signal peptide (59,300 daltons).

The binding of $[^3H] \text{L-AP4}$ to the deglycosylated receptor was also assessed. $[^3H] \text{L-AP4}$ binding to samples treated with PNGase F was 99% of control samples (average of two independent determinations) that were not treated with the enzyme (Figure 10, panel B), indicating that agonist binding to truncated mGluR4 is not dependent upon asparagine-linked carbohydrates. Approximately 0.15 μg of m4Tr-Y548 was incubated with 100 μl of wheat germ agglutinin-sepharose in 50 mM Tris-HCl and 10 mM NaCl (pH 7.0) at 4°C for 16 hours, and followed by elution with N-acetyl-D-glucosamine, which often used to replace the bound glycoprotein on lectins. Immunoblotting experiment indicated that small amount of the truncated receptor was bound to the lectin, but most of the protein remained unbound. Moreover, N-acetyl-D-glucosamine failed to elute the bound receptors off the lectin-coupled sepharose (data not shown).

3.6 Expression of Truncated mGluR4 and mGluR8 in E. coli

The amount of the soluble receptor m4Tr-Y548 secreted from HEK cells was estimated to be 0.15 μg/ml of culture medium. Since the amount of purified protein required for crystallization experiment is typically in the milligram range, an alternative expression system producing higher amounts of the soluble protein would be desirable. To obtain high level of expression for soluble truncated mGluRs on a large scale, two bacterial expression systems were tested.
Figure 10  Enzymatic deglycosylation of m4Tr-Y548. Panel A: lane 1, cell culture medium from HEK cells transfected with m4Tr-Y548 and incubated at 37°C for 1 hour; lane 2, cell culture medium from HEK cells transfected with m4Tr-Y548 and treated with PNGase F at 37°C for 1 hour. Panel B: histogram summarizing the results of [3H] L-AP4 binding (30 nM) to m4Tr-Y548 with and without PNGase F. Each column is the average of 2 independent experiments.
The DNA fragments encoding the mGluR4 ATD up to Gln549 and the equivalent construct containing the mGluR8 ATD up to Gly543 (Peltekova et al., 2000) were used as the coding sequences for expressions in E. coli. The DNA fragments were inserted into the pBAD/Myc-His vector. The pBAD plasmids are pUC-derived expression vectors designed for inducible, dose-dependent recombinant protein expression and purification in E. coli. The level of expression can be controlled by the L-arabinose concentration in the culture media. Recombinant proteins expressed in this vector were tagged at the carboxyl-terminus with a Myc epitope tag and a 6X His tag (Table 2). The insertions were designed so that the truncated receptors would be translated in-frame with the vector for both the 5' start codon and the 3' sequences of the Myc and His tags.

The expression plasmids were transformed into the TOP10 E. coli strain. This strain is capable of L-arabinose uptake, but not metabolizing it. This is important for expression studies as the level of L-arabinose will be constant inside the cell and not decrease over time. To find the optimal expression condition, L-arabinose was added at final concentrations of 0.2%, 0.02%, 0.002%, 0.0002% and 0.00002%. Immunoblotting showed that the level of expression increased initially as the concentration of L-arabinose increased. However, very little difference in the expression levels was found between the samples induced with 0.02% L-arabinose and that with 0.2% L-arabinose. Therefore, the subsequent expression test were done using 0.02% as the final concentration of L-arabinose.

To test the solubility of the truncated receptors, bacterial cells were sonicated in 100 μl of bacteria lysis buffer and centrifuged at 100,000 x g for 45 minutes to separate the soluble and insoluble proteins. No immuno-reactive band was found in negative control samples (E. coli transformed with pBAD/Myc-His vector). Immunoblotting analysis revealed that immuno-
reactive bands were present only in the insoluble portions of the samples from the m4Tr-pBAD/Myc-His and m8Tr-pBAD/Myc-His transformants (Figure 11, panel A). The immuno-reactive proteins from m4Tr-pBAD/Myc-His and m8Tr-pBAD/Myc-His both migrated at an estimated $M_r = 63,000$, which was very close to the predicted molecular masses for the truncated mGluR4 and mGluR8 (62,700 daltons for truncated mGluR4 and 63,000 daltons for truncated mGluR8). Results from the radioligand binding assay using $[^3]$H] L-AP4 failed to detect specific binding in either the soluble or the insoluble samples.

The cytoplasmic space of E. coli is known to be in a reduced state that prevents the proper formation of disulfide bond. The truncated mGluR4 and mGluR8, each contains 14 conserved cysteines, may therefore be misfolded when expressed intracellularly in E. coli. The two constructs using pET22b vectors, m4Tr-pET22b and m8Tr-pET22b, produced truncated mGluR4 (amino acids 1 to 549) and mGluR8 (amino acids 1 to 543) with an E. coli signal peptide attached to their amino-termini. The signal peptide may localize the truncated receptors to the periplasmic space, which is a more oxidative environment than the cytoplasmic space. Both truncated receptors had a 6X His tag attached to their carboxyl-termini for affinity purification (Table 2). After the addition of IPTG to the growth media, the expressions of these proteins were observed in the E. coli strain BL21 (DE3) transformed with the plasmids. Immunoblotting analysis revealed that m4Tr-pET22b and m8Tr-pET22b proteins were expressed in both cytoplasmic/membrane fractions and periplasmic extracts (Figure 11, panel B). The truncated receptors in the cytoplasmic/membrane fractions migrated at a $M_r$ of 62,400, which is similar to the predicted molecular masses for truncated receptors. The receptors in the periplasmic extracts migrated on SDS-PAGE at $M_r = 61,000$, which is slightly smaller than the predicted molecular masses. In both the cytoplasmic/membrane fractions and periplasmic extracts, the expression
Figure 11  Immunoblotting analysis of truncated mGluR4 and mGluR8 expressed in E. coli. Panel A: Intracellular expression of truncated mGluR4 and mGluR8 in E. coli (TOP10) using pBAD/Myc-His vector. Lanes 1, 2 and 3 contain insoluble intracellular fractions from the transformed E. coli cells. Lane 1: pBAD/Myc-His transformant (negative control); lane 2: m4Tr-pBAD/Myc-His transformant; lane 3: m8Tr-pBAD/Myc-His transformant. Lanes 4, 5 and 6 contain the soluble intracellular fractions from the transformed E. coli cells. Lane 4: pBAD/Myc-His transformant (negative control); lane 5: m4Tr-pBAD/Myc-His transformant; lane 6: m8Tr-pBAD/Myc-His transformant. Samples contained 8 µg of total insoluble proteins or 1 µg of total soluble proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and labeled with an anti-c-myc mouse monoclonal antibody. Resemble immunoblotting results were obtained from 2 independent experiments. Panel B: Periplasmic expression using the pET22b vector. Lanes 1, 2 and 3 contain cytoplasmic and membrane fractions from transformed E. coli cells. Lane 1: pET22b transformant (negative control); Lane 2: m4Tr-pET22b transformant; Lane 3: m8Tr-pET22b transformant. Lanes 4, 5 and 6 contain periplasmic extractions from the transformed E. coli cells. Lane 4: pET22b transformant (negative control); Lane 5: m4Tr-pET22b transformant; Lane 6: m8Tr-pET22b transformant. Samples contained 8 µg of total cytoplasmic/membrane proteins or 0.8 µg of total periplasmic proteins from the m4Tr-pET22b transformant and 0.1 µg of total periplasmic proteins from the m8Tr-pET22b or pET22b transformant were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and labeled with an anti-mGluR4/8 rabbit polyclonal antibody. The blots shown are representative of 3 independent experiments. Arrow indicates the periplasmic expressions of m4Tr-pET22b and m8Tr-pET22b.
level of m4Tr-pET22b was much higher than that of m8Tr-pET22b. In the cytoplasmic/membrane fraction of the bacteria expressing m4Tr-pET22b, multiple immunoreactive bands migrating at smaller Mₐₜ than the predicted receptor molecular mass were observed (Figure 11, panel B). These smaller bands may represent the fragments produced by protease degradation of the receptor protein. The periplasmic extracts were subjected to [³²P]L-AP4 binding analysis. No significant specific binding of the radiolabeled agonist was seen in either of the truncated receptors (data not shown).

3.7 Expression of Truncated mGluR4 and mGluR8 in Insect Cells using the Baculovirus Expression System

DNA fragments encoding truncated mGluR4 containing amino acids 1 to 549 and mGluR8 containing amino acids 1 to 543 were inserted into baculovirus transfer vector pBlueBac4.5 (Table 2). After obtaining the recombinant viruses, they were used to infect three different insect cell lines: SF-9, MG-1, and High 5. At 72 hours post-infection, whole cells and culture media were collected separately and subjected to immunoblotting analysis. Immunoreactive proteins were found in cells infected with the recombinant viruses, but not in the uninfected cells. Immunoreactive bands were present at high level in the cellular portions of all cell types, but not in the culture media (Figure 12, panel A). The truncated mGluR4 migrated at a Mᵦ = 64,900, which is slightly larger than the predicted molecular mass for the nonglycosylated truncated mGluR4 (62,700 daltons). The Mᵦ of the truncated mGluR8 appeared to be approximately 68,000, which is also larger then the predicted molecular mass for the nonglycosylated truncated mGluR8 (63,000 daltons). Multiple immunoreactive bands with lower molecular weights were
Figure 12 Immunoblotting analysis of truncated mGluR4 and mGluR8 expressed in sf-9 insect cells. Panel A: expression analysis on samples from sf9 cells infected with m4Tr-pBlueBac4.5 and m8Tr-pBlueBac4.5. Lane 1: whole cell fraction of uninfected sf9 cells. Lane 2: whole cell fraction of cells infected with m4Tr-pBlueBac4.5. Lane 3: whole cell fraction of the cells infected with m8Tr-pBlueBac4.5. Lane 4: medium fraction of uninfected sf9 cells. Lane 5: medium fraction of cells infected with m4Tr-pBlueBac4.5. Lane 6: medium fraction of cells infected with m8Tr-pBlueBac4.5. Panel B: expression analysis on samples from sf9 cells infected with m4Tr-pK503-19 and m8Tr-pK509-8. Lane 1: whole cell fraction of uninfected sf9 cells. Lane 2: whole cell fraction of cells infected with m4Tr-pK503-19. Lane 3: whole cell fraction of the cells infected with m8Tr-pK509-8. Lane 4: medium fraction of uninfected sf9 cells. Lane 5: medium fraction of cells infected with m4Tr-pK503-19. Lane 6: medium fraction of cells infected with m8Tr-pK509-8. Samples contained 8 μg of total cellular proteins or 1 μg of total medium proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and labeled with an anti-mGluR4/8 rabbit polyclonal antibody. The blots shown are representative of 3 independent experiments.
Table 2. Features of the Expression Constructs for Truncated mGluR4 and mGluR8 in *E. coli* and Insect Cells

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Expression Host</th>
<th>Host Strain</th>
<th>Vector System</th>
<th>Signal Sequence</th>
<th>Expected Localization</th>
<th>Tag*</th>
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<td><strong>E. coli</strong></td>
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<td>Truncated mGluR4</td>
<td>TOP10</td>
<td>pBAD/Myc-His</td>
<td>Rat</td>
<td>Intracellular</td>
<td>M: C-terminal, H: C-terminal</td>
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<td>Truncated mGluR8</td>
<td>TOP10</td>
<td>pBAD/Myc-His</td>
<td>Rat</td>
<td>Intracellular</td>
<td>M: C-terminal, H: C-terminal</td>
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* M = Myc epitope tag; H = His tag; F = FLAG epitope tag.
detected in the cellular fractions of cells expressing truncated mGluR8; these bands most likely resulted from protease degradation. Infected cells were collected and sonicated in PBS. After high-speed centrifugation in an ultracentrifuge at 100,000 x g for 45 minutes to separate the soluble proteins from the insoluble proteins, all samples were analyzed by immunoblotting experiments. Only low amounts of the receptor proteins were detected in the supernatants, whereas higher levels were found in the insoluble samples (data not shown). Cell membranes, soluble cytoplasmic fractions and cell culture media from infected Sf-9, MG-1 and High5 cells were prepared and subjected to radioligand binding assay. However, no specific binding of [3H] L-AP4 was detected in any of the samples.

The original cDNAs of rat mGluR4a and mGluR8a encode signal peptides of the receptors, which were 32 and 33 amino acids in length respectively. To test whether replacing the rat signal sequence with a signal peptide originating from baculoviral protein AcNPV ecdysone glucotransferase would facilitate the secretion of the truncated receptors from insect cells, DNA fragments encoding truncated mGluR4 amino acids 33 to 548 and mGluR8 amino acids 34 to 543 were generated by PCR and subsequent subclonings. These DNA fragments, lacking the rat receptor signal sequences, were inserted into pK503-19 (truncated mGluR4) and pK509-8 (truncated mGluR8), both derivatives of pFastBac vector. These vectors contained the sequence encoding the baculoviral signal peptide upstream of their multiple cloning regions (Table 2). As the result, the rat receptor signal peptides were replaced by the baculoviral signal peptide when the truncated receptors were expressed in sf9 cells. The recombinant proteins were tagged with FLAG epitope at the amino-termini and 6X His tag at the carboxyl-termini.

Immunoblotting analysis detected the expression of the truncated receptors in samples from sf9 cells transfected with m4Tr-pK503-19 and with m8Tr-pK509-8. The truncated receptors
were detected in the both culture media and the whole cell fractions (Figure 12, panel B). In the whole cell fractions, both the truncated mGluR4 and the truncated mGluR8 migrated at a $M_r = 64,900$. The approximately 2,000 dalton differences between the relative molecular weights and the predicted molecular masses for the truncated receptors are likely due to the glycosylation of the receptors. The truncated mGluR4 and mGluR8 receptors were detected in the cell media. They migrated at $M_r = 63,800$, which is approximately $M_r = 4,000$ larger than the predicted sizes for the nonglycosylated truncated receptors with the signal peptides cleaved (59,700 daltons). Samples of the cell culture media were dialyzed against binding buffer and subjected to [$^3$H]L-AP4 binding analysis. However, in two experiments, no specific binding was detected (data not shown).
4.0 Discussion

4.1 Pharmacology of the Soluble Truncated mGluR4

The cDNA construct encoding m4Tr-Y548 produced a protein that extended from the amino terminus to Tyr548, which is located 39 amino acids upstream from the junction of the ATD and the first putative TMD of mGluR4. This region of ATD includes the entire LIVBP homology region and part of the cysteine-rich region. The protein was secreted into the cell culture medium, indicating that the protein did not possess regions of sufficient hydrophobicity to cause retention in the endoplasmic reticulum or plasma membrane of the cell and that it was soluble in an aqueous environment.

A pharmacological analysis of the m4Tr-Y548 was conducted by assessing the ability of various mGluR4 ligands to compete with the binding of the radiolabeled agonist [3H]L-AP4. The results indicate that the binding properties displayed by the soluble m4Tr-Y548 reflect the unique pharmacological profile of the group III mGluRs. Ligands at group I mGluRs or ionotrophic glutamate receptors showed little or no affinity for either the full-length mGluR4a or m4Tr-Y548. Except for the antagonist CPPG, the rank order of potency for a series of group III mGluR ligands at m4Tr-Y548 was the same as at the full-length membrane-bound mGluR4a. These observations suggest that the primary determinants of [3H]L-AP4 binding are conferred by residues present in the ATD of mGluR4. However, the inhibition constants for L-glutamate, L-AP4, L-SOP, and cyclobutylene AP5 were lower than those of the full-length receptor, indicating that the truncated soluble receptor displayed higher affinities for agonists compared with the full-length receptor. The differences in affinity of the ligands were likely not caused by differences in assay conditions for the soluble and membrane-bound receptors, since the affinities of the
soluble receptor for antagonists were either similar (to MPPG and MAP4) or lower (to CPPG) compared with the full-length receptor.

Strikingly similar results were reported in the study of a truncated mGluR8 receptor (m8Tr-G543), which terminates 40 amino acids upstream of the first putative TMD of mGluR8 (Peltekova et al., 2000). This receptor displayed a similar pharmacological profile to the full-length mGluR8. However, as observed with the soluble mGluR4 receptor, the affinities of the truncated mGluR8 for agonists were higher than that of the full-length mGluR8 receptor. The antagonist CPPG had 10-fold decrease in affinity for the soluble receptor, whereas another antagonist, MAP4, displayed similar affinities for the soluble and full-length mGluR8 receptors. These observations suggest that the increase in affinities for agonists and the decrease in affinity for CPPG are shared features of the soluble truncated mGluR4 and mGluR8.

Interestingly, in a recent study of a soluble truncated GABA_B1 receptor, the agonist-binding affinities of a soluble GABA_B1 receptor were also increased compared with the full-length GABA_B1 (Malitschek et al., 1999). This increase was also observed with a truncated GABA_B1 receptor that contained a single TMD and was therefore membrane-anchored. Moreover, despite the increased affinity to agonists, the rank order of affinities was unchanged by the truncations. These observations are remarkably similar to our findings with mGluRs. However, unlike our results on mGluR4, the affinities of antagonists at the truncated GABA_B1 receptors did not differ from those at the full-length receptor.

The change in the ligand affinities after the truncation suggests that regions of the full-length receptor excluded from m4Tr-Y548 and m8Tr-G543 might influence the affinities. These regions include the TMDs, the extracellular loops between the TMDs, and the 39 amino acids between Tyr548 and the first TMD. In the full-length receptor, the ATD may possibly interacts
with one or more of these regions after ligand binding, which in turn induces changes in the affinity for the ligand. In a model proposed by Pin and Bockaert (1995), it has been suggested that agonist binding to the mGluRs may induce a conformational change that allows the ATD to interact with a region within the TMDs. Such an interaction may result in a decrease in agonist affinity. According to this model, the TMDs do not influence the affinity of antagonists because antagonists are not expected to promote the interaction between the ATD and the TMDs. Pin and Bockaert's model explains the higher agonist affinities observed with the m4Tr-Y548, m8Tr-G543 and the truncated GABA<sub>6</sub>R1s, since the TMDs do not exist in these receptors. The observation that antagonists MAP4 and MPPG displayed similar affinities for the m4Tr-Y548 and the full-length mGluR4 is also supported by the model. However, the model fails to address the 7-fold decrease in the affinity of CPPG for the soluble receptor compared to the full-length mGluR4. An alternative explanation is that the high-affinity agonist site observed in the truncated receptors represents an alternate conformational state of the lower-affinity site present in the full-length receptor. A possible reason for the increase in agonist affinity is that the binding domain of the soluble mGluR4 is locked in a slightly changed state due to the structural constraints imposed by the truncation. The new conformation may also have reduced affinity for larger molecules such as CPPG.

Whether or not ligand binding in other mGluRs is affected by regions outside the ATDs remains to be determined. Although the pharmacological results reported by Okamoto et al. (1998) on the binding of [³H]quisqualic acid to the soluble mGluR1 receptor are difficult to compare with ours because they did not report IC<sub>50</sub> values for L-glutamate or other compounds that were assessed in this study, it appears that the affinity for [³H]quisqualic acid for the soluble receptor was very similar to the affinity measured for the full-length mGluR1 receptor. One
difference between the truncated mGluR1 receptor and the m4Tr-Y548 is that the former contained the entire ATD of mGluR1, whereas the latter did not include the 39 amino acids immediately upstream from the first TMD. Our results demonstrated that this region is not required for high affinity binding of [3H]L-AP4 to mGluR4. This observation is consistent with mutational and chimeric studies conducted on mGluR1 and mGluR4, which showed that several residues located in the more amino-terminal regions of the ATDs of the receptors are required for ligand binding (O'Hara et al., 1003; Takahashi et al., 1993; Hampson et al., 1999). However, we cannot rule out the possibility that these 39 amino acids of mGluR4 may affect the affinity of ligands for the binding site.

Of interest in this regard are studies on the receptor for the glycoprotein hormones luteinizing hormone and choriogonadotropin. The luteinizing hormone/choriogonadotropin receptor is a member of the G-protein-coupled receptor family and, like mGluRs, it has a large ATD that can be separated from the seven TMDs and still retain ligand binding activity. Radioligand binding studies using 125I-labeled human choriogonadotropin have shown that the exodomain (equivalent to the ATD) alone has a higher affinity for the glycoprotein hormone than the full-length receptor (Xie et al., 1990; Ryu et al., 1998). A structural analysis of this receptor using site-directed mutagenesis has shown that several amino acids in the second extracellular loop within this TMD region affect the affinity of the ligand for the receptor. It was suggested that residues within this loop constrain the affinity of the hormone for the receptor (Ryu et al., 1998). Although the amino acid sequences of the mGluRs are not homologous with the glycoprotein hormone receptors, it is possible that in mGluR4 the extracellular loops or the regions immediately upstream of the TMDs are involved in a similar mechanism to constrain the affinities of the agonists for the binding site located in the first 548 amino acids of the receptor.
Many GPCRs display biphasic binding curves indicative of the presence of high and low affinity binding sites. The commonly accepted explanation for high and low affinity binding sites is that they reflect receptors with bound and unbound G-protein, respectively. However, some GPRCs, such as muscarinic acetylcholine receptors, show multiple states of affinity in the absence of G-proteins (Perterson et al., 1984; Wreggett and Wells, 1995). Conversely, other GCRPs such as the cloned mGluR4 receptors display only a single class of sites in radioligand binding assays (Eriksen and Thomsen, 1995; Thomsen et al., 1997); it is possible that lower affinity sites may exist but cannot be detected with binding assay. Alternatively, it is possible that the unitary nature of the binding state of the cloned membrane-bound mGuR4 receptor reflects only a low affinity state due to the absence of the appropriate G-proteins in the host cell lines. However, it has been observed that co-expression of full-length mGluR4 with various mammalian G-protein subunits including Gi1, Gi2, Gi3, and Go in insect sf9 cells does not significantly increase the affinity or the capacity (Bmax) of [3H]L-AP4 for mGluR4 (L. Blythe and D. R. Hampson, unpublished observation). Moreover, [3H]L-AP4 binding experiments conducted in rat (Hudtloff and Thomsen, 1998) and mouse (Thomsen and Hampson, 1999) brain have detected only a single class of sites with a K_D value very similar to that seen with the cloned receptor. Thus, the binding site in the full-length receptor with an affinity for [3H]L-AP4 in the 400 – 500 nM range may represent the high affinity state of mGluR4. If this is true, the comparable affinity for [3H]L-AP4 at m4Tr-Y548, which lacks G-protein coupling region, suggests that the G-protein coupling may not be required for the high affinity state of the receptor.

Although the radioligand binding assays conducted in this study and a number of previous studies revealed only one binding site in mGluR4, the possible existence of a second binding site
is suggested by a recent study on a mGluR4 variant that functions as a taste receptor in lingual tissue (Chaudhari et al., 2000). Compared to the brain form mGluR4a, this receptor starts at Met308 and lacks about 50% of the ATD (amino acids 1 to 588 in brain mGluR4a). Interestingly, none of the amino acid residues that are believed to be critical in ligand binding to the brain form mGluR4, including Arg78, Ser159 and Thr182, are present in this taste form mGluR4. A functional assay measuring the inhibition of cAMP formation revealed that the taste receptor has a ~100-fold lower sensitivity to L-AP4 than the full-length brain form. The EC₅₀ value of L-AP4 for taste form mGluR4 was about 100 μM, whereas that for the brain form mGluR4 was between 0.5 to 1.0 μM. The low binding affinity displayed by the taste form receptor may represent the binding of agonists to a second binding site in mGluR4. This low affinity binding site could be in the carboxyl-terminal half of the ATD that remained in the taste form mGluR4. Alternatively, it could reside in the TMDs or extracellular loops. In the radioligand binding assay, a binding site with such a low affinity would not be detected.

4.2 Minimal Sequence in mGluR4 Ligand Binding

A primary objective of this study was to probe the limits of truncation of the mGluR4 ATD in terms of producing truncated proteins that retained the ability to be secreted and bind ligands. Since the shortest construct analyzed in this study (m4Tr-V413) contains a truncated LIVBP homology domain, it was not surprising that this protein did not possess binding activity. The second shortest construct (m4Tr-Y489) also failed to display any binding activity in the soluble or membrane fractions, despite the fact that this protein does include the entire LIVBP homology region. This observation is in agreement with the results obtained with a mGluR1 receptor, which is truncated at a site equivalent to 8 amino acids downstream of the carboxyl-terminus of
m4Tr-Y489. This truncated mGluR1 (mGluR103), like m4Tr-Y489, was incapable of binding agonists (Okamoto et al., 1998). The low expression level of mGluR103 was suggested as the possible reason for the protein to lack binding ability. It is possible that the LIVBP homology region by itself is sufficient for imparting ligand-binding capabilities to mGluRs. However, the segment of amino acid sequence between the site of truncation at 39 amino acids and 98 amino acids upstream from the first TMD, contains information that greatly facilitates the secretion of the truncated receptors. This region contains 6 of the 17 cysteine residues that are conserved in the ATDs of all 8 mGluRs and the homologous calcium-sensing receptor; it is likely that one or more of these 6 cysteines are required for maintaining the 3-dimensional structure of the receptor in its native conformation.

Further comparisons between our results and those obtained with chimeric and truncated GABA\textsubscript{B}R1 receptors are informative. Of particular interest are experiments conducted using chimeric receptors possessing portions of the ATD of the GABA\textsubscript{B}R1a subunit fused to downstream sequences of the mGluR1b receptor. A chimeric receptor containing the ATD of the GABA\textsubscript{B}R1a subunit encompassing all of the ATD except the last 40 amino acids immediately upstream of the first TMD (termed aN550) bound a GABA\textsubscript{B} receptor photoaffinity ligand, whereas a construct fused 60 amino acids upstream of the first TMD (termed aN530) did not bind the photoaffinity label (Malitschek et al., 1999). These observations are strikingly similar to our results with truncated mGluR4 receptors.

We also examined the construct that encoded the entire ATD of mGluR4 (m4Tr-P586). This protein was truncated at the junction of the ATD and the first putative TMD. Surprisingly, although it contained no TMD, the protein was not secreted from transfected cells and did not bind \[^{3}H\]L-AP4. This observation suggested that one or more residues in the intervening
sequence between the first TMD and the truncation site 39 amino acids upstream, inhibited protein secretion. The lack of binding ability may be directly due to the presence of the 39 amino acid segment, or alternatively, as a consequence of the intracellular retention of the protein. One study on a mGluR8 receptor terminated at the junction of the ATD and the first TMD reported results resemble our observations on m4Tr-P586 (Peltekova et al., 2000). This 39 amino acid segment of polypeptide contains 3 conserved cysteines. The 3 cysteines are also present in the truncated mGluR8. It is possible that one or more of these 3 cysteines might inhibit secretion and binding by forming aberrant intramolecular disulfide bonds with other proteins in the endoplasmic reticulum and therefore inhibit the normal folding of the receptor.

In support of this speculation, a cysteine-to-serine mutation in the calcium-sensing receptor was required in a conserved cysteine residue located immediately upstream of the first TMD for secretion of the ATD (Goldsmith et al., 1999). These results, however, differ from those obtained from the analogous construct of mGluR1. A truncated mGluR1 containing the entire ATD was secreted and bind agonists when expressed in insect cells with a baculovirus expression system (Okamoto et al., 1998). Although the reason for this difference in secretion is not obvious, one possibility may be the presence of N-linked carbohydrate side chains near the carboxyl-terminal end of the truncated proteins. mGluR4 and mGluR8 both have a consensus sequence for N-glycosylation 19 residues upstream from the first TMD; this site is missing in mGluR1 and the GABA<sub>B</sub>R1 receptor subunit in which the entire ATD can also be expressed as a secreted protein (Malitschek et al., 1999). Although this carbohydrate side chain, if present in the wild-type receptor, might confer some useful function, the presence of the carbohydrate close to the carboxyl terminus of the truncated receptors might impair secretion from the cell.
Together these results suggest that the critical boundaries for secretion and activity in group III mGluRs and in GABA<sub>B</sub>Rs are similar. Our results suggest that there are strict limitations on the proper folding of truncated versions of the ATDs of mGluR4 and mGluR8. Importantly, the LIVBP homology region in conjunction with part of the cysteine-rich region is required for optimal secretion in a soluble form that retains ligand binding activity.

4.3 Biochemical Characterization of the Soluble Truncated mGluR4

The soluble truncated mGluR4, m4Tr-Y548, contains four consensus sequences for asparagine-linked glycosylation. The decrease in the molecular weight of the truncated protein by about \( M_r = 8,000 \) after complete deglycosylation indicates that one or more of these sites are glycosylated. An analysis of the effects of deglycosylation on the binding of \([^{3}H]L-AP4\) to the soluble receptor demonstrated that the bound carbohydrate is not required for agonist binding. Although our data show that asparagine-linked oligosaccharides are not required for ligand binding to mGluR4, it is possible that the bound carbohydrates may be important in the subcellular targeting of this receptor in the nervous system. Despite the presence of oligosaccharides in the truncated receptor protein, wheat germ agglutinin did not effectively remove the receptor protein from cell culture medium, indicating that wheat germ agglutinin is not the most suitable lectin for the purification of m4Tr-Y548. The type of lectin suitable for protein purification may depend on the type of oligosaccharides on the protein. For example, RCA-1 lectin was determined to be the most efficient lectin to qualitatively purify a soluble, truncated human CaR (Goldsmith et al., 1999). Pilot test such as screening with a lectin affinity chromatography screening kit may identify the most efficient lectin to isolate m4Tr-Y548 from the cell culture medium.
The soluble m4Tr-Y548 exists as a monomer in the presence of high concentrations of a reducing reagent and as a dimer and trimer in the presence of low concentrations of the reducing agent. Dimeric forms of all the mGluR subtypes have been observed on immunoblots of brain tissue and transfected cells (Pickering et al., 1993; Hampson et al., 1994; Petralia et al., 1996; Pekhletski et al., 1996; Romano et al., 1996; Nomura et al., 1994; Shigemoto et al., 1997). Romano et al. (1996) have shown that mGluR5 migrated on SDS-PAGE as a dimer under nonreducing conditions and as a monomer under reducing conditions, indicating that the dimers are formed by intermolecular disulfide bonds. Analysis of the electrophoretic mobility of a truncated construct of mGluR5 indicated that the intermolecular disulfide bonds are formed between cysteine residues located in the ATD of mGluR5 (Romano et al., 1996). Modulation of monomer and dimeric forms by reducing agents was also seen with the soluble truncated form of the mGluR1 receptor (Okamoto et al., 1998; Robbins et al., 1999). The dimerization of soluble truncated mGluR1 ATD occurred in the endoplasmic reticulum, and is not dependent on protein glycosylation as it was not prevented by treatment of the cells with tunicamycin.

Despite the studies outlined above providing evidence for dimers of mGluRs, no data exist demonstrating a direct link between the ability to bind ligands or activate signal transduction pathways and a particular oligomeric configuration of an mGluR. However, in this study the [3H]L-AP4 radioligand binding assay was carried out in the absence of reducing agents, whereas the addition of reducing agent DTT decreased binding activity in a concentration-dependent fashion. Despite the observation that at a concentration of 1mM or higher DTT caused the soluble truncated mGluR4 to migrate on SDS-PAGE as a monoer, the bindings of [3H]L-AP4 to samples containing DTT at concentrations of 0mM, 0.01mM, 0.1mM, 1mM, 10mM and 100mM decreased sequentially. The effect of DTT in reducing [3H]L-AP4 binding is likely due to the
disruption of both the intramolecular and the intermolecular disulfide bonds. It is likely that in the typical oxidation/reduction environment of the extracellular space in nerve tissue, mGluRs exist as oligomeric complexes. The oligomeric configurations of mGluRs as they exist in nerve cells in vivo are not known. However, our observations on the ATD of mGluR4 together with the results on mGluR1 and mGluR5 noted above all suggest that intermolecular disulfide bonds are a general structural feature of the mGluR family of receptors and that the active form of the receptors is likely to be a multimeric complex.

4.4 Expression of Truncated mGluR4 and mGluR8 in E. coli

To investigate the possibility of higher level expression, m4Tr-Y548 and m8Tr-G543 were expressed in bacteria using two different expression systems. Compared to mammalian cell culture, bacterial expression systems have a number of advantages, including the rapid cell growth, low cost of growth medium, and high expression level. In some cases yields of purified recombinant proteins in E. coli from expression vectors containing T7 promoter have typically been in the range of 10 – 100 mg per liter of culture (Chang et al., 1999). However, expression of mGluRs in a prokaryotic system has not been reported to date. In this study we found that the truncated versions of mGluR4 and mGluR8 maintained ligand binding ability when expressed in mammalian cells, but not when expressed in E. coli.

One common problem in bacterial expression systems is that proteins are often expressed in an inactive form and require refolding. The production of inactive protein may be due not only to the properties inherent in the protein expressed, but also to the rate of accumulation of the protein. The extraordinary protein yields of T7-based systems such as pET system may often be achieved at the expense of solubility (Sun et al., 1997). In many cases, such as the
crystallization of the GluR2 subtype of iGluR, the inactive protein can be denatured by chaotrophic agents such as urea and guanidine–HCl and then refolded into active form with proper conditions (Armstrong et al., 1998). Sometimes the denatured recombinant protein could not be refolded into the active form using conventional methods, but was reactivated by direct solubilization of the inclusion bodies with Triton X-100 (Sunitha et al., 2000). However, in other cases, proteins may resist all attempts to renature them.

The first bacterial expression system I examined was the araB expression system in E. coli. In this system, recombinant protein expression is tightly controlled and readily induced with L-arabinose. The expression level of the recombinant protein with araB system is typically lower than that of T7-based system. However, the truncated mGluR4 and mGluR8 were found insoluble and inactive. Therefore, I expressed the truncated receptors with a bacterial signal peptide for potential periplasmic localization of the recombinant proteins. Since the ATDs of mGluRs are homologues of PBPs that naturally expressed in the bacterial periplasmic space, periplasmic space might provide a better environment for the correct folding of the truncated ATDs. The processes mediating translocation across the cell membrane of E. coli are incompletely understood. The signal peptide is known to be necessary, but not sufficient for export proteins into the periplasm. Periplasmic localization may also depend on determinants within the mature protein (Wickner et al., 1991; Lory, 1992). In the expression of GluR2 subtype iGluR subunit in E. coli with a bacterial signal peptide, the recombinant protein was successfully translocated into the periplasmic space and demonstrated ligand binding activity (Arvola and Keinanen, 1996). However, the level of the receptor was low when expressed in periplasmic space, and cytoplasmic expression was ultimately used for generating crystal (Armstrong et al., 1998). In our study, the truncated receptor proteins found in periplasmic
extracts were at very low levels, whereas most of the proteins remained in the cytoplasmic/membrane fractions. Furthermore, the small amount of proteins present in the periplasmic extracts were incapable of binding to [3H]L-AP4. One possible reason that the mGluR ATDs lack binding activity when expressed in the bacterial periplasmic space is that the ATDs of mGluRs contain cysteine rich regions and the disulfide bonds formed between these cysteines are necessary for the correct folding of the ATDs. The periplasmic space, although more oxidative than the cytoplasmic compartment, may still be too reduced for the proper formation of the disulfide bonds in mGluRs.

4.5 Expression of Truncated mGluR4 and mGluR8 using the Baculovirus Expression System

The third expression host I tested in this study was insect cells. Compared with mammalian cell lines, insect cells typically express recombinant proteins at higher levels. Protein insolubility is generally not a concern when expressing proteins in insect cells, and only simple N-linked glycosylation will be present in the protein.

I have used two different vector systems to express the truncated mGluR4 and mGluR8 in sf9 cells. In the first system, the rat receptor signal peptide was used, whereas in the second system, the rat signal peptide was replaced by a baculoviral signal peptide. Immunoblotting analysis indicated a better secretion of the expressed proteins with the baculoviral signal peptide than that with the rat signal peptide. These observations are different from what Okamoto et al. (1998) reported for mGluR1 ATD, which was secreted into the culture medium from the insect cells by utilizing its original (rat) signal peptide. Our results, however, do bear some similarity to the results obtained in the study of soluble truncated GABABR1 (Malitschek et al., 1999). In that
study, the ATD of \( \text{GABA}_B R_{1a} \) with the rat signal peptide was secreted from the insect cells and into the culture medium. However, the soluble secreted \( \text{GABA}_B R_{1a} \) was detected on the immunoblot at a lower level than the soluble secreted \( \text{GABA}_B R_{1b} \) (the other alternatively spliced form of \( \text{GABA}_B R_1 \)), which had a baculoviral signal peptide. It was not reported whether \( \text{GABA}_B R_{1b} \) with the rat signal peptide was secreted into the culture medium. Interestingly, the two truncated receptors that were secreted into the culture media with their original signal peptides have shorter signal sequence (18 amino acids for mGluR1 and 17 amino acids for \( \text{GABA}_B R_{1a} \)) than that of \( \text{GABA}_B R_{1b} \) (29 amino acids). The lengths of the signal peptides for mGluR4a (32 amino acids) and mGluR8a (33 amino acids) are close to that of \( \text{GABA}_B R_{1b} \). Therefore, one could speculate that the lengths of the original signal peptides for these receptors might affect their secretion. With some proteins, a short mammalian signal peptide might be sufficient for the secretion in insect cells, whereas a longer one may interfere with the proper secretion.

In my study, replacing the rat signal peptides of truncated mGluR4 and mGluR8 with the baculoviral signal peptide did facilitate the secretion of the receptors into cell media. Our observation on the truncated mGluR4 and mGluR8 with the baculoviral signal sequence, however, differs from that of the truncated \( \text{GABA}_B R_{1b} \) in terms of binding activity. With a baculoviral signal sequence, \( \text{GABA}_B R_{1b} \) was secreted from the insect cells and pharmacologically active (Malitschek et al., 1999). The m4Tr-pK503-19 and m8Tr-pK509-8, on the other hand, failed to display binding to \([^3]H\)L-AP4. One possible reason for these truncated receptors to lack binding activity is that, glycosylation on the ATDs of mGluR4 and mGluR8 may affect the proper folding of the proteins. Compared with mammalian cells, insect cells are not capable of producing protein with complex polysaccharides. While our results in this study
indicated that, after m4Tr-Y548 was properly folded, N-linked carbohydrates were not required for binding to $[^3H]$L-AP4, it is still possible that these polysaccharides are involved in protein folding. Another possible explanation for the lack of binding activity seen in the insect cell medium samples containing truncated mGluR4 and mGluR8 is the low levels of proteins present in the samples. As in the study of the truncated mGluR103, the soluble protein was detected in the culture medium with immunoblotting but was not detected by binding assay using $[^3H]$quisqualate (Okamoto et al., 1998)
5.0 Conclusions and Future Considerations

Previous studies using site-directed mutagenesis and chimeric receptors have suggested that the ligand binding pockets of mGluRs are located in the ATDs. My results on truncated mGluR4 receptors (Han and Hampson, 1999, Peltekova et al., 2000) provide a direct evidence that the first 548 amino acids of mGluR4 ATD contain all of the structural information that is necessary and sufficient for ligand selectivity and affinity for the receptor.

A truncated mGluR4 receptor containing only the first 548 amino acids of the full-length mGluR4a was successfully produced in HEK cells as a soluble, secreted protein. The results from [3H]L-AP4 binding assay indicate that this soluble truncated mGluR4 receptor displays a pharmacological profile very similar to that of the full-length wild-type mGluR4a. The affinities for agonists to the soluble receptor were slightly higher compared to that of the full-length mGluR4a. We therefore conclude that the first 548 amino acids of mGluR4, which contain the entire LIVBP homology region and part of the cysteine-rich region, are sufficient to maintain the ligand binding activity and ligand selectivity. Other regions of the receptor may also regulate the ligand binding to mGluR4 through their interactions with the ATD and the subsequent conformational changes in the ligand binding pocket.

The second conclusion from this study is that, there are strict limitations on the proper folding of truncated versions of mGluR4 ATD. Shorter receptors lacking the cysteine-rich region and a longer truncated receptor containing the entire ATD failed to be secreted from the cells and display binding to the agonist [3H]L-AP4. Therefore, when expressed in mammalian cells, the LIVBP homology region and part of the cysteine-rich region are both required for optimal secretion in a soluble form of mGluR4 that retains ligand binding activity.
In the third part of my study, I attempted to express the soluble truncated mGluR4 and its mGluR8 analog in *E. coli* and insect cells. The expressed proteins did not bind $[^{3}\text{H}]$L-AP4. These results suggest that these expression systems may lack the cellular mechanisms for protein modifications, such as disulfide bonding formation and/or glycosylation, which could be crucial for the correct folding of the ATDs of mGluRs.

Future studies could attempt to express the truncated receptor with protein disulfide isomerase in a mutant strain of *E. coli* (FA113) that has a more oxidative cytoplasmic environment. In *E. coli*, disulfide isomerases such as DsbA and DsbC could enhance the rate of disulfide bond isomerization and greatly assist the folding of proteins containing multiple disulfide bonds. This system has been reported to facilitate folding of intracellularly expressed proteins with multiple disulfide bonds (Bessette et al., 1999). If the lack of stable disulfide bonds accounts for the receptor proteins being inactive in *E. coli*, this mutant system could provide the suitable machinery to produce functional truncated receptors. The second approach is to establish a stable mammalian cell line expressing the soluble truncated receptors. Since we have demonstrated that m4Tr-Y548 is soluble and active when expressed in HEK cells, this approach may be the most promising one for the production of a functional soluble receptor. However, as in the study of the soluble truncated human CaR (Goldsmith et al, 1999), a large bioreactor may be required to produce sufficient amount of protein for purification.

In summary, work conducted in this study has (1) demonstrated a role of ATD in ligand binding to mGluR4, and (2) produced a soluble receptor that pharmacologically closely resembles the full-length mGluR4. This soluble receptor provides a starting point for conducting further detailed structural analysis, such as X-ray crystallization, on the binding site of mGluR4.
6.0 References


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