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Structural characterization of intracellular C-terminal domains of group III metabotropic glutamate receptors

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ABSTRACT

Metabotropic glutamate receptors (mGluRs) are regulated by interacting proteins that mostly bind to their intracellular C-termini. Here, we investigated if mGluR6, mGluR7a and mGluR8a C-termini form predefined binding surfaces or if they were rather unstructured. Limited tryptic digest of purified peptides argued against the formation of stable globular folds. Circular dichroism, ^{1}H NMR and $^{1}H^{15}N$ HSQC spectra indicated the absence of rigid secondary structure elements. Furthermore, we localized short linear binding motifs in the unstructured receptor domains. Our data provide evidence that protein interactions of the analyzed mGluR C-termini are mediated rather by short linear motifs than by preformed folds.

1. Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system, acting on ionotropic (ion channel-associated) and metabotropic (G-protein-coupled) glutamate receptors. Metabotropic glutamate receptors (mGluRs) are subdivided into three groups and regulate intracellular signal cascades that modulate neuronal excitability, synaptic plasticity, memory function and neurodegeneration [1]. Group III receptors (mGluR4, mGluR6-8) are negatively coupled to adenylyl cyclase and, with the exception of mGluR6, are suggested to function as presynaptic auto-receptors and low-pass filters [1,2].

Neurotransmission requires a tightly controlled interplay between synaptic proteins, both in space and time. This is accomplished by the formation of synaptic signal complexes that integrate functionally related proteins such as neurotransmitter receptors, enzymes and scaffold proteins. The highest variability between mGluR types is present within their intracellular C-terminal domains and indeed, most described mGluR binding proteins interact with these C-termini (CT) [3]. Importantly, mutations in mGluR binding proteins can lead to neuropsychiatric disorders including addiction, depression, epilepsy and schizophrenia [4].

While the three-dimensional structure of several mGluR binding partners is known, the conformation of the mGluR-CT themselves is poorly characterized. Thus, it is not clear if mGluR-CT contain predefined binding surfaces for interacting proteins, or if these domains are rather unstructured. Therefore, in this study we analyzed the structure of these domains combining biochemical, biophysical and bioinformatic methods.

2. Materials and methods

2.1. Peptide preparation

C-termini of mGluR6, mGluR7a and mGluR8a from rat (Fig. 1) were tagged with glutathione-S-transferase by cloning in pET-41a, expressed in *Escherichia coli* and immobilized on glutathione-Sepharose under native conditions, as described [5]. Peptides were released into the supernatant with Enterokinase (8 U; Novagen, Darmstadt, Germany) for 18 h at 21 °C and analyzed by SERVA-Blue stained Tricine-SDS gels. Because of major side products, the mGluR7a-CT was chemically synthesized with its N-terminus acetylated (AG Henklein, Charité Berlin, Germany).

Abbreviations: CD, circular dichroism; CT, C-terminus; DPC, dodecylphosphocholine; FHA, forkhead associated; HSQC, heteronuclear single quantum coherence; ITSM, immunoreceptor tyrosine-based switch motif; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; mGluR, metabotropic glutamate receptor; NMR, nuclear magnetic resonance; PAH, paired amphiphatic helix; PP1, protein phosphatase 1; SH2/3, Src-homology 2/3; SLiM, short linear motif

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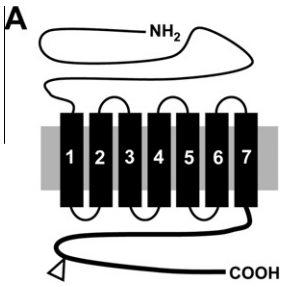

B

Receptor	Source	Weight	Sequence
mGluR6 - CT	E. coli	3.72 kDa	**S**HPEQNVQKRKRSLKKTSTMAAPPQNENAEDAK
mGluR7a-CT	chemical synthesis	7.25 kDa	HPELNVQKRKRSFKAVVTAATMSSRLSHKPSDRPNGEAKTELCENVDP NSPAAKKKYVSYNNLVI
mGluR8a-CT	E. coli	7.39 kDa	**S**HPEQNVQKRKRSFKAVVTAATMQSKLIQKGNDRPNGEVKSELCESLE TNTSSTKTTYISYSNHSI

Fig. 1. Amino acid sequences of mGluR C-termini used in this study. (A) Membrane topology of mGluRs. A grey box represents the membrane, transmembrane regions 1–7 are indicated by black rectangles. The intracellular C-terminus (bold) is alternatively spliced (triangle). (B) Sequences of mGluR-CT generated for this study. Serine residues encoded by the polylinker sequence of the expression vector that remain after Enterokinase cleavage are highlighted in bold. Except for underlined amino acids in mGluR7a, all sequences were predicted to be disordered.

For further purification of mGluR6-CT and mGluR8a-CT, the flow-through from centrifugal filter devices (Millipore, Billerica, MA) with molecular weight cut-offs of 30 kDa (mGluR6) and 50 kDa (mGluR8a) was dialyzed against 20 mM sodium phosphate buffer (pH 7.4) which yielded 500 µl of 80 µM mGluR6-CT and 40 µM mGluR8a-CT. To obtain ^{15}N isotopically labeled mGluR8a-CT, M9 minimal medium with $^{15}NH_4Cl$ (Euriso-top, Saint-Aubin Cedex, France) as sole nitrogen source was used.

2.2. Mass spectrometry

Purified mGluR-CT were prepared for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry performed on an Autoflex (Bruker Daltonics, Bremen, Germany) in the positive-ion mode as described previously [6]. A peptide standard mix (Bruker Daltonics) served as external calibrant. Fifty individual spectra were averaged and analyzed using the FlexAnalysis software (Bruker Daltonics).

2.3. Limited proteolysis

Hen egg-white lysozyme (0.5 µg/ml in 25 mM NH_4HCO_3; Carl Roth, Karlsruhe, Germany) or mGluR-CT were incubated at 37 °C with trypsin (Promega, München, Germany) at an enzyme to substrate ratio of 1:250. After 10 min, 4 h and 18 h the reaction was quenched with 0.1% trifluoroacetic acid, heated for 5 min at 80 °C, vacuum dried and subjected to MALDI-TOF mass spectrometry. Peak masses were identified by comparing with theoretical masses generated from Protein-Prospector MS Digest (http://prospector.ucsf.edu).

2.4. Circular dichroism (CD) and nuclear magnetic resonance (NMR) techniques

CD spectra of mGluR6-CT (80 µM), mGluR7a-CT (30 µM) and mGluR8a-CT (40 µM) were recorded between 260 nm and 190 nm at 25 °C employing a 0.5 mm cuvette in a Jasco J-810 CD spectrophotometer (Jasco, Groß-Umstadt, Germany). Calculated molar ellipticities were utilized to predict secondary structure elements with CONTINLL and CDSSTR [7]. ^1H NMR spectra of mGluR6-CT (80 µM), mGluR7a-CT (200 µM) and mGluR8a-CT (40 µM) were recorded in the presence of 5% (v/v) D_2O with 256 scans and a recycling time of 3 s at 25 °C using 600 MHz or 800 MHz Unity INOVA spectrometers, equipped with HCN-coldprobes (Varian, Palo Alto, CA), multiplied with a Lorentzian–Gauss window function and Fourier-transformed. $^1H^{15}N$ HSQC spectra were recorded with 8 scans and 128 increments in F1 with or without 50 mM dodecylphosphocholine at 15 °C or 25 °C using the 600 MHz spectrometer, multiplied with a cosine window function in both dimensions and Fourier-transformed.

2.5. Bioinformatic methods

Short linear motifs (SLiMs) were detected by pattern searches [8] using the pattern curated in MiniMotif Miner and the Eukaryotic Linear Motif resource. To estimate the functional relevance of identified motifs, we defined an *E*-value that represents the probability of each motif-associated consensus pattern to be present by chance within any given protein, being low for strictly and high for fuzzy annotated patterns.

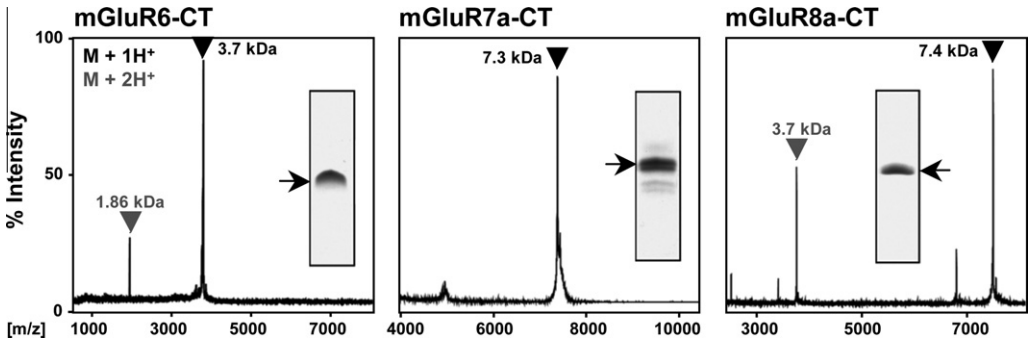

Fig. 2. Characterization of generated mGluR C-termini. MALDI-TOF mass spectra and protein gels (insets) show identity and purity of generated mGluR-CT. Average-isotopic peaks are labeled with observed peptide masses in Dalton. Mass peaks corresponding to mono- or di-protonated masses are marked as $M+1H^+$ (black arrowheads) or $M+2H^+$ (grey arrowheads).