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GDP-bound Gαi2 regulates spinal motor neuron differentiation through interaction with GDE2

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ABSTRACT

Gαi proteins play major roles in the developing and mature nervous system, ranging from the control of cellular proliferation to modulating synaptic plasticity. Although best known for transducing signals from activated seven transmembrane G-protein coupled receptors (GPCRs) when bound to GTP, key cellular functions for Gαi-GDP are beginning to emerge. Here, we show that *Gαi2* is expressed in motor neuron progenitors that are differentiating to form postmitotic motor neurons in the developing spinal cord. Ablation of *Gαi2* causes deficits in motor neuron generation but no changes in motor neuron progenitor patterning or specification, consistent with a function for Gαi2 in regulating motor neuron differentiation. We show that Gαi2 function is mediated in part by its interaction with GDE2, a known regulator of motor neuron differentiation, and that disruption of the GDE2/Gαi2 complex in vivo causes motor neuron deficits analogous to Gαi2 ablation. Gαi2 preferentially associates with GDE2 when bound to GDP, invoking GPCR-independent functions for Gαi2 in the control of spinal motor neuron differentiation.

Introduction

The generation of neuronal diversity in the central nervous system is critical for the formation of functional neural circuits. This complex process involves the integration of signals that trigger neuronal differentiation with those specifying neuronal fates to regulate the timely differentiation of distinct neuronal subtypes (Jessell, 2000; Kintner, 2002). Perturbation of the regulatory networks that control the transition from cell proliferation to differentiation can have serious consequences such as the depletion of progenitor pools, imbalances in neuronal components, tumor formation and ultimately, the disruption of neural networks (Bertram, 2000; Kintner, 2002). However, the molecular mechanisms that regulate the process of neuronal differentiation are still not well understood.

The G-protein subunit, Gα plays diverse roles in the central and peripheral nervous system that include controlling key cognitive and sensory processes such as synaptic plasticity, pain, taste, and olfaction (Wettschurek et al., 2004; Malbon, 2005). These functions primarily involve their ability to mediate signals from a varied and large number of seven transmembrane G-protein coupled receptors (GPCRs) (Neer, 1995; Neves et al., 2002). Gα proteins bind quiescent GPCRs in an inactive GDP-bound form that associates in a complex with Gβ and Gγ subunits. However, upon activation of GPCRs through chemical or physical stimulation, the Gα subunit undergoes a GDP to GTP exchange that causes the Gα/β/γ heterotrimer to release from the GPCR, and dissociate into an active Gα-GTP monomer and a Gβ/Gγ dimer. The Gα-GTP subunit subsequently binds to downstream targets or effector proteins, thereby functioning as a central component in GPCR signaling pathways (Neer, 1995; Neves et al., 2002). The Gα subunit also controls the duration of the GPCR-mediated signal by its intrinsic GTPase activity. Cleavage of GTP to the GDP form inactivates Gα, and results in the reassociation of the α/β/γ heterotrimer with the GPCR. Although initially thought to facilitate Gα binding to GPCRs, Gβ/Gγ heterodimers also mediate GPCR signaling, albeit to a lesser extent than Gα (Neer, 1995; Neves et al., 2002).

Interestingly, emerging studies provide evidence that Gαi proteins have additional functions that are distinct from their roles in transducing GPCR signals. In *C. elegans*, *Drosophila* and vertebrates, Gαi proteins function as important regulators of asymmetric cell division through their ability to orientate and position the mitotic spindle (Gotta and Ahringer 2001; Schaefer et al., 2001; Hampoelz and Knoblich, 2004; Afshar et al., 2004; Du and Macara, 2004). They mediate this function by associating with Pins (LGN in vertebrates) through the Pins GoLoco domain. In contrast to its function in GPCR signaling, the active form of Gαi in spindle positioning is Gαi-GDP, which binds to Pins/LGN instead of Gαi-GTP (Schaefer et al., 2001; Hampoelz and Knoblich, 2004; Du and Macara, 2004). Pins/Gαi complexes are also implicated in vesicle trafficking and localizing NMDA receptors to the cell membrane, a process central to synaptic plasticity (Sans et al., 2005; Knoblich, 2005). In this paradigm, Gαi-GDP elevates the levels of the NMDA receptor subunit NR2B on the membrane in the presence of Pins, thereby increasing synaptic

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transmission. These two examples thus indicate that GDP-bound forms of Gαi have important regulatory functions in the nervous system that are distinct from their direct roles in GPCR-mediated signaling.

Given the importance of Gα proteins in nervous system function, we considered the possibility that Gαi proteins may be required for regulating neuronal diversity. One model system where the molecular pathways that regulate neuronal differentiation and subtype specification are relatively well characterized is in developing spinal motor neurons (Jessell, 2000; Price and Briscoe, 2004). Spinal progenitors located in the ventricular zone (VZ) of the spinal cord are patterned into discrete dorsal–ventral domains through integrating sonic hedgehog (shh), fibroblast growth factor (FGF), and retinoic acid signals (RA) (Diez del Corral et al., 2003; Novitch et al., 2003). Each progenitor domain expresses a unique profile of transcription factors that ultimately regulates their capacity to generate a particular neuronal subtype (Jessell, 2000; Price and Briscoe, 2004). In the case of motor neurons, RA signals induce the bHLH protein Olig2 in ventral progenitors, which acts as a key determinant of motor neuron identity by priming cells to implement motor neuron fate specification programs (Mizuguchi et al., 2001; Novitch et al., 2001; Novitch et al., 2003; Lee et al., 2005). RA subsequently initiates the differentiation of $Olig2^+$ progenitors into postmitotic motor neurons through upregulating the expression of GDE2, a six transmembrane protein containing an extracellular glycerophosphodiester phosphodiesterase (GDPD) domain (Nogusa et al., 2004; Rao and Sockanathan, 2005; Yanaka, 2007; Yan et al., 2009). GDE2 GDPD activity triggers Olig2 downregulation, and synchronizes Ngn2-dependent neurogeneic pathways and motor neuron fate specification networks to drive the differentiation of postmitotic motor neurons (Rao and Sockanathan, 2005; Yan et al., 2009).

Here, we investigate the expression of Gαi proteins in spinal motor neurons and the function of Gαi2 in motor neuron differentiation with respect to known regulators of motor neuron differentiation. We find that different members of the Gαi family are expressed in differentiating and postmitotic motor neurons, suggesting sequential roles for Gαi proteins in motor neuron development. Using loss of function and overexpression assays in the chick spinal cord, we show that Gαi2 plays roles in regulating motor neuron differentiation, and that its function is mediated in part through its interaction with GDE2. Strikingly, Gαi2 preferentially interacts with GDE2 when bound to GDP. These findings identify a role for Gαi2 in spinal motor neuron development, and invoke GPCR-independent functions for Gαi2 in regulating motor neuron differentiation.

Materials and methods

In situ hybridization and immunohistochemistry

Embryos were prepared for immunohistochemistry and in situ hybridization as described (Sockanathan and Jessell, 1998). Tissues were embedded in Tissue-Tek O.C.T. (Sakura Finetek) and 12-μm serial sections were obtained. Primary antibodies used are as follows: K5 (rabbit anti-Isl1/2), 1:2500; guinea pig anti-Isl1/2, 1:10,000 (provided by T.M. Jessell); 4H9 (anti-Isl2), 1:100; 81.5C10 (anti-HB9/MNR2), 1:100 (Developmental Studies Hybridoma Bank, [DHSB]); rabbit anti-MNR2, 1:8000 (provided by B. Novitch); goat anti-βGal, 1:3000 (Arnel); rabbit anti-GFP, 1:2000 (Molecular Probes); mouse anti-PCNA, 1:2000 (SIGMA); rat anti-BrdU 1:100 (Abcam); rabbit anti-phospho-Histone H3(Ser10), 1:200 (Milipore); mouse anti-Nkx6.1 F55A10, 1:50 (DHSB); mouse anti-Pax6 1:250 (DHSB); rabbit anti-Nkx2.2, 1:4000 (provided by T.M. Jessell); rabbit anti-Irx3, 1:8000 (provided by T.M. Jessell); mouse anti-Lim1/2 (1:1; DHSB). Images were captured using a Zeiss LSM 5 Pascal confocal microscope. In situ hybridization was performed as described (Shaeren-Wiemers and Gerfin-Moser, 1993). Quantitation of neuronal numbers was carried out using 5–10 sections/embryo from 5 embryos.

In ovo electroporation and siRNAs

All cDNAs were derived from the chick and subcloned into pCAGGS or a 250 bp fragment from the mouse HB9 promoter-based vector for in ovo electroporation (MN_e; Lee et al., 2004). For siRNA experiments, *Gαi2* siRNA duplexes were electroporated as previously described (Rao et al., 2004). *Gαi2* siRNA sequences (Dharmacon) are as follows: 5′ ACAUCCAGAGCAAGUUUGAUU 3′; 3′UCAAACUUGCUCUGGAUGUUU 5′ Control DsRed siRNA sequences are as published in Rao et al. (2004).

Co-immunoprecipitation assays

Flag or 6XHis epitope tags were fused to the N-terminus of GDE2 or C-terminus of Gαi2 and subcloned into pCAGGS or pCS2 vectors. Transiently transfected HEK293T cells were harvested and homogenized in lysis buffer using standard procedures (Yan et al., 2009). Lysates were incubated with anti-Flag M2 (Sigma) antibody and GammaBind G Sepharose beads (GE Healthcare) or anti-Flag M2 (Sigma)-bound agarose beads overnight at 4 °C under constant rotation. After centrifugation and extensive washing, the precipitated proteins were analyzed by SDS-PAGE and western blot using rabbit anti-His (Santa Cruz), anti-FLAG M2 or rabbit anti-GDE2 antibodies (Yan et al., 2009). For the GDP/AlF_4^- experiments, HEK293T cells were separately transfected with either Flag-GDE2 or Gαi2-6xHis plasmids. Cells were lysed, using 0.1% TritonX lysis buffer (0.1% Triton-X100; 25 mM Tris-Cl, pH7.5; 150 mM NaCl; 10 mM MgCl2; 1/200 SIGMA Proteinase Inhibitors), spun to remove debris, and supernatants transferred to fresh tubes. Gαi2 lysates were split into aliquots, and water, GDP (10 μM final), or NaF (10 mM final) + AlCl3 (30 μM final) were added. Gαi2 lysates were incubated for 30 min at RT, prior to mixing with GDE2 lysates. Anti-Flag antibody was added to each tube, rotated at 4 °C for 3 h, spun, and supernatants were transferred to fresh tubes containing gamma-bind slurry (pretreated with 2% BSA and washed in lysis buffer). Beads and lysates were rotated 1 h at 4 °C, and washed 4 times with lysis buffer supplemented with appropriate concentrations of GDP or NaF/AlCl3. Samples were eluted in sample buffer and run on 10% SDS/polyacrylamide gels, transferred to PVDF membranes, and bands visualized with ECL+ chemiluminiscence autoradiography (Amersham).

Large scale co-immunoprecipitation experiments and LC–MS/MS analysis were performed as described in Yan et al. (2009). Rabbit anti-mGDE2 CT antibodies were used at dilutions 1:1000.

BrdU labeling

To calculate the proliferative index, electroporated embryos were exposed to BrdU for 30 min before dissection and processing as described above (Yan et al., 2009).

Results

Gαs are expressed in developing spinal motor neurons

As a first step to define the function of Gαi proteins in neuronal development, we examined the expression of three members of the *Gαi* family in the ventral spinal cord of Hamburger Hamilton stage (St) 20 chick embryos: *Gαi2*, *Gαi3*, and *Gαi1*. At this stage of development, cells at different stages of differentiation are present; progenitor cells are located medially in the VZ, newly differentiating cells are present in the intermediate zone (IZ), and fully differentiated motor neurons are beginning to settle in the lateral marginal zone (MZ) of the ventral horn (Hollyday, 2001). In situ hybridization analysis shows that *Gαi2* is expressed medially in ventral progenitors, newly differentiating and newly born motor neurons (Fig. 1B). In contrast, *Gαi3* is expressed in newly differentiating and postmitotic motor neurons, while *Gαi1* is