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THE SYK KINASES ORCHESTRATE CEREBELLAR GRANULE CELL TANGENTIAL MIGRATION

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Abstract—The tyrosine kinases of the Syk family are essential components of the well-characterized immunoreceptor ITAM-based signaling pathway. However, ITAM-based signaling typically does not function in isolation. Instead, it is enmeshed in the molecular network controlling cellular adhesion and chemotaxis. Consistent with the increasing number of data involving ITAM-bearing molecules in neuronal functions, we previously depicted a role for Syk kinases in the establishment of neuronal connectivity. In the developing cerebellum, we found that Syk is essentially expressed in the granule cells (GC) and more importantly, phosphorylated on tyrosine residues representative of an active form of the kinase in tangentially migrating GC. In light of these findings, experiments were performed to establish the implication of Syk in this process. We showed that Syk state of phosphorylation is spatiotemporally regulated during GC ontogeny. Moreover, the analysis of external granular layer microexplants treated with a Syk pharmacological inhibitor together with the quantification of ectopic GC in Syk$^{+/-}$; ZAP-70$^{-/-}$ mutant mice brought evidence of a requirement of Syk in GC tangential migration. Syk phosphorylation was induced by EphB2 engagement and locally turned down by a not yet identified factor that could in part explain the restricted pattern of Syk phosphorylation observed along GC migratory route. Whereas Syk kinase activity appeared not essential for ephrin/Eph-mediated axon extension, it might provide polarization signals required for proper nucleus translocation during GC migration. In conclusion, Syk kinase acts downstream of receptors controlling GC tangential migration.

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INTRODUCTION

Tyrosine kinases of the Syk family, Syk and ZAP-70, are essential components of the well-characterized immunoreceptor ITAM-based signaling pathway. Upon immunoreceptor engagement, tyrosines within the ITAM are dually phosphorylated by Src family kinases, providing a docking site for the tandem SH2 domains of the Syk family kinases. These proximal events allow the recruitment and the activation of both kinase families and serve as a critical link to adapter molecules capable of nucleating multiple signaling pathways (Smith-Garvin et al., 2009). Importantly, many adhesion molecules whose expression is not restricted to hematopoietic cells have been shown to signal through an immunoreceptor-like pathway, using an ITAM-transducing subunit in a co-optive manner to recruit and activate Src/Syk kinases (Abram and Lowell, 2007; Ivashkiv, 2009; Letellier et al., 2010; Kazerounian et al., 2011). In this way, ITAM-based signaling intersects with the molecular network controlling cellular adhesion and motility.

Consistent with a growing body of data demonstrating a role for ITAM-based signaling in the establishment of neuronal connectivity (Chuang and Lagenaur, 1990; Yamada et al., 2001; Kitano et al., 2002; Kaifu et al., 2003; Hamada et al., 2004; Ogawa et al., 2007; Baudouin et al., 2008; Boulanger, 2009; Xu et al., 2010; Angibaud et al., 2012; Louveau et al., 2013), in previous studies we depicted activated forms of Syk kinases in specialized populations of migrating neurons or projecting axons. Notably this applied for tangentially migrating cerebellar granule cells (GC) (Hatterer et al., 2011). Moreover we identified ephrin/Eph as adhesion molecules utilizing ITAM-bearing molecules and associated Syk kinases for growth cone collapsing response (Angibaud et al., 2011; Noraz et al., 2016). Finally, we brought *in vivo* evidence of the involvement of Syk kinases in commissural neuron axon guidance during spinal cord midline crossing (Noraz et al., 2016). On the basis of these findings, in this paper, we have studied the role of the Syk kinases in driving GC tangential migration in the developing cerebellum.

GC originate from a germinal zone in the upper rhombic lip (Miale and Sidman, 1961; Alder et al., 1996). In mice, GC precursors leave the rhombic lip around embryonic day 13 (E13) and migrate rostrally over the surface of the cerebellar anlage to form a mitotically

active region called the external granular layer (EGL) (Hatten and Heintz, 1995). Between E18.5 and P16.5 GC clones divide actively in the outer part of the EGL (oEGL) and then differentiate and migrate over a three-day period into mature GC (Komuro et al., 2001; Espinosa and Luo, 2008). Post-mitotic GC first go through an initial tangential migration, extending axons parallel to the pia surface along the medial–lateral axis in the inner part of the EGL (iEGL) (Komuro et al., 2001). Subsequently, GC migrate radially through the molecular layer (ML) and the Purkinje cell layer (PCL) to a final position in the internal granular layer (IGL) (Komuro and Rakic, 1995). Whereas several adhesion or guidance molecules have been implicated in GC migration (Chedotal, 2010; Kilpatrick et al., 2012), their downstream intracellular components are still largely unknown.

Here, we brought evidence of Syk kinases acting downstream of receptors controlling GC tangential migration. The involvement of Syk kinases in this process was established *ex vivo* using EGL microexplants and *in vivo*, on Syk$^{+/-}$; ZAP-70$^{-/-}$ double mutant mice. The ephrinB2/EphB2 pathway was identified as responsible for the modulation of Syk state of activation in this system.

EXPERIMENTAL PROCEDURES

Animals

Wild-type C57BL/6 mice (Janvier Labs) were used in this study. Syk heterozygous and ZAP-70 homozygous mutant mice (Syk$^{+/-}$; ZAP-70$^{-/-}$), on a C57BL/6 background (at least 10 backcrossing), were a kind gift from V.L. Tybulewicz (Division of Immune Cell Biology, MRC National Institute for Medical Research, London). Syk$^{+/-}$; ZAP-70$^{-/-}$ and Syk$^{+/+}$; ZAP-70$^{-/-}$ mice were generated by intercrossing and genotyped as described previously (Noraz et al., 2016). Mice were bred and maintained in Transgenic Animal Care Facility (ALECS, Lyon, France). Animal care and procedures have been conducted according to the European Community Council Directive 2010/63/UE and the French Ethics Committee.

Antibodies

The description of antibodies is presented in Table 1.

Tissue section immunofluorescent labeling

Tissue sections were obtained from mouse embryos (E18) as well as postnatal (P1, P6 and P13) and adult animals (6–10 weeks) as previously described (Hatterer et al., 2011). For immunostaining, sections were first incubated in blocking buffer, PBS 0.3% Triton X-100 (PBS-T) containing 3% BSA for 1 h at RT and then with primary antibodies overnight at 4 °C. After three washes in PBS-T, sections were incubated with appropriate Alexa Fluor-conjugated Abs diluted in blocking buffer for 1 h at RT. After three washes in PBS-T and two washes in PBS, sections were treated with 0.1 μg/ml DAPI (4′, 6-Diamidine-2-phenylindole dihydrochloride) for 5 min to label the nuclei and then mounted in Fluor Preserve reagent (Merk Millipore). For control experiments, samples were treated following the same procedure but primary Abs were omitted.

Quantification of ectopic GC in the ML

Sagittal sections prepared from the cerebellar vermis of adult mice were stained with anti-Pax6 Abs to label GC nucleus. A mosaic image containing a section of the whole cerebellum was acquired using the Axiovision software 4.7. A region of interest corresponding to the ML total area was defined using ImageJ and the number of Pax6-positive nuclei was counted. Results were expressed as the number of GC per mm^2 of ML. Four sagittal sections were analyzed per animal.

Culture of EGL microexplants

Cerebella were dissected out from postnatal P4-P5 mice, placed in cold HBSS (Gibco) containing 0.5% glucose (Sigma–Aldrich) and freed from meninges. The cerebellar vermis was taken out with a razor blade and cut into 300 × 300 -μm fragments using a McIlwain tissue-chopper. Such obtained microexplants were collected in complete medium, BME medium (Gibco) supplemented with 1% N2 (Gibco), 0.1% BSA (Sigma–Aldrich) 1.2 mM L-Glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco). Microexplants containing the EGL were then selected under the microscope and put onto 24 × 24-mm-coated glass coverslips (200 μg/ml poly-L-lysine and 20 μg/ml laminin, Sigma–Aldrich) placed in 60-mm Petri dishes. After 10 min at room temperature, the time for microexplants to attach to the substrat, 2 ml of complete medium was added and microexplants were cultured at 37 °C for 48 h. Microexplants were then washed in PBS and fixed in PBS 4% PFA 2% sucrose for 20 min.

Microexplants immunofluorescent labeling

Immunostaining of fixed microexplants was essentially similar to tissue sections. Co-immunostaining of EphB2 receptor and ephrinB2 cell-bound fraction was realized on live cells at room temperature. Microexplants were treated with ephrinB2-Fc and control Fc recombinant proteins (R&D Systems) that had been previously preclustered with an anti-human Fc biotinylated Ab at a 1:2 ratio in plain BME medium for 2 h at RT. Preclustered ephrinB2-Fc and control Fc (1 μg/ml) were applied in bath and incubated for 15 min at RT. After 2 washes in PBS microexplants were incubated for 15 min with goat anti-EphB2 Ab diluted in complete medium, washed twice in PBS and further incubated for 15 min in complete medium containing Alexa Fluor 488-conjugated anti-goat secondary Ab and Alexa Fluor 546-conjugated streptavidin to visualize ephrinB2 cell-bound fraction. After 2 washes in PBS, microexplants were fixed. For the co-immunostaining of ephrinB2 cell-bound fraction and phospho-Syk, microexplants treated as above with ephrinB2-Fc and control Fc were washed twice in PBS, fixed and permeabilized in blocking buffer for 1 h. Anti-phospho-SykY348 Ab were then added overnight at 4 °C and after 3 washes in PBS-T,