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FAK

Dynamic integration of guidance signals at the growth cone

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During the formation of neural circuitry, axons are known to be guided to their specific targets by a relatively small arsenal of guidance signals. However, the molecular integration of this guidance information inside the axonal growth cone (GC) is still baffling. Focal adhesion kinase (FAK) is a cytosolic kinase which interacts with a complex molecular network via multiple phosphorylation sites. Paradoxically, FAK activation is required by both attractive and repulsive cues to control respectively axon outgrowth and disassembly of adhesive structures together with cytoskeletal dynamics. It was suggested that FAK might work as a versatile molecular integrator switching to different functions depending on its activation state. Two studies published recently by our group and Woo et al. shed light on this issue: for the first time, these works report a detailed molecular analysis of FAK activation and phosphorylation pattern in primary neuronal cultures in response to the repulsive cues Semaphorin3A and ephrinA1 respectively. Here we comment on the major novelties provided by these papers in the context of previous literature and we speculate on the future avenues of investigation opened by these works.

Key words: FAK, growth cone, semaphorin, ephrin, netrin

Abbreviations: GC, growth cone; FAK, focal adhesion kinase

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Summary

During axon navigation, growth cones read guidance signals in the extracellular environment to reach their specific targets. Many studies in the last decades showed that neural wiring is regulated by a surprisingly small number of guidance cues. Depending on the receptor repertoire and on the signaling machinery expressed by a given neuron, these cues trigger multiple signaling cascades in the GC. However, the molecular integration of these signals is still poorly understood. A plausible model envisions that the diverse signaling cascades may converge on master regulators of GC motility acting as molecular integrators.

FAK is a signaling molecule that controls cell motility and maturation of adhesive structures downstream to disparate cues such as integrins, growth factors, semaphorins, ephrins and netrins.[1-4] Various studies in cell lines showed that FAK is a complex molecular switch that functions both as cytosolic kinase and as scaffold protein with multiple tyrosine phosphorylation sites. Nevertheless, this vast literature leaves many key questions unanswered. In particular, (1) how can we explain the apparent paradox that FAK activation is required for both attraction and repulsion? (2) How does FAK modulate the response to graded low and high signal intensities sensed by the GC in physiological conditions? Two novel studies published by our group[2] and by Woo[3] et al. provide interesting insights to these fundamental questions.

It Is Not What You Activate but How You Do It!

FAK is a biosensor that links external stimuli to multiple intracellular pathways to control the maturation of adhesive structures, cell shape and motility.[1] Several studies in non-neuronal cells revealed that FAK interacts with many

Table 1. Multiple regulation of FAK activation state

Cue	Y397	Y407	Y576	Y577	Y861	Y925	Time	Ref.
Netrin1	=	+	++	++	+	=	5'	4
Netrin1	++	na	na	++	+++	na	5' and longer times	5
Netrin1*	+	+	++	+++	++	=	ns	6
Sema3A	++	--	+3'; =5'	++3'; +5'	--	++	3' and 5'	2
ephA1	++	--	=	na	++	na	5'	3

Resume of FAK phosphorylation pattern under Netrin1, Sema3A and eprhin-A1 stimulation. FAK Y397 is the autophosphorylation site and is the priming step in FAK activation; it also permits the interaction with Src kinase. Y407 negatively regulates FAK activity. Y576 and Y577 are in the catalytic loop and their phosphorylation unleashes the maximal enzymatic activity of the kinase. Y861 is in a scaffold domain and its phosphorylation was linked to increased motility. Y925 is a binding site for Grb2 and this phosphorylation cause the dissociation of Paxillin from FAK and adhesion points. =, unchanged; +, increased; -, decreased. *Biochemical assay performed in HEK293 cells. na, not addressed; ns, not specified.

partners in different cell types and cellular compartments. We summarize here some of the key findings of this huge literature (reviewed in ref. 1). The best-characterized FAK phosphorylation event is the autophosphorylation at Tyr397. This phosphorylation event is the first step in FAK activation for integrins, growth factors and G-protein-linked stimuli that promote cell motility and it creates a binding site for SRC-FAMILY KINASES (SFKs). Additionally, this motif is recognized also by other SH2-DOMAIN containing proteins, such as the Shc adaptor protein, p120-RasGAP, the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and others. Within the FAK/Src complex, Src phosphorylates FAK at Y861 which is located in the C-terminal domain. This promotes the association with p130 Cas and facilitates Rac activation, lamellipodia formation and cell migration. Y407 is associated with the negative regulatory function on FAK in cell lines and is phosphorylated upon serum starvation, contact inhibition as well as netrin[4-6] and VEGF stimulation.[7] Y925 phosphorylation creates a binding site for Grb2, which overlaps with the paxillin binding site at the C-terminal, and it was suggested to be involved in the turnover of focal contacts. The complexity of FAK is function is further enhanced by its capacity to interact with both activators and/or inhibitors of various small GTPase proteins (Rho, Rac, Cdc42 and Ras), which link FAK to cell migration via the control of adhesions and the polymerization or stabilization of actin and microtubules filaments. Notably, whereas $FAK^{-/-}$ cells show slow migration speed and directional motility, FAK overexpression or activity promotes of the migration and the invasive phonotype of carcinoma cells.[1] Considering all these controversial results and different roles of FAK depending on the cellular type or environment, in this commentary we discuss the last evidences of FAK function in axon guidance.

Since FAK activation is required by both attractive[4-6] and repulsive[8] cues, we may infer that not the activation per se but how FAK is activated determines its function. In our work, we tested this hypothesis using primary hippocampal cultures as a model.

It was previously reported that Netrin1[4-6] stimulation induces FAK phosphorylation in multiple tyrosine residues. On the other hand, FAK plays a crucial role in the signaling of the repulsive cue Sema3A in cortical neurons.[8] In our study,[2] we scrutinize the mechanism of FAK activation in Sema3A signaling. We show that after addition of Sema3A, FAK is activated and highly phosphorylated in Y925 (see **Table 1**) and this phosphorylation leads to Paxillin dissociation from the adhesion points. In addition, Sema3A treatment causes the phosphorylation of two residues in the catalytic loop of FAK (Y576, Y577), which results in the full activation of FAK kinase activity. Concurrently, the addition of Sema3A provokes the phosphorylation of α-actinin, a direct substrate of FAK, which leads to the disorganization of actin filaments in focal contacts.[9] Besides, we observed that, upon Sema3A stimulation, Y861 was dephosphorylated. Interestingly, under Netrin1 stimulation Y861 is highly phosphorylated while Y925 does not rise notably (see **Table 1**). Therefore, we suggest that FAK might have an on/off mechanism of these tyrosines in response to attractive or repulsive cues, which affects the scaffolding properties of its carboxy-terminal domain. Another remarkable difference between the response to Sema3A and Netrin1 is the regulation of Y397 and Y407. Since it was suggested that Y407 is a site of negative regulation of FAK,[10] Y407 phosphorylation is expected to decrease in conditions in which Y397 phosphorylation increases. Consistently, in our study we show that Sema3A induces FAK activation by increasing of Y397 phosphorylation and concurrently decreasing Y407 phosphorylation. Netrin1 was reported to increase Y397 phosphorylation in different ways[4-6] (we suppose that diverse culture conditions most likely account for these discrepancies; e.g., Liu and colleagues observe a high level of phosphorylated Y397 already in basal conditions). What's more, Netrin1 also induced Y407 phosphorylation. We might speculate that this unexpected increase in Y407 under Netrin1 stimulation concomitant with the phosphorylation of Y861, could be explained as a negative feedback system to avoid FAK overactivation in these experimental conditions. In fact, FAK overactivation[1] and the phosphorylation of Y861 were implicated in cell migration/invasion and oncogenic transformation in cell lines.[11] In sum, we propose that FAK controls the axonal response to both growth and repulsion signals by switching to different functional states revealed by differences in its phosphorylation pattern. Future work will be required to understand the intricate dynamic of these FAK phosphorylation events.

But, what happens if we compare the level of phosphorylation of FAK in response to molecules with similar action

as Sema3A and ephrin-A1? In a recent work, Woo et al.[3] analyzed the effect of ephrin-A1 and FAK in retinal ganglion neurons. On one hand, they studied the phosphorylation pattern of FAK and the adhesion point dynamic under ephrin-A1 subcollapsing doses; on the other, they analyzed the effect of ephrin-A1 on axonal growth in two neuronal types, nasal and temporal retinal ganglion cell.

The effect of ephrin-A1 largely overlaps with that of Sema3A (see **Table 1**). Treatment with ephrin-A1 caused a dose-dependent increase in the intensity of p-Y397 FAK within growth cones and also caused a significant decrease of p-Y407. However, in contrast to Sema3A stimulation, Y576 FAK phosphorylation was not significantly effected by ephrin-A1 treatment. Besides the possible differences due to the use of antibodies on fixed tissue[3] or protein lysate,[2] this might depend on the fact that Woo et al. looked at Y576 phosphorylation at 5 min. In fact, we observe that Sema3A induces a quick phosphorylation of Y576 at 3 min which is rapidly reversed at 5 min. Unfortunately, Woo et al. did not check Y577, the other kinase activation site that is phosphorylated by Sema3A. Moreover, ephrin-A1 also increased FAK phosphorylation at Y861. Noteworthy, Woo et al. observed that, at low doses, ephrin decreases adhesion turnover but does not induce GC collapse. Instead, at high doses, they observed GC collapse as after Sema3A stimulation in Chacón et al. work. Considering that Y861 phosphorylation is linked to the activation of Rac pathway, which positively regulates lamellipodia formation in GC,[12] we hypothesize that Y861 dephosphorylation might be involved in GC collapse. It will be interesting to test this hypothesis and to compare FAK phosphorylation pattern at low and high doses of ephrin-A1: this will reveal whether these differences between Sema3A and ephrin-A1 are related to the cue or to the growing conditions of the GC.

Interestingly, Woo et al. found opposite responses to ephrin-A1 addition between nasal and temporal neurons (see **Table 2**): before ephrin-A1 addition, temporal axon outgrowth was significantly faster than nasal axon outgrowth. However, upon the addition of ephrin-A1, temporal axons significantly slowed, while nasal axons accelerated. To check FAK activation, the authors look at Y397 phosphorylation. Notably, they found that the basal level of FAK phosphorylation at Y397 is higher in temporal than in nasal axons; in addition, ephrin-A1 stimulation increased Y397 phosphorylation in both neuronal types (see **Table 2**). Therefore, Woo et al. proposed that after ephrin-A1 addition Y397 phosphorylation in nasal axons reaches a level that promotes maximal outgrowth: levels of p-Y397 above this level, as is the case in temporal axons upon ephrin-A1 stimulation, or below this level, which is the case of nasal axons in basal conditions, decrease outgrowth.

Table 2. FAK activation controls axonal speed

Neuronal type	ephrin	p-Y397 FAK	Axon speed
Nasal neurons	-	+	++
	+	++	+++
Temporal neurons	-	++	+++
	+	+++	+

Summary of the levels of FAK Y397 phosphorylation and axonal speed in nasal and temporal retinal neurons pre- and post-ephrin-A1 induction.

FAK and SFKs Connection in Axonal Response

As FAK, Src and SFKs were shown to be involved in both attractive and repulsive response to guidance cues. Binding to FAK Tyr 397 results in the activation of Src which in turn phosphorylates key FAK residues as Tyr576, Tyr577, Tyr861 and Tyr 925. Hence, the association of SFKs with FAK is crucial to FAK signaling activation. Consistently, kinases of the Src family are involved in FAK dependent axonal responses to the chemoattractants as Netrin-1 and NGF[4,6,13] and to repulsive cues as Sema3A[8] and ephrin-A1.[9] Interestingly, activation of the FAK/Src signaling cascade distinguishes attractive from repulsive axonal responses of neurons forming the anterior commissure. Sema3B induced the membrane relocalization of FAK and the recruitment of Src kinases to FAK in neurons with attractive but not repulsive responses.[14] Surprisingly, the pharmacological inhibition of Src transformed the effect of Sema3B from attractive into repulsive. On the other side, Src is not required for the role of Fak is in RGM/neogenin repulsive signaling.[15] In basal conditions, FAK mediates the interaction between neogenin and Ras-specific GTPase-activating protein (GAP) p120GAP, which binds to FAK Y397. RGM stimulation induced dephosphorylation of FAK at Y397 and dissociation of p120GAP from FAK, thereby enabling the association of p120GAP with GTP-RAS and its inactivation, which is required for the RGMa-mediated repulsive function in cortical neurons. Notably, Src does not seem necessary for this function since pharmacological inhibition of Src did not affect Y397 phosphorylation and the association of FAK with p120GAP in this model.

Conclusions and Perspectives

It was hypothesized that FAK might integrate multiple guidance cues by specifically switching on and off different signaling routes depending on the various inputs.[1] The short time-course analysis of Sema3A induced FAK activation that we performed revealed two major facts: the high dynamicity of FAK activation pattern and its specificity compared to the Netrin1 induced pattern. While the phosphorylation state of some tyrosines increases or decreases linearly between 3 and 5 min (e.g., Y397 and Y407) the phosphorylation of other residues peaks at 3 min and remains at the same level or decreases at 5 min (e.g., Y576, Y577 and Y925). As this time course was reported for the first time, we cannot say if this high dynamicity is specific of the neurons, maybe linked to the high motility of the GCs, or if it is a general feature of FAK activation. We speculate that these different phosphorylation patterns could be related to different FAK roles over the time-course of GCs collapse and retraction. For instance, a short stimulation by Sema3A might cause

FAK to inhibit the assembly new adhesion points thus blocking axonal outgrowth. A prolonged stimulation might then put FAK in a state where it actively promotes the disruption of existing adhesion points leading to GC retraction. In fact, the dynamic of Sema3A induced GC stop and retraction observed in time lapse experiments is consistent with this hypothesis (M.R.C., unpublished data).

The idea of different functional activation states of FAK is further corroborated by the comparison between Netrin1 and Sema3A induced phosphorylation patterns which differ in some key residues as Y407 or Y925. In particular, we show that Sema3A causes a two-fold increase in Y925 phosphorylation (which remains stable or slightly increases upon Netrin1 induction) and this is crucial for FAK driven and axonal retraction, most likely via the dissociation of Paxillin from adhesion points.

On the other side, Woo et al. showed that the function of FAK can also vary depending on intensities of the input. At low non-collapsing doses, ephrinA1 induces the stabilization of retinal ganglion neuron GCs via FAK activity. Moreover, a balanced activity of FAK in nasal and temporal neurons is required for their optimal axonal outgrowth. Consistently, Woo et al. showed that FAK is required for the proper topographic connections of nasal and temporal axons in vivo.

Taken together, these works prompt us to review the traditional way of looking at FAK activation. If Y397 phosphorylation remains a good biochemical read out for FAK activation, we now know that this parameter alone gives us little information on the functional outcome of this key molecular switch. Future studies will reveal how the dynamic regulation, in time and intensity, of FAK phosphorylation pattern controls FAK activity and the interaction with its multiple partners. Moreover, the multifaceted function of FAK likely depends on the different cells types and/or developmental stages, which imply the superimposition of FAK signaling on diverse signaling contexts.

In addition, an important issue was never tackled to our knowledge: how does concomitant stimulation with diverse guidance cues, as it happens in physiological conditions, impact on FAK activity. In fact, many signaling molecules involved in FAK function, such as SFKs and Rho family GTPases, are also regulated by various guidance cues. In our opinion, a linear additive model, where an attractive molecule simply counterweight a repulsive one, would hardly explain how a few dozens of guidance molecules can precisely master the wiring of billions of neurons. Hence, it would be interesting to test whether simultaneous stimulation with multiple cues can reveal more complex synergistic and/or permissive interactions between guidance cues.

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