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The cytoplasmic domain of neuropilin-1 regulates focal adhesion turnover

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ABSTRACT

Though the vascular endothelial growth factor coreceptor neuropilin-1 (Nrp1) plays a critical role in vascular development, its precise function is not fully understood. We identified a group of novel binding partners of the cytoplasmic domain of Nrp1 that includes the focal adhesion regulator, Filamin A (FlnA). Endothelial cells (ECs) expressing a Nrp1 mutant devoid of the cytoplasmic domain (*nrp1*$^{cyto\Delta/\Delta}$) migrated significantly slower in response to VEGF relative to *the cells expressing wild-type Nrp1* (*nrp1*$^{+/+}$ cells). The rate of FA turnover in VEGF-treated *nrp1*$^{cyto\Delta/\Delta}$ ECs was an order of magnitude lower in comparison to *nrp1*$^{+/+}$ ECs, thus accounting for the slower migration rate of the *nrp1*$^{cyto\Delta/\Delta}$ ECs.

Structured summary of protein interactions:
NRP1 physically interacts with **alpha enolase**, **Myh10**, **Myh9**, **EEF1alpha1** and **FlnA** by anti bait coimmunoprecipitation (View interaction)
FlnA and **NRP1** colocalize by fluorescence microscopy (View interaction)
NRP1 and **rab11** colocalize by fluorescence microscopy (View interaction)
NRP1 physically interacts with **Myh10**, **Dync1h1**, **Myh9** and **EEF1alpha1** by anti bait coimmunoprecipitation (View interaction)
NRP1 and **FlnA** bind by isothermal titration calorimetry (View interaction)
NRP1 physically interacts with **p130Cas** by anti bait coimmunoprecipitation (View interaction)
NRP1 and **p130Cas** colocalize by fluorescence microscopy (View interaction)
NRP1 binds to **FlnA** by surface plasmon resonance (View interaction)

1. Introduction

Nrp is a single-pass transmembrane protein, which has a highly conserved short cytoplasmic domain that lacks catalytic activity [1,2]. While the downstream signaling of the tyrosine kinase receptor of vascular endothelial growth factor (VEGF)-A, VEGF receptor 2 (VEGFR2), has been the subject of numerous studies and its major elements have been established [3], the contribution of Nrp1 to VEGF signaling is less well understood. Several studies suggested that Nrp1 might have a VEGFR2-independent function in mediating VEGF signaling [4–7]. Indeed, Nrp1 has binding partners other than VEGFR2, such as receptors of the plexin family [8], but it remains unclear how the cytoplasmic domain of Nrp1 mediates signaling. Presumably, the cytoplasmic domain could recruit other proteins that may be able to transduce VEGF signaling. The best studied Nrp1 cytoplasmic binding protein is synectin, also named Nrp1-interacting protein (NIP) [9]. Synectin contains a single PDZ (postsynaptic density 95, disk large, zona occludens-1) domain, through which it binds to the C-terminus of Nrp1. Synectin recruits the molecular motor myosin VI to uncoated endocytic vesicles, thus facilitating the trafficking of endocytosed membrane receptors [10], such as VEGFR2 in complex with Nrp1, to promote arteriogenesis [11].

Focal adhesions are large aggregates of proteins that anchor the cell to the extracellular matrix and that undergo cycles of assembly and disassembly during cell movement [12,13]. Among other signals, the dynamics of FAs are regulated by growth factors [14],

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including VEGF-A [15], a major stimulant of vasculogenesis and angiogenesis during development and in the adult organism [16,17]. Treatment with VEGF increased FA density in ECs [18]. The signaling of VEGF to FAs appeared to follow the canonical VEGFR2-dependent pathway, and to be mediated by the non-receptor FA kinase (FAK), and by a closely related kinase, Pyk2 [19] The VEGF-induced increase in FA assembly was accompanied by an increase in EC migration. Though Nrp1 had not been observed directly in FAs [19], proteomics studies identified the VEGF co-receptor Nrp1 as a FA component [20].

The regulation of FA turnover is not fully understood, and it is likely to involve multiple molecular mechanisms [13]. One of these invoked FlnA [18], a large scaffold protein that binds filamentous actin (F-actin) and numerous other proteins, including transmembrane receptors [21,22]. FlnA is composed of 24 IgG repeats including a C-terminal dimerization domain [21]. Most of its ligands, excluding F-actin, bind to the last three C-terminal IgG repeats.

Here, we investigated if the Nrp1-mediated intracellular signaling of VEGF originates from a protein complex bound to the cytoplasmic domain of Nrp1. We first combined immunoprecipitation and mass spectroscopy to identify several novel Nrp1-associated proteins, including FlnA. Separately, we found by immunoprecipitation and immunoblotting that the scaffold protein p130Cas also associates with Nrp1. Both proteins are known to be involved in the dynamics of FAs [23]. We tested, therefore, if Nrp1 is involved in FA turnover. Our results support this premise.

2. Materials and methods

See Supplemental materials.

3. Results

3.1. Identification of novel ligands to the cytoplasmic domain of Nrp1

We used a proteomics approach to identify cytoplasmic proteins that associate with Nrp1. Immunoprecipitates of Nrp1 from quiescent and from VEGF-A_{164}-treated (20 ng/ml, 5 min) primary mouse heart ECs were resolved by SDS–PAGE. The band patterns of the two samples were partially different (Fig. 1ote, Table 1). The proteins were identified by liquid chromatography–tandem mass spectroscopy (LC–MS). In the quiescent ECs, these proteins were cytoplasmic dynein heavy chain 1 (Dync1h1), myosin heavy chains 9 and 10 (Mhy9, Mhy10), and eukaryotic translation elongation factor 1 α1 (EEF1α1). Mhy9, Mhy10, and EEF1α1 were present in the immunoprecipitate from both VEGF-treated and quiescent ECs. Mhy9 and Myh10 are subunits of non-muscle myosin II, known also as heavy chains IIa and IIb. They are involved in endosomal trafficking [24], cytokinesis [25], and cell shape remodeling [26], and are localized to early endosomes, stress-fibers, and FAs [27]. The proteins unique to the VEGF-treated sample were α-enolase and Filamin-A (FlnA). Due to its connection to the actin cytoskeleton and protein transport, the involvement of FlnA was investigated further.

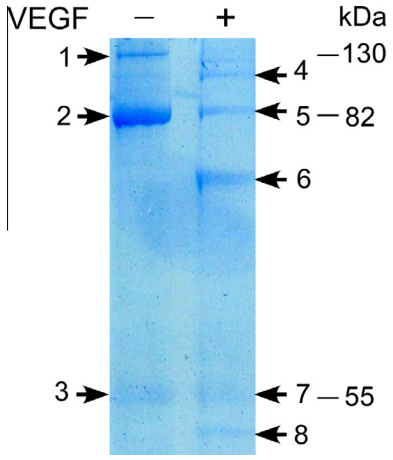

Fig. 1. Identification of novel binding partners for the cytoplasmic domain of Nrp1. Coomassie-stained tris-glycine acrylamide (10%) showing bands of proteins immunoprecipitated by Nrp1 from primary mouse heart ECs incubated without or with 20 ng/ml VEGF-A_{165} for 10 min. (1) Dynein heavy chain; (2) myosin heavy chains 9 and 10; (3) eukaryotic translation elongation factor 1 α1; (4) filamin A; (5 and 6) myosin heavy chains 9 and 10; (7) eukaryotic translation elongation factor 1 α1; (8) a enolase. None of these bands were present in a sample that was immunoprecipitated by non-immune goat IgG (not shown). Table 1 listing the proteins that were coimmunoprecipitated together with Nrp1, and whose corresponding bands were identified by LC–MS.

Table 1
Protein ligands of the Nrp1 cytoplasmic domain that were identified by LC–MS. The protein name, gene name, peptide coverage, and Mascot scores are listed.

Protein	Band number	Gene name	Peptides	Mascot score
Non-muscle myosin IIa heavy chain	2, 5, 6	*myh9*	83	5871
Non-muscle myosin IIb heavy chain	2, 5, 6	*myh10*	40	2479
Cytoplasmic dynein heavy chain 1	1	*dyhc1*	4	148
Filamin A	4	*flnA*	2	85
Eukaryotic translation elongation factor 1 α1	3, 7	*ef1α1*	5	275
α enolase	8	*enoa*	3	175

3.2. FlnA binds Nrp1 directly

Given that FlnA binds directly several classes of transmembrane receptors [21,22], we sought to determine if the association of FlnA to Nrp1 is similarly direct. As shown by the MS analysis, we identified Nrp1 as an FlnA-associated protein by co-immunoprecipitation from the lysate of *nrp1*$^{+/+}$ ECs (Fig. 1). We used two independent in vitro approaches to test if FlnA and Nrp1 are capable to bind directly to each other. First, we used surface plasmon resonance (SPR) to determine the equilibrium binding kinetics between FlnA and the Nrp1 cytoplasmic domain. FlnA is a modular protein consisting of 24 immunoglobulin-like (Ig) repeats, which bind several proteins including F-actin. We tested the interaction of Nrp1 cytoplasmic region with the most common binding sites of FlnA, the repeats 10–11, 18, 19, 20, 21, 22 and 23–24 of FlnA and found that only repeats 23–24 of FlnA associates with the Nrp1 cytoplasmic region with an appreciable affinity (KD of 9.6 ± 1.2 μM) (Fig. 2A–C). This was confirmed by isothermal titration calorimetry, which showed a closely similar dissociation constant of 12.1 ± 2.0 μM (Fig. 2D).

3.3. Nrp1 colocalizes with FlnA in vesicles in response to VEGF-A

To gain insight into the functional significance of the interaction between Nrp1 and FlnA, we tracked their localization in VEGF-A-treated *nrp1*$^{+/+}$ ECs. Whereas there was little or no colocalization in quiescent ECs (Fig. 3A), the two proteins colocalized extensively in vesicular punctae in the cytoplasm 5 min after the introduction of VEGF-A (Fig. 3B). The extent of colocalization decreased at the later time points of 10 and 30 min (Fig. 3C–E).

To test for the dependence of the colocalization of Nrp1 and FlnA on the cytoplasmic interaction of the two proteins, we probed