Neuropilin-1 exerts co-receptor function for TGF-beta-1 on the membrane of cancer cells and enhances responses to both latent and active TGF-beta

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Introduction

Neuropilin (Nrp)-1 and Nrp-2 are multifunctional proteins frequently expressed by cancer cells and contribute to tumor progression by mechanisms that are not well understood. They are co-receptors for vascular endothelial growth factor and class 3 semaphorins, but recently we found that Nrp1 also binds latent and active transforming growth factor (TGF)-β1, and activates the latent form latency-associated peptide (LAP)–TGF-β1. Here, we report that Nrp1 has affinity for TGF-β receptors TβRI and TβRII, the signaling TGF-β receptors, as well as TβRIII (beta-glycan), as determined in binding assays, pull down assays and confocal microscopy. Nrp1 had a higher affinity for TβRI than TβRII and could form a complex with these receptors. In breast cancer cells, Nrp1 and TβRII colocalized in the presence of TGF-β1. Nrp1 acted as a TGF-β co-receptor by augmenting canonical Smad2/3 signaling. Importantly, Nrp-positive cancer cells, unlike negative cells, were able to activate latent TGF-β1 and respond. We examined two other membrane proteins that bind LAP–TGF-β, i.e. an RGD-binding integrin (αvβ3) and Glycoprotein A repetitions predominant (GARP) or LRRC32; expressed by activated 90 regulatory T cells. In vitro, these receptors did not activate LAP–TGF-β1, but subsequent addition of Nrp1 activated the cytokine. Thus, Nrp1 might collaborate with other latent TGF-β receptors in TGF-β capture and activation. We also show that Nrp2 has activities similar to Nrp1. We conclude that Nrp1 is a co-receptor for TGF-β1 and augments responses to latent and active TGF-β. Since TGF-β promotes metastasis, this is highly relevant to cancer biology.

Materials and methods

Nrp-Fc, TGF-β receptor-Fc fusion proteins and antibodies

Rat Nrp1 extracellular domain conjugated to an Fc fragment of human IgG1 (Nrp1-Fc) was from R&D Systems (Minneapolis, MN). There is 98% homology between mouse and rat Nrp1, and 93% homology between mouse and human Nrp1. An IgG1-Fc construct of identical sequence (R&D Systems) was used as a control (referred to as Fc). Extracellular domains of rat Nrp2, mouse TβRI (ALK5) and TβRII conjugated to the same Fc were also from R&D Systems, as was soluble human RII (not fused to Fc). Monoclonal antibodies against all these proteins, all recombinant TGF-β component proteins, mouse anti-human LAP and anti-TGF-β1.23 (clone 1D11) antibodies and anti-Nrp1 goat polyclonal antibody were from the same company. Anti-TβRII extracellular domain (clone 141231) and anti-Nrp1 (clone 130603) monoclonal antibodies bind to both the mouse and human antigens. For confocal microscopy, antibodies or LAP were conjugated to AlexaFluor rhodamines (Molecular Probes/Invitrogen, Carlsbad, CA). For immunoprecipitation, we used anti-TβRI and anti-TβRII affinity purified rabbit polyclonal antibodies against the cytosolic tails of the receptors (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Nrp1 monoclonal antibody (clone 130604) from R&D Systems. Human GARP-Fc was from Enzo Life Sciences (Plymouth Meeting, PA), and human integrin αvβ3 from R&D Systems.

Origin and features of cells

MDA-MB-453, MCF-7 and MDA-MB-231 were from ATCC (Manassas, VA). MDA-MB-453 is negative for both Nrp1 and Nrp2 or any VEGF receptor (33). MDA-MB-231 expresses high levels of Nrp1 and low levels of Nrp2, and MCF-7 expresses only Nrp1 (34). We confirmed these patterns of expression by western blotting and flow cytometry analysis (data not shown). Cells...
were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Before the experiments, they were subjected to serum deprivation for 16 h, by growth in serum-free medium AIM V medium (Invitrogen). The later medium was used in the experiments of cell treatment with TGF-β1 or L-AP-TGF-β1. Cell proliferation was measured in the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT; 21).

**Transfection**
Expression of Nrp1 by MDA-MB-231 and MCF-7 cells was suppressed by Nrp1-targeted small interfering RNA (siRNA) transfection using oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA) and Eugene-6 (Roche Biosciences, Palo Alto, CA) as a transfection reagent. MDA-MB-453 cells were transfected with pBLAST vector encoding the complete human Nrp1 sequence (Invivogen, San Diego, CA). Efficiency of transfection was tested by cell enzyme-linked immunosorbent assay (ELISA) and immunofluorescence analysis. Positive or negative magnetic sorting of Nrp1 cells was performed using anti-Nrp1 antibodies, as described (21).

**Coprecipitation (pull down) assays**
MDA-MB-231 cells were lysed in the extraction buffer. Anti-TJRI or anti-TJRII antibodies at a final dilution 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA), premixed with the secondary anti-rabbit biotinylated antibody (Biologend, San Diego, CA), were added to the extract and incubated overnight at 4°C. Streptavidin-ferrofluid (R&D Systems) was added to the mixture in the ratio 1:1 by volume and incubated on ice for 20 min. The proteins labeled with biotin were pulled down by magnetic separation. The eluted proteins were subjected to western blot, using anti-Nrp1 antibody. The membrane was stripped and restained with antibodies for TGF-β receptors. In a similar way, mouse anti-Nrp1 antibody (clone 130604) was used to coprecipitate TGF-β receptors using anti-mouse-IgG1 agarose beads (Sigma-Aldrich Canada, Oakville, ON, Canada).

**Cell-free ELISA binding assays**
These assays were performed as we have described previously (21). Briefly, Nrp1-Fc or other proteins were bound to Nunc Maxisorb plates (Nilge Nunc International-Fisher Scientific, Nepean, ON, Canada). Soluble ligands were incubated in the pre-coated blocked plate for 2 h at room temperature or at 4°C overnight. The binding was performed in phosphate-buffered saline (PBS), except for integrin. In the latter case, PBS was replaced with Mg2+-containing Hanks’ balanced salt solution. Unbound proteins were collected, the plates were washed and ELISA assays were performed with specific antibodies to detect bound proteins. Non-specific binding for every ligand concentration was determined in uncoated wells treated with the blocking solution and subtracted from the optical density at 450 nm (OD450) for total binding to give the values of specific binding. The binding was expressed in arbitrary units defined as OD450 for the specific binding. The assays were performed in duplicates.

**Cell-surface ELISAs**
This was performed as described (35), with some modifications. The cells in a 96-well plates were fixed for 20 min in 4% paraformaldehyde (BD Biosciences, Mississauga, ON, Canada) at 4°C. In preliminary experiments, this fixative did not permeabilize the cell membrane, permitting quantification exclusively of membrane antigens. The fixed cells were examined by ELISA with specific antibodies (OD450). The background staining was measured in the wells containing cells and the secondary, but no primary antibodies (negative control) and was subtracted from all readings to measure the specific staining. To control the cell loss due to multiple washes, the cells were then stained with crystal violet, and cell-bound crystal violet was measured at 540 nm. These values were used to normalize the OD450 readings. Cell-surface expression is presented as arbitrary units = (OD450 for the specific staining)/OD450 or as a percentage to the level of their expression at the beginning of the incubation. For internalization studies, the cells in a 96-well plates were serum-starved overnight and treated with a constant concentration of LAP, or other protein, in the same medium. The cells were kept either at 4°C to prevent internalization (time 0 samples) or at 37°C over the time course as required for the experiment, as described (35). The treatment was terminated at various time points by removal of the medium, rinsing with PBS and adding ice-cold fixative, and the cells were assayed by ELISA. Measurements were performed at a minimum in triplicates.

**Signal SMAD reporter assay**
We used the TGF-β Signal kit from SABiosciences (Fredrick, MD) for the quantification of TGF-β-induced SMAD2/3 signaling. pSMAD-dependent expression of the firefly luciferase was quantified with a Dual-Luciferase Reporter assay system (Promega Corporation, Madison, WI) according to the manufacturer’s protocol. All transfections were performed in triplicate. The level of the firefly luciferase activity was normalized by the corresponding level of the Renilla luciferase activity and the values for the negative control were subtracted.

In some experiments, the Luc-transfected cells were seeded onto the 96-well plates, which were pre-coated with GARP-Fc (10 nM) or integrin (3.5 nM), rinsed and treated (or not) with 1 nM LAP-TGF-β1 in Hanks’ balanced salt solution for 1 h at 37°C. Unbound LAP-TGF-β1 was washed out, and the plates were blocked in serum-free medium containing 1% bovine serum albumin before seeding the cells at 2 × 10^4 cells per well in AIM V medium. Luciferase activity was measured 24 h later.

**Confocal microscopy**
For internalization assays, the cells were serum-starved overnight, rinsed and treated with TGF-β1, LAP or left untreated for the specified time intervals. Cells were either kept at 4°C to prevent internalization or at 37°C over the time course indicated. At the end of the incubation, the medium was removed, the cells washed three times in PBS and fixed in ice-cold fixative. The fixed cells were rinsed in 1% bovine serum albumin–PBS, PBS alone, and mounted using Permafluor (LabVision Corporation, Fremont, CA). Recombinant LAP directly labeled with AlexaFluor 488 was used to study its binding and internalization by MDA-MB-231 cells. Irrelevant protein (gelatin or Fc) labeled with AlexaFluor 488 was used as a negative control. Colocalization analysis was performed in ImageJ software using an algorithm described previously (36).

**Statistical analysis**
Analyses were performed with the GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA). The significance of differences between experimental and control results was determined by either Student’s t-test or analysis of variance. P < 0.05 was considered significant.

**Results**

1. **TJRI, TJRII and TJRIII bind to Nrp1**
Soluble recombinant TJRI-Fc, TJRII-Fc and TJRIII were retained on the plate coated with Nrp1-Fc (Figure 1A–C). Nrp1-Fc bound TJRI-Fc with the highest affinity (K_D = 3.2 nM) independent of mature TGF-β1, however, binding of TJRII-Fc was considerably increased by this cytokine (Figure 1B). In the absence of TGF-β1, Nrp1 bound to TJRII-Fc with a K_D of 39 nM, but the addition of 0.1 nM TGF-β1 decreased K_D to 3.2 nM. This range was in the same range with TJRI-Fc. Recombinant TJRIII bound to Nrp1-coated plate in a TGF-β1-independent way with the K_D = 6.8 nM (Figure 1C). In the reverse assay, soluble Nrp1-Fc was retained on the plates coated with TJRII-Fc or TJRIII-Fc without a notable difference in the affinity (data not shown).

Soluble Fc did not compete with Nrp1-Fc for binding to any of the receptors (Figure 1D). We observed competition in the binding of soluble Nrp1-Fc between soluble and plate-bound TGF-β receptor protein for each of the three receptors but not between the different receptors (data not shown). Importantly, the interaction of the receptors with the extracellular domain of Nrp1 was blocked by an anti-Nrp1 antibody (Figure 1A–C). The binding of the receptors to Nrp1 was not affected by free LAP (data not shown).

The binding of TJRIII (a heparan sulfate proteoglycan) (35) to Nrp1-Fc was prevented by heparin (Figure 1E), suggesting that Nrp1 was binding the heparan sulfate groups of TJRIII. Indeed, Nrp1 has affinity for heparin and heparan sulfate (1,2). Heparin failed to block binding of TJRII-Fc in the same experiment (Figure 1E).

We also performed coprecipitation assays. We could pull down all three recombinant receptors with Nrp1-Fc (Figure 1F; the data for TJRII is not shown). In the same way, native Nrp1 coprecipitated with either TJRI or TJRII from the cell extracts of naturally Nrp1 MDA-MB-231 cells (Figure 1G). This confirms that Nrp1 in its native form could bind to the RI and RII receptors in their native forms (without Fc).

**Nrp1 can link TJRI and TJRII**
TJRI and TJRII have low affinity for each other, but in the presence of TGF-β, they bind together tightly (37,38). Here, we could ELISA plates with TJRI-Fc and examined the retention of TJRII-Fc when it was added in soluble form premixed (or not) with either TGF-β1 or Nrp1. As expected, TGF-β1 increased the retention of TJRII-Fc (Figure 2A). Importantly, Nrp1-Fc also increased the retention of TJRII-Fc, and this occurred either in the presence or absence of TGF-β1 (Figure 2A), although retention was slightly higher without the cytokine. These results provide evidence that Nrp1 is capable of...
Nrp-1 exerts co-receptor function

Simultaneously binding both TβRI and TβRII, independent of TGF-β1. However, TGF-β1 retention was markedly increased by Nrp1-Fc (Figure 2B). These assays confirm that TGF-β1, TβRI-Fc and Nrp1-Fc were concurrently bound to the TβRII-Fc on the plate.

Colocalization and cointernalization of Nrp1 and TβRI

We observed colocalization of naturally-expressed Nrp1 and TβRI on the plasma membrane. Colocalization occurred in cells not treated with TGF-β1 (Figure 3A). Percent colocalization = 96% as calculated using MacBiophotonics ImageJ.

In cells treated with TGF-β1, we report cointernalization of Nrp1 and TβRI (Figure 3B). Following the treatment with TGF-β1 for 15 min at 37°C, most of the TβRI- and Nrp1-specific staining was found in the cytoplasm. Cointernalization of TβRI, TβRII and Nrp1 was confirmed by cell ELISA, which showed that up to 60% of the receptor proteins expressed on the cell surface were internalized within 10 min of exposure to TGF-β1 (Figure 3C). Internalization was not seen over that time period in cells not treated with TGF-β1 (Figure 3D). These results suggest that Nrp1 cointernalizes with the TβRI–TβRII receptor complex.

Nrp2 interactions with TGF-β components are similar to Nrp1

We observed remarkable similarity between Nrp2 and Nrp1 (supplementary Figure 1A–F is available at Carcinogenesis Online). Thus, plate-bound Nrp2-Fc captured both TβRI and TβRII (supplementary Figure 1A and B is available at Carcinogenesis Online), showing higher affinity for TβRI than TβRII and improved binding of TβRII in the presence of TGF-β1. Similarly to our published results with...
Nrp1 can link T\[RI\] and T\[RII\]. A—The ELISA plate was first coated with T\[RI\]-Fc, before addition of other (soluble) components. TGF-β of 1 nM enhanced the retention of T\[RII\]-Fc to the plate (+TGF-β1). The addition of Nrp1-Fc also increased the retention of T\[RII\]-Fc (+Nrp1-Fc), in TGF the presence or absence of TGF-β1. 1 nM T\[RI\] was premixed (or not) with either 1 nM TGF-β1, or 1 nM Nrp1-Fc or TGF-β1 and Nrp1-Fc together. Retention of T\[RI\] was quantified by ELISA. B—The ELISA plate was first coated with T\[RI\]-Fc (as in A), and then incubated with T\[RI\] premixed with TGF-β1 (or not), and with Nrp-Fc (concentration = 0.1 nM, 1 nM or no Nrp1-Fc). Bound TGF-β1 was detected with the 1D1 anti-TGF-β monoclonal antibody. The data show that an increased amount of TGF-β1 was retained on the plate in the presence of Nrp1-Fc (P < 0.05). Two experiments yielded similar results.

Nrp1 (21), we found that Nrp2 binds to both active TGF-β1 (supplementary Figure 1C is available at Carcinogenesis Online) and its LAP component (supplementary Figure 1D is available at Carcinogenesis Online). LAP binding was inhibited by anti-LAP antibody (supplementary Figure 1E is available at Carcinogenesis Online). Finally, in a pull down assay, T\[RI\]-Fc coprecipitated with Nrp2-Fc (supplementary Figure 1F is available at Carcinogenesis Online).

The binding constants for T\[RI\] with Nrp1 and Nrp2 fall into the same range, whereas T\[RII\] had a higher affinity for Nrp2 that for Nrp1. Nrp2-Fc also bound T\[RII\] with high affinity in the same range as Nrp1 (data not shown).

Nrp1 augments canonical SMAD signaling

Nrp1 expression augmented TGF-β1-induced responses, as shown by either knockdown or forced expression (Figure 4A–F). To detect SMAD2/3-dependent signaling, we utilized the TGF-β Cignal Reporter assay. Knockdown of Nrp1 by siRNA in MDA-MB-231 breast cancer cells (residual Nrp1+ cells were depleted by magnetic sorting) greatly reduced signaling in response to active TGF-β1 (Figure 4A). Note that Nrp1 knockdown was highly effective with our reagents (see below). Interestingly, these cells express Nrp2 but at a much lower level than Nrp1 (34). It appears that Nrp1 exerts most of the TGF-β co-receptor activity in these cells. We repeated the experiment in MCF-7 cells (Nrp1+ and Nrp2+), and Nrp1 knockdown abolished SMAD-responsive luciferase expression (Figure 4C). Furthermore, the proliferation of these cells was inhibited by mature TGF-β1, but this was markedly impaired by Nrp1 knockdown (Figure 4D).

Nrp1/Nrp2 double-negative MDA-MB-453 cells responded to TGF-β1 stimulation with an increase in signaling (Figure 4B). However, when transfected to express Nrp1 (Nrp1+ cells were isolated by magnetic sorting), the response was significantly increased (P < 0.05). These findings in MDA-MB-231 and MDA-MB-453 cells support the conclusion that Nrp1 exerts a costimulatory effect in the TGF-β signaling pathway.

Nrp1 induced responsiveness to LAP–TGF-β1 in MDA-MB-453

When MDA-MB-453 cells were incubated with LAP–TGF-β1, there was only a minimal decrease in proliferation (Figure 4E) and no increase in SMAD signaling (Figure 4F). However, transfected Nrp1+ MDA-MB-453 cells exposed to the latent cytokine showed markedly decreased proliferation (Figure 4E) and increased SMAD signaling (Figure 4F). These findings suggest that the Nrp1+ cells were activating LAP–TGF-β1.

Interactions of Nrp1 with LAP

We examined binding of free LAP on the membrane of MDA-MB-231 cells. By confocal microscopy (Figure 5A), we observed that cells incubated with labeled LAP captured LAP and that it colocalized with Nrp1. Nrp1 knockdown with siRNA eliminated LAP binding to these cells. The efficiency of Nrp1 knockdown is demonstrated by western blotting in Figure 5B. It was also evident that LAP was internalized. To study the rate of internalization, we used a cell-surface ELISA assay. With this assay, we could detect internalization of LAP with Nrp1 within a few minutes of its incubation with cells (Figure 5C). These results suggest that Nrp1 binds LAP on the membrane of MDA-MB-231 cells and internalizes it rapidly.

Nrp1 can activate LAP–TGF-β1 bound to other receptors

We examined the capacity of Nrp1 to activate LAP–TGF-β1 bound to either GARP-Fc (Figure 6A–C) or αvβ3 integrin (Figure 6D–F). Soluble Nrp1-Fc bound only weakly to plate-bound GARP-Fc (Figure 6A) and even more weakly to plate-bound αvβ3 (Figure 6D). However, when soluble LAP–TGF-β1 was added first, followed by Nrp1-Fc, then binding was greatly increased in both cases (Figure 6A and D). This indicates LAP–TGF-β1 was bridging the plate-bound proteins to Nrp1-Fc. To address whether LAP–TGF-β1 activation was occurring, we used both a cell-free and a cell-based assay. In the first assay (Figure 6B), we incubated LAP–TGF-β1 with plate-bound GARP-Fc, followed by incubation with Nrp1-Fc (or not). We then tested the binding of the 1D1 anti-TGF-β antibody, which binds only to the activated form of the cytokine. This antibody did not bind to GARP-bound LAP–TGF-β1 (Figure 6B). However, when Nrp1 was added we observed subsequent reactivity of the 1D1 antibody to the bound complex. We repeated these assays on αvβ3 integrin-coated plates (instead of GARP) (Figure 6E) and obtained identical results. This suggests that in both cases, a conformational change was induced by Nrp1-Fc, exposing an epitope of active TGF-β1.

To examine activation directly, we coated tissue culture wells with either GARP-Fc (Figure 6C) or αvβ3 integrin (Figure 6F) and bound LAP–TGF-β1 (or not) to the wells. We then added MDA-MB-453 cells as wild-type or Nrp1+t–transfected cells, also transfected with the TGF-β-responsive luciferase-expressing plasmid (Cignal assay), and measured TGF-β-induced SMAD2/3 signaling. Wild-type MDA-MB-453 cells (Nrp1/2 negative) generated only a weak SMAD2/3 signal, whereas Nrp1-transfected cells signaled significantly more strongly (Figure 6C and F). Thus, Nrp1 on the cell membrane appears able to activate latent TGF-β1 that is attached to either GARP or αvβ3 integrin.

Discussion

Most of the cancer-promoting effects of Nrps have been attributed to VEGF (1,2). However, since some tumors express one of the Nrps but...
neither VEGFR1 nor VEGFR2 (5,34,39), it seems probably non-VEGF interactions are also involved. We have examined Nrp1 interactions with TGF-β and found it is a high affinity receptor for both latent and active TGF-β1 (21). Importantly, Nrp1 was able to activate the latent form, i.e. LAP–TGF-β1.

Here, we hypothesized that Nrp1 contributes to the responsiveness of cancer cells to TGF-β1. We report the novel finding that the extracellular domain of Nrp1 has high affinity for the extracellular domains of TβRI, TβRII and TβRIII. Its affinity for TβRI was higher than TβRII. The binding of Nrp1-Fc to TβRI-Fc-coated plates was unaffected by the presence of mature TGF-β1, whereas the binding to TβRII-Fc-coated plates was considerably increased by this cytokine. These results are consistent with the fact that TβRII binds TGF-β1 directly, whereas TβRII does not or has very low affinity (37,38). We speculate that TGF-β1 strengthens the interaction between Nrp1 and TβRII through its ability to bind to both of these molecules.

Nrp1-Fc was able to pull down either soluble TβRII-Fc, TβRII-Fc or TβRIII. These interactions are with the extracellular domains only, but our work does not exclude intracellular interactions. Indeed, both Nrp1 and TβRIII bind to the cytoplasmic PDZ protein GIPC. The interactions of Nrp1 with the TGF-β signaling receptors (RI and RI) also occurred with native proteins (no Fc fragments). Furthermore, by confocal microscopy we observed colocalization and cointernalization of Nrp1 and TβRI.

Because TβRII has a low affinity for each other in the absence of active TGF-β (37,38), we examined whether Nrp1 could link these components. Indeed, we could demonstrate that the retention of TβRII-Fc on TβRI-Fc-coated plate was improved by adding either active TGF-β1 or Nrp1-Fc. This finding suggests that Nrp1 was forming a bridge between TβRI and TβRII. In the presence of Nrp1-Fc, the retention of TβRII-Fc was slightly higher without TGF-β1 than with TGF-β1. The reason for this has not been elucidated but is difficult to determine because of multiple potential molecular interactions. Interestingly, under these conditions, there was increased retention of TGF-β1 to the TβRII-coated plate. We conclude from all these assays that Nrp1 is capable of binding specifically to both major components of the TGF-β signaling receptor. Importantly, Nrp1 did not prevent binding of TGF-β1 to the receptor complex, but possibly facilitated it.

We then showed that Nrp1 acts as a co-receptor for the TGF-β pathway. Compared with Nrp1−/− cells, Nrp1−/− cells responded more strongly to mature TGF-β1 in a luciferase SMAD2/3 reporter assay. Similarly, we observed increased SMAD2/3 phosphorylation in Nrp1−/− cells in a cell-based ELISA assay (data not shown). Thus,
the knockdown of Nrp1 in MDA-MB-231 or MCF-7 cells greatly reduced or abolished SMAD signaling in response to the active cytokine. MCF-7 cells (Nrp1+ and Nrp2−) following Nrp1 knockdown are deficient in both Nrps. MDA-MB-231 express much higher levels of Nrp1 than Nrp2, and it appears that in these cells, the response to TGF-β1 is altered primarily by Nrp1. In the reverse experiment, forced expression of Nrp1 in Nrp-negative MDA-MB-453 cells significantly increased TGF-β1-induced SMAD-responsive promoter induction in Nrp1-transfected MDA-MB-453 cells, as compared with sham-transfected cells. MDA-MB-453 (wild-type) cells do not express Nrp1 or Nrp2 (data not shown). These cells were transfected with Nrp1 or sham-transfected and were treated as above. In A and B, *P < 0.05 versus all other bars, and two experiments yielded similar results. C—MCF-7 cells, which express Nrp1 but not Nrp2, were treated as in (A) for Nrp1 knockdown and reporter assay. Nrp1 knockdown abolished SMAD-responsive luciferase expression. D—Active TGF-β1 suppressed the proliferation of wild-type MCF-7 cells (Nrp1+); but this effect was markedly diminished by Nrp1 knockdown (Nrp1−). E and F—Nrp1 induces activation of LAP-TGF-β1. E—Proliferation of Nrp1-transfected but not sham-transfected MDA-MB-453 was suppressed by LAP-TGF-β1. F—LAP-TGF-β1 induced SMAD2/3-responsive luciferase expression in Nrp1 transfected but not in the sham-transfected MDA-MB-453 cells. *P < 0.01 versus all other bars. Two experiments yielded similar results.

Fig. 4. Nrp1 enhances SMAD2/3 signaling. A—Knockdown of Nrp1 with siRNA decreased the level of pSMAD2/3-responsive luciferase signal (signal reporter assay) in MDA-MB-231 and MCF-7 cells treated with active TGF-β1 (0.25 nM), compared with control scrambled siRNA-transfected cells. The cells were first transfected with Nrp1-targeted siRNA for knockdown, and 72 h later with the SMAD2/3-responsive Cignal reporter plasmids. Twenty hours after the Cignal reporter transfection, they were treated with TGF-β1 for another 24 h before the assay. The assay was performed in triplicate. B—Nrp1 expression significantly increased TGF-β1-induced SMAD-responsive promoter induction in Nrp1-transfected MDA-MB-453 cells, as compared with sham-transfected cells. MDA-MB-453 (wild-type) cells do not express Nrp1 or Nrp2 (data not shown). These cells were transfected with Nrp1 or sham-transfected and were treated as above. In A and B, *P < 0.05 versus all other bars, and two experiments yielded similar results. C—MCF-7 cells, which express Nrp1 but not Nrp2, were treated as in (A) for Nrp1 knockdown and reporter assay. Nrp1 knockdown abolished SMAD-responsive luciferase expression. D—Active TGF-β1 suppressed the proliferation of wild-type MCF-7 cells (Nrp1+); but this effect was markedly diminished by Nrp1 knockdown (Nrp1−). E and F—Nrp1 induces activation of LAP-TGF-β1. E—Proliferation of Nrp1-transfected but not sham-transfected MDA-MB-453 was suppressed by LAP-TGF-β1. F—LAP-TGF-β1 induced SMAD2/3-responsive luciferase expression in Nrp1 transfected but not in the sham-transfected MDA-MB-453 cells. *P < 0.01 versus all other bars. Two experiments yielded similar results.
LAP–TGF-β1 by increased SMAD signaling and a significant reduction in proliferation.

By confocal microscopy, we observed colocalization of soluble LAP added to cultures and cell-bound Nrp1, and there was rapid internalization of both molecules. Membrane expression of Nrp1 declined to its lowest point at 7–8 min and then increased. We attribute this to receptor re-expression. However, we have not performed a detailed analysis of receptor recycling, and this is an interesting subject for future study.

Some cancer cells express only the Nrp2 isoform. We now report that Nrp2 can also bind latent and active TGF-β1 and interact with the TβRI and TβRII receptors, in a way quite similar to Nrp1, although differences in affinity were noted. We also observed that Nrp2-Fc can activate LAP–TGF-β1 (data not shown). Thus, Nrp1 and Nrp2 might be largely interchangeable in their interactions with TGF-β components, but this question requires further study.

There are two forms of latent TGF-β, i.e., the small latent complex (LAP–TGF-β) and the large latent complex consisting of the LAP–TGF-β covalently bound to a latent TGF-β-binding protein. Large latent complex attaches to the membrane of some cell types, where it can be activated by a variety of mechanisms. Receptors that can capture LAP–TGF-β, in addition to Nrp1, include the RGD-binding integrins (LAP has an RGD motif), especially the α5 subfamily and GARP. In this role, integrins have been the most studied (30,41,42), whereas GARP (43–46) has only been recently identified. Some RGD-binding integrins activate latent TGF-β, whereas GARP (43–46) has only been recently identified. 

Some RGD-binding integrins activate latent TGF-β (30,41,42), but in vitro they cannot activate without other molecules. In vivo, activation is thought to occur by one of two mechanisms (42). In the first case, typified by α5β6, traction forces cause conformational changes in LAP. In the second case, typified by αvβ8, activation requires the proteolytic action of matrix metalloproteinase enzymes. However, because LAP has both an integrin-binding site (RGD) and Nrp-binding sites (21), we hypothesize there is a third mechanism. In this putative model, Nrp1 or Nrp2 contribute to the activation of latent TGF-β that is bound to an integrin, GARP or other receptor. Indeed, here we show that Nrp1 can activate LAP–TGF-β1 after it attaches to either α5β3 integrin or GARP. These interactions might be most relevant on the membrane of cancer cells where Nrp5 and RGD-binding integrins are often coexpressed. GARP is expressed by regulatory T cells (43–46) that have been linked to depressed anticancer immunity, and we speculate this might depend (at least in part) on the activation of latent LAP–TGF-β1 bound to GARP.

A question of major interest is how Nrp1 captures LAP. Our previous binding studies suggest that there is more than one site of interaction (21). We identified the arginine-rich C-terminal segment of LAP as a probably binding site to Nrp1 (21). Indeed, a soluble C-terminal LAP peptide (QSSRHRR) inhibited the binding of either free LAP or LAP–TGF-β1 to Nrp1. Furthermore, we found that a short peptide derived from the sequence of the b2 domain of Nrp1 (RKFK) could activate LAP–TGF-β1, suggesting that this is a second site of interaction. It should also be noted that mature TGF-β1 binds directly to Nrp1 (21), but the binding site is unknown. Further studies are required to completely elucidate the interactions between Nrp and TGF-β components.

Recently, some authors reported that cell-penetrating peptides that bind to Nrp1 have a C-terminal consensus R/KXXR/K sequence,
usually with a terminal arginine (R) (47). They coined the term C-end rule (CendR) to describe this type of binding. Subsequent publications (48, 49) revealed an Nrp1-dependent cell/tissue penetration process (48, 49). The C-terminal motif of LAP (RHRR) follows the C-end rule, and we have shown it binds Nrp1 (21). Our confocal studies revealed rapid cointernalization of Nrp1 and LAP. This raises the question of whether LAP was internalized by the same mechanism(s) as CendR peptides, and this warrants further studies.

Clathrin-mediated endocytosis appears to promote TGF-β signaling (50) although this is not fully elucidated (27, 51). Nrp1 acts as a general adapter by binding GIPC, through a short terminal motif (SEA) of its cytoplasmic tail (14, 15, 52). GIPC, Dab2 and other proteins link vesicles of the clathrin pathway with molecular motor myosin VI (Myo6), to promote endosomal trafficking (53). In our studies, Nrp1 and TβRII appeared to be colocalized in cytoplasmic vesicles. Although extensive additional studies are required, this suggests that Nrp1 can influence the internalization or TβRI.

In conclusion, we show that Nrp1 can contribute to TGF-β capture, activation and signaling in cancer cells. We report the novel finding that Nrp1 has affinity for the three classical TGF-β signaling receptors (TβRI, TβRII and TβRIII). Importantly, our study demonstrates that Nrp1 is a co-receptor in the TGF-β pathway. We also report that Nrp2 has many similar properties. These interactions of Nrp with components of the TGF-β pathway may explain, at least in part, how they contribute to cancer progression.

Supplementary material

Supplementary Figures A–F can be found at http://carcin.oxfordjournals.org/

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References

Nrp-1 exerts co-receptor function


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