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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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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 (betaglycan), 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 TβRI cointernalized 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 (CLRRC32). RGD-binding integrins are frequently expressed by cancer cells, and glycoprotein A repetitions predominant is expressed by activated regulatory T cells that appear linked to poor tumor immunity. *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.

Introduction

Neuropilin (Nrp)-1 is a transmembrane protein expressed by endothelial cells, dendritic cells, regulatory T cells, several other normal cell types, and malignant tumor cells (1–8). Nrp2 shares many properties with Nrp1 (1,2,9). Both Nrp1 and Nrp2 have been linked to tumor progression (5–9). The Nrps are co-receptors for the class 3 semaphorins (SEMA3) (10,11) and vascular endothelial growth factor (VEGF) (1,12,13). The short cytoplasmic tail of Nrp1 has no signaling motif, but binds to the PDZ protein synectin (also denoted GIPC) (14,15), which associates with several proteins. Recent studies revealed Nrp1 can bind several other growth factors, and/or their receptors, such as hepatocyte growth factor, the hepatocyte growth factor receptor (c-Met), some fibroblast growth factors and platelet-derived growth factor (16–19). It also interacts with β1 integrins (20). Recently, we found that Nrp1 is a receptor for both the latent and active forms of transforming growth factor (TGF)-β1 (21). Importantly, Nrp1 activated latency-associated peptide (LAP)–TGF-β1, i.e. the latent form of TGF-β1.

TGF-β can suppress early neoplastic disease but later promotes metastasis (22–26). The three TGF-β isoforms use the same receptor (TβR) consisting of TβRI (ALK5), TβRII and TβRIII (betaglycan) (27,28). TβRIII binds TGF-β and recruits TGF-β to RII, but it also has other functions (28,29). TβRII/TGF-β1/TβRI form a complex with serine/threonine kinase activity, in which TβRII phosphorylates TβRI. Then, TβRI phosphorylates Smad2 and Smad3, and the latter form a heteromeric complex with Smad4, which translocates to the nucleus, binds to DNA and regulates transcription. TβRI, TβRII and TβRIII bind active (mature) TGF-β, but not the latent cytokine, and the mechanisms of TGF-β activation by cancer cells are not well understood. TGF-β is maintained in a latent form by non-covalent association with LAP and must be released for activation. This can occur through several mechanisms dependent on either proteolysis, integrins (30), thrombospondin-1 (31) or, as we have recently shown, Nrp1 (21).

The pro-tumor effects of Nrp1 have often been attributed to interactions with VEGF. Interactions with SEMA3 can have either protective or negative effects (32). However, the remarkable variety of ligands identified suggests a more general function for the Nrps. In this study, we hypothesized that Nrp1 plays a key role in TGF-β processing on the membrane of cancer cells. Here, we compared Nrp-positive and -negative breast cancer cell lines (33,34). We found that Nrp1 has affinity for TGF-β1 signaling receptor components (TβRI and TβRII), and exerts co-receptor function. $Nrp1^+$ breast cancer cells, unlike $Nrp1^-$ cells, also captured and activated LAP–TGF-β1. Futhermore, Nrp1-activated LAP–TGF-β1 after it had attached to other receptors, i.e. either an integrin or glycoprotein-A repetitions predominant [glycoprotein A repetitions predominant (GARP) or LRRC32; expressed by activated 90 regulatory T cells] From these results, we conclude that Nrp1 has co-receptor and activating functions for TGF-β1.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GARP, Glycoprotein A repetitions predominant; Nrp, neuropilin; LAP, latency-associated peptide; OD, optical density; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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 RIII (not fused to Fc). Monoclonal antibodies against all these proteins, all recombinant TGF-β component proteins, mouse anti-human LAP and anti-TGF-β1,2,3 (clone 1D11) antibodies and anti-Nrp1 goat polyclonal antibody were from the same company. Anti-TβRI 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 chromophores (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 LAP–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 Fugene-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-TβRI or anti-TβRII antibodies at a final dilution 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA), premixed with the secondary anti-rabbit biotinylated antibody (Biolegend, 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 (Nalge 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 Mg^{2+}- 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 (OD_{450}) for total binding to give the values of specific binding. The binding was expressed in arbitrary units defined as OD_{450} 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, Missisauga, 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 (OD_{450}). 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 OD_{450} readings. Cell-surface expression is presented as arbitrary units = (OD_{450} for the specific staining)/OD_{540} 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.

Cignal SMAD reporter assay

We used the TGF-β Cignal 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

TβRI, TβRII and TβRIII bind to Nrp1

Soluble recombinant TβRI-Fc, TβRII-Fc and TβRIII were retained on the plate coated with Nrp1-Fc (Figure 1A–C). Nrp1-Fc bound TβRI-Fc with the highest affinity (K_D = 3.2 nM) independent of mature TGF-β1; however, binding of TβRII-Fc was considerably increased by this cytokine (Figure 1B). In the absence of TGF-β1, Nrp1 bound to TβRII-Fc with a K_D of 39 nM, but the addition 0.1 nM TGF-β1 decreased K_D to 8.8 nM, bringing it in the same range with TβRI-Fc. Recombinant TβRIII 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 TβRI-Fc or TβRII-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 TβRIII (a heparan sulfate proteoglycan) (35) to Nrp1-Fc was prevented by heparin (Figure 1E), suggesting that Nrp1 was binding the heparan sulfate groups of TβRIII. Indeed, Nrp1 has affinity for heparin and heparan sulfate (1,2). Heparin failed to block binding of TβRI-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 TβRIII is not shown). In the same way, native Nrp1 coprecipitated with either TβRI or TβRII 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 TβRI and TβRII

TβRI and TβRII have low affinity for each other, but in the presence of TGF-β, they bind together tightly (37,38). Here, we coated ELISA plates with TβRI-Fc and examined the retention of TβRII-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 TβRII-Fc (Figure 2A). Importantly, Nrp1-Fc also increased the retention of TβRII-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

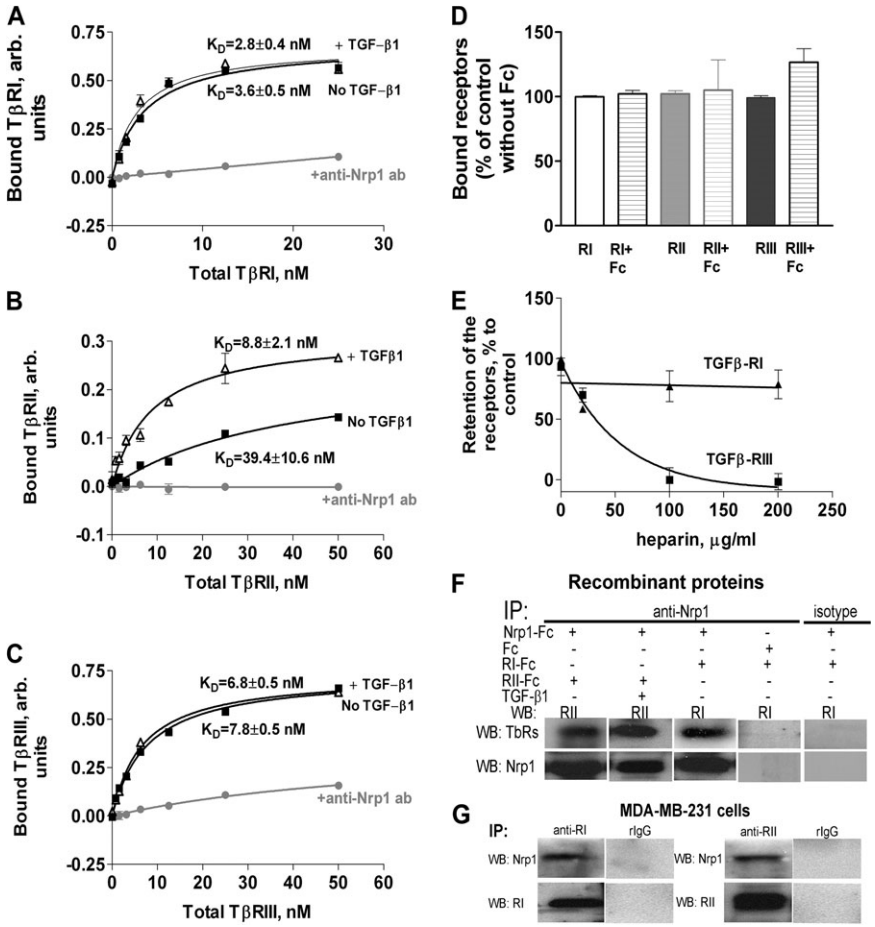

Fig. 1. Binding of TβRI, TβRII and TβRIII to Nrp1. **A**–**E** ELISA plates were coated with recombinant Nrp1-Fc as described in Materials and Methods. Recombinant TGF-β1 receptors were incubated on the plate with or without 1 nM TGF-β1. To test the specificity of binding the anti-Nrp1 antibody (clone 130603, 20 μg/ml) was added together with the receptor proteins in some wells. The anti-Nrp1 antibody blocked binding of all three receptors. A–C—squares, no TGF-β1; triangles, with TGF-β1; circles, with anti-Nrp1 antibody. A—Binding of TβRI-Fc to Nrp1-Fc. B—Binding of TβRII-Fc to Nrp1-Fc. The addition of TGF-β1 increased the binding affinity. C—Binding of TβRIII to Nrp1-Fc. D—Soluble Fc did not inhibit the binding of either soluble TβRI-Fc (RI), TβRII-Fc (RII) or TβRIII (RIII), when it was premixed in equal amounts (1 nM each) to these receptors, and tested on Nrp1-Fc-coated plates. These concentrations of soluble receptor are in the low range (ascending part of the binding curve), and competition would be apparent if it was occurring. The results are expressed as percent of binding without the competitor Fc fragment (± standard error of the mean). E—Retention of TβRIII, but not TβRI-Fc, on the Nrp1-Fc-coated plate was decreased by heparin. 10 nM TβRI-Fc or TβRIII was premixed with the indicated concentrations of heparin before incubation on the Nrp1-Fc-coated plate. In Figure 1A–E, representative results are shown in each case at least three experiments yielded similar results. **F** and **G**—Coprecipitation of TβRI and TβRII with Nrp1. F—Recombinant proteins TβRI-Fc (RI) or TβRII-Fc (RII) were mixed with Nrp1-Fc before adding mouse anti-Nrp1 antibody (clone 130604) or isotype control. Proteins binding to anti-mouse-IgG-Agarose (which does not bind to human IgG-Fc) were subjected to western blotting. The figure shows that both TβRI-Fc and TβRII-Fc are pulled down with Nrp1-Fc. The RI and Fc control represents RI mixed with the human Fc fragment instead of Nrp1-Fc, and no binding was observed. Isotype control did not precipitate Nrp1 and neither RI (shown) nor RII (data not shown). IP identifies the antibody used for immunoprecipitation; WB identifies the antibody used for western blotting. G—Cell extracts of MDA-MB-231 cells were treated with affinity purified rabbit anti-TβRI or anti-TβRII antibodies (Santa Cruz Biotechnology), or rabbit IgG (rIgG) as a control, and biotinylated anti-rabbit secondary antibody. The proteins were bound to streptavidin-ferrofluid and pulled down using magnetic separation. Western blotting (WB) was performed with monoclonal antibodies against either TβRI (anti-RI pull down), TβRII (anti-RII pull down) or Nrp1. WB with an isotype control (same isotype as anti-Nrp1) was negative (data not shown). The figure shows that anti-TβRI precipitated both native TβRI and native Nrp1, and anti-TβRII precipitated both native TβRII and native Nrp1. This indicates that wild-type Nrp1 binds to both receptors in their wild-type form. In panels F and G, two experiments yielded similar results.

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, TβRII-Fc and Nrp1-Fc were concurrently bound to the TβRI-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

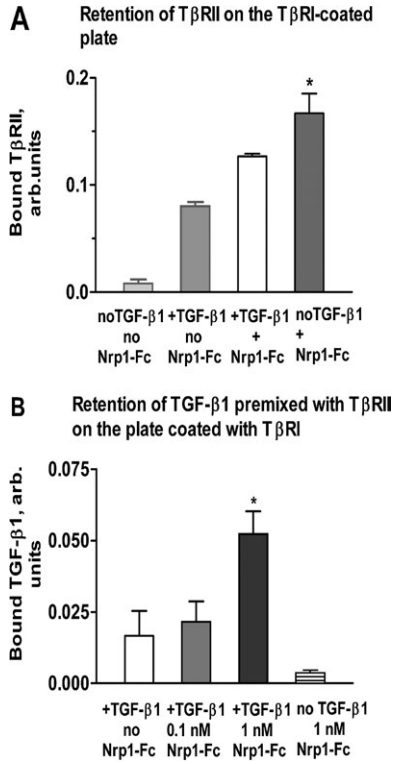

Fig. 2. Nrp1 can link TβRI and TβRII. **A**—The ELISA plate was first coated with TβRI-Fc, before addition of other (soluble) components. TGF-β1 of 1 nM enhanced the retention of TβRII-Fc to the plate (+TGF -β1). The addition of Nrp1-Fc also increased the retention of TβRII-Fc (+Nrp1-Fc), in TGF the presence or absence of TGF-β1. 1 nM TβRII was premixed (or not) with either 1 nM TGF-β1, or 1 nM Nrp1-Fc or TGF-β1 and Nrp1-Fc together. Retention of TβRII was quantified by ELISA. **B**—The ELISA plate was first coated with TβRI-Fc (as in A), and then incubated with TβRII 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 1D11 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, TβRI-Fc coprecipitated with Nrp2-Fc (supplementary Figure 1F is available at *Carcinogenesis* Online).

The binding constants for TβRI with Nrp1 and Nrp2 fall into the same range, whereas TβRII had a higher affinity for Nrp2 that for Nrp1. Nrp2-Fc also bound TβRIII 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 knockown 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 ($Nrp1^+$ cell 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 1D11 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 1D11 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 $Nrp1^+$-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

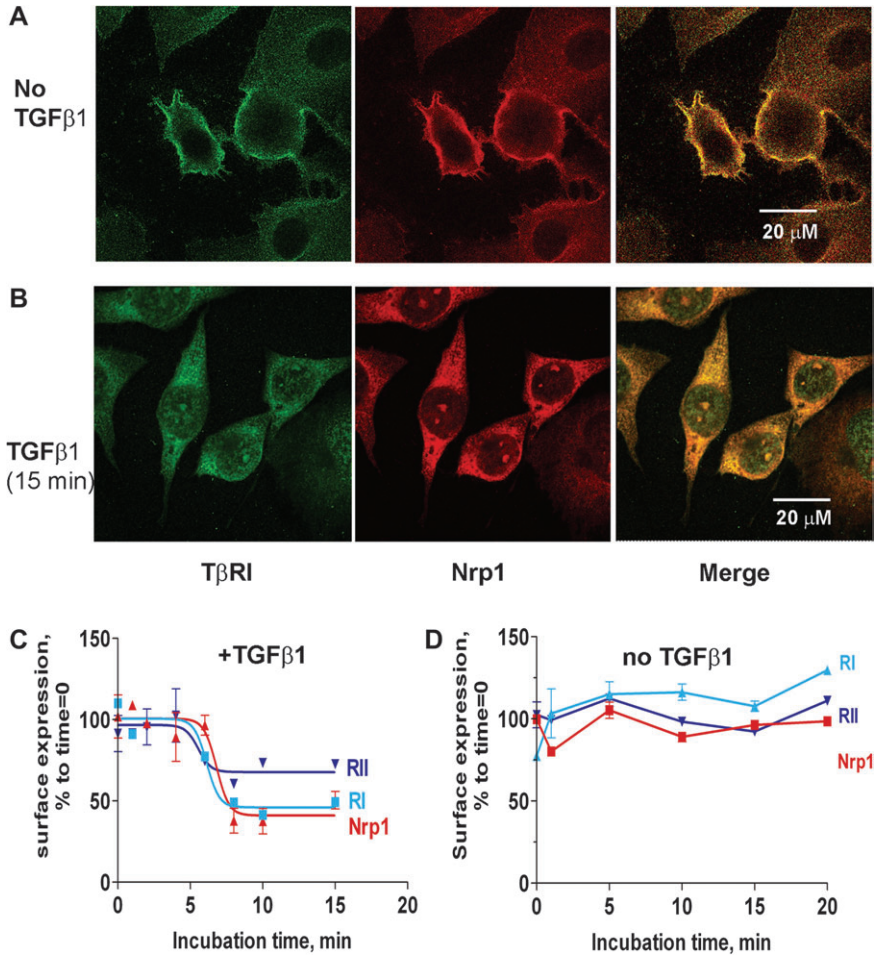

Fig. 3. Colocalization and cointernalization of Nrp1 and TβRI. **A**—Nrp1 colocalizes with TβRI on the surface of MDA-MB-231 cells (confocal microscopy). **B**—Nrp1 cointernalizes with TβRI after incubation with 0.5 nM active TGF-β1 (confocal microscopy). In A and B, the cells were serum-starved overnight, treated or not with TGF-β1 for 15 min at 37°C (B), fixed and permeabilized and stained with the directly labeled antibodies. **C**—Following incubation with active TGF-β1, there is rapid synchronous internalization of TβRI, TβRII and Nrp1 in MDA-MB-231 cells, as detected by cell-surface ELISA. MDA-MB-231 cells were serum-starved overnight, rinsed and incubated with 1 nM TGF-β1 in a serum-free medium for the specified time. D—No internalization of Nrp1 and the receptors is observed under the same conditions in the absence of TGF-β1. Quantification of the surface expression of the receptors (± standard error of the mean) was performed as described in the Materials and Methods using specific primary and biotinylated secondary antibodies. The surface expression of the receptors at any given time is expressed as a percentage to their expression at the time 0 determined at 4°C. The assay was performed in triplicate and the experiment was repeated twice with similar results.

neither VEGFR1 nor VEGFR2 (5,34,39), it seems probably non-VEGF interactions are also involved. We have examined Nrp1 interactions with TGF-β1 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 TβRI 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 TβRI-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 RII) also occurred with native proteins (no Fc fragments). Furthermore, by confocal microscopy we observed colocalization and cointernalization of Nrp1 and TβRI.

Because TβRI and TβRII have 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 TβRI-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,

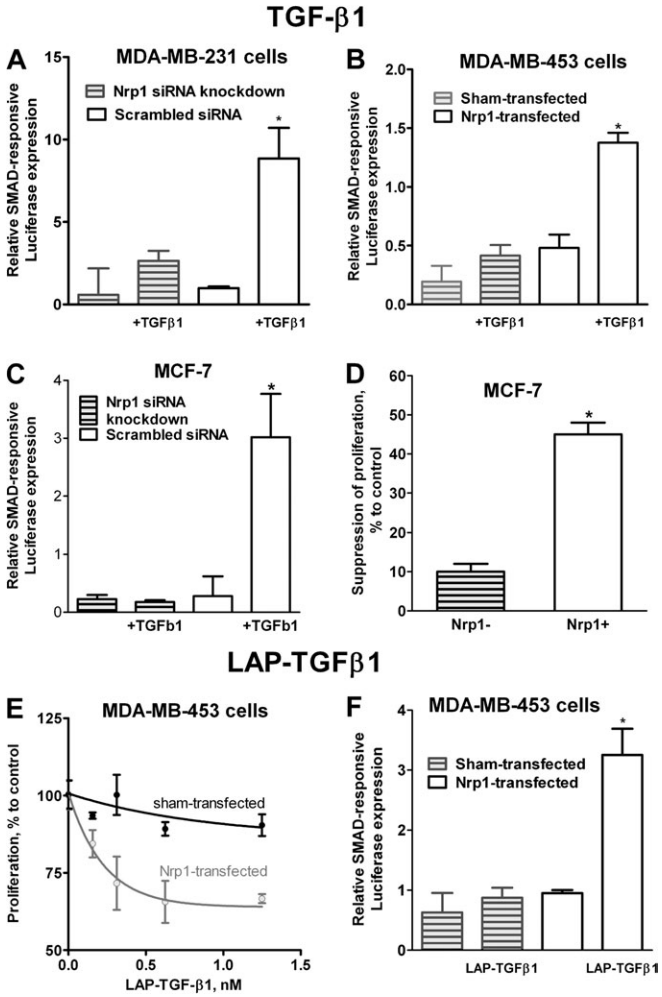

Fig. 4. Nrp1 enhances SMAD2/3 signaling. **A**, **C**—Knockdown of Nrp1 with siRNA decreased the level of pSMAD2/3-responsive luciferase signal (cignal reporter assay) in MDA-MB-231 and MCF-7 cells treated with active TGF-β1 (0.25 nM), compared with control scrambled siRNA-transfected cells. The cells 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.

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 SMAD2/3 signaling. Our data show that a Nrp1-dependent increase in Smad-responsive promoter activation is highly reproducible, as observed in three of three breast cancer cell lines we tested. Furthermore, we show that Nrp1 knockdown impaired both Smad signaling and the antiproliferative effect of active TGF-β1 on MCF-7 cells. This confirms in a different assay (proliferation), that Nrp1 enhances the response of breast cancer cells to active TGF-β1.

Interestingly, while we were preparing this manuscript, another group (40) reported that in stromal fibroblasts, Nrp1 interacts with TβRII in coprecipitation assays, and that elimination of Nrp1 suppressed canonical SMAD2/3 signaling. Nrp1 knockdown also impaired SMAD1/5 signaling in response to bone morphogenetic proteins, but not to TGF-β (where it appeared slightly increased). SMAD1/5/8 is the canonical morphogenetic protein signaling pathway (28), and perhaps Nrp1 also costimulates that pathway. In contrast to that report (40), here we show that Nrp1 interacts with all three classical receptors, and that RI rather than RII is the high affinity ligand. In our case, to confirm these interactions, we have performed both coprecipitation studies and quantitative affinity binding studies. In addition, we demonstrate that the interaction involves extracellular domains of these proteins. Despite some differences, both studies suggest that Nrpl can be integrated into the TGF-β receptor complex, and increase signaling in the classic SMAD2/3 pathway.

We show that Nrp1, expressed by human breast cancer cells, can capture and activate LAP–TGF-β1. In accord with this, MDA-MD-453 breast cancer cells, lacking Nrp1 and Nrp2 failed to respond to LAP–TGF-β1. MDA-MD-453 cell proliferation was suppressed by active but not latent TGF-β1 (data not shown). In contrast, when these cells were induced to express Nrp1 by transfection, they responded to

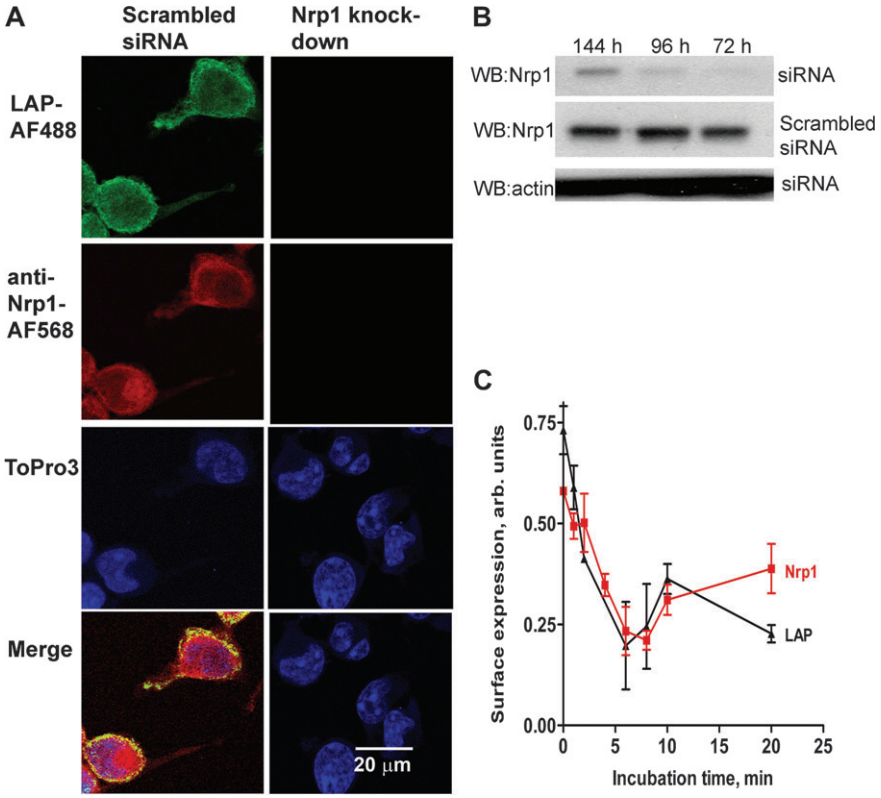

Fig. 5. Naturally-expressed Nrp1 binds LAP on the cell surface and cointernalizes with it. **A**—Nrp1 binds LAP on the surface of MDA-MB-231 cells: confocal microscopy analysis shows that LAP, directly labeled with AF 488, colocalizes and cointernalizes with Nrp1. Nrp1 expression was knocked down with siRNA (or not, with scrambled siRNA), and the cells were incubated with LAP-AF 488 for 15 min at 37°C before the fixation. AF 488-labeled gelatin used as a negative control did not stain the cells (data not shown). Isotype antibody control (instead of Nrp1 antibody) did not stain the cells (data not shown). **B**—The figure shows knockdown of Nrp1 in MDA-MB-231 cells, as detected by Western blotting (WB) at the indicated time points after siRNA treatment. **C**—Cointernalization of LAP with Nrp1 over time measured by cell surface ELISA. Serum-starved and rinsed MDA-MB-231 cells were incubated with 1 nM LAP at 37°C for the specified time in serum-free medium. Surface expression of both LAP and Nrp1 was assayed as described in Materials and Methods. The assay was performed in quadruplicate and performed three times with 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 α_V 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 (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 $\alpha_V\beta6$, traction forces cause conformational changes in LAP. In the second case, typified by $\alpha_V\beta8$, 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 $\alpha_V\beta3$ integrin or GARP. These interactions might be most relevant on the membrane of cancer cells where Nrps 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,

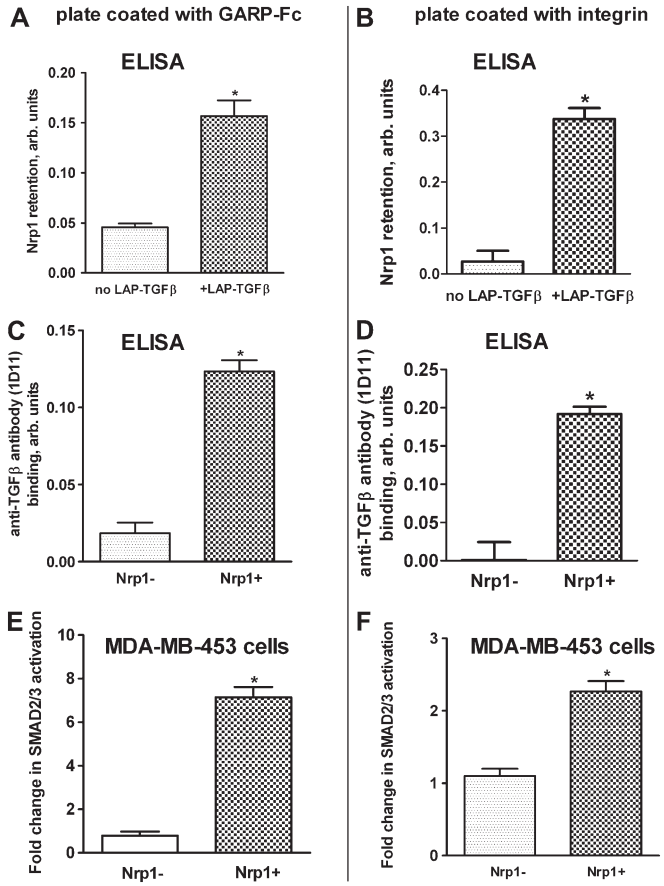

Fig. 6. Nrp1 activates LAP–TGF-β1 bound to GARP-Fc or integrin. **A–C**—GARP-Fc-coated plate. A—Nrp1-Fc binds to GARP only weakly, but binding is markedly increased by LAP–TGF-β1 that appears to link Nrp1-Fc to the GARP-Fc-coated plate. B—Anti-TGF-β antibody (clone 1D11), which binds only active TGF-β, binds to LAP–TGF-β1 retained on the GARP-Fc-coated plate in the presence, but not in the absence of Nrp1-Fc. This suggests that LAP–TGF-β1 has undergone a conformational change allowing binding of 1D11 to an epitope of mature TGF-β1. Concentration of LAP–TGF-β1 = 1.4 nM, of Nrp1-Fc = 1 nM. C—LAP–TGF-β1 was first attached to matrix-bound GARP-Fc and, after washing, cells were added. SMAD2/3-responsive luciferase expression is induced in the Nrp1-transfected, but not in the sham-transfected, MDA-MB-453 cells. **D–F**—αvβ3 integrin-coated plate. These experiments were performed in the same way as A–C, except that the plate was coated with αvβ3 instead of GARP-Fc and in Mg^{2+}-Hanks' balanced salt solution instead of PBS. The results obtained with GARP-Fc and integrin were highly similar. D—LAP–TGF-β1 links Nrp1-Fc to the integrin-coated plate. E—1D11 monoclonal antibody binds to LAP–TGF-β1 retained on the integrin-coated plate in the presence of Nrp1-Fc, but not in its absence. F—SMAD2/3-responsive luciferase expression is induced in the Nrp1 transfected, but not in the sham-transfected, MDA-MB-453 cells. *In panels A–F, $P < 0.01$ for bars with Nrp1-Fc or $Nrp1^+$ cells versus bars lacking Nrp1-Fc or with Nrp1-negative cells, respectively. Three experiments yielded similar results.

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 that could be used to deliver therapeutic compounds (49,50). 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 TβRI 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-β1 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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