Differential phosphorylation of NG2 proteoglycan by ERK and PKCα helps balance cell proliferation and migration

Irwan T. Makagiansar, Scott Williams, Tomas Mustelin, and William B. Stallcup

Cancer Center, The Burnham Institute for Medical Research, La Jolla, CA 92037

Two distinct Thr phosphorylation events within the cytoplasmic domain of the NG2 proteoglycan help regulate the cellular balance between proliferation and motility. Protein kinase Cα mediates the phosphorylation of NG2 at Thr^{2256}, resulting in enhanced cell motility. Extracellular signal–regulated kinase phosphorylates NG2 at Thr^{2314}, stimulating cell proliferation. The effects of NG2 phosphorylation on proliferation and motility are dependent on β1-integrin activation. Differential cell surface localization of the two distinctly phosphorylated forms of NG2 may be the mechanism by which the NG2–β1-integrin interaction promotes proliferation in one case and motility in the other. NG2 phosphorylated at Thr^{2314} colocalizes with β1-integrin on microprotrusions from the apical cell surface. In contrast, NG2 phosphorylated at Thr^{2256} colocalizes with β1-integrin on lamellipodia at the leading edges of cells. Thus, phosphorylation and the resulting site of NG2–integrin localization may determine the specific downstream effects of integrin signaling.

Introduction

To colonize new areas and establish cell masses sufficient for further development, both normal progenitor cells and malignant tumor cells must be able to migrate and proliferate. There is evidence to suggest that cells may not be able to engage in both of these activities simultaneously. In the rat brain, invading glioma cells are observed to halt their migration along blood vessels during periods of mitosis (Farin et al., 2006), mimicking the saltatory pattern of normal glial progenitor migration (Suzuki and Goldman, 2003). Correspondingly, the most highly invasive glioma cells in human tumors are often found to exhibit the lowest rates of proliferation and vice versa (Giese et al., 2003). We have investigated the role of the NG2 proteoglycan in regulating the choice between glioma cell proliferation and motility.

NG2 is expressed by a variety of immature progenitor cell types and also by several types of tumors (Stallcup, 2002). Several studies suggest a role for NG2 in promoting the proliferation and motility that are characteristic of both normal progenitor cells and malignant tumor cells (Nishiyama et al., 1996; Burg et al., 1997; Grako et al., 1999; Ozerdem and Stallcup, 2004). As a membrane-spanning molecule, NG2 affects proliferation and migration via interactions with both extracellular and intracellular binding partners. The NG2 ectodomain has the ability to sequester growth factors and bind to growth factor receptors (Nishiyama et al., 1996; Goretzki et al., 1999; Grako et al., 1999), influence the processing of kringle domain proteins (Goretzki et al., 2000; Chekenya et al., 2002), interact with extracellular matrix ligands such as collagen VI (Burg et al., 1996; Tillet et al., 1997, 2002), and form signaling complexes with α3β1-integrin and galectin-3 (Fukushi et al., 2004; Wen et al., 2006). The cytoplasmic domain of NG2 is involved in activation of the Rho family GTPases Rac and Cdc42 (Eisenmann et al., 1999; Majumdar et al., 2003; Yang et al., 2004) as well as in anchorage via the PDZ-containing scaffolding proteins MUPP1 and GRIP1 (Barritt et al., 2000; Stegmuller et al., 2003).

Although NG2 exhibits some signal-transducing capabilities of its own (Iida et al., 1995; Fang et al., 1999; Tillet et al., 2002), its ability to enhance signaling by growth factor receptors (Nishiyama et al., 1996; Grako et al., 1999) and β1-integrins (Iida et al., 1995; Eisenmann et al., 1999; Fukushi et al., 2004; Yang et al., 2004) greatly expands the proteoglycan's scope of action. Posttranslational modifications of NG2 provide an

Correspondence to Irwan T. Makagiansar: irwan.makagiansar@emdbiosciences.com

I.T. Makagiansar's present address is EMD Biosciences, Inc., San Diego, CA 92121.

S. Williams' present address is Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121.

T. Mustelin's present address is Amgen, Inc., Seattle, WA 98119.

Abbreviations used in this paper: ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; MEK, MAPK kinase; P-ERK, phosphorylated ERK.

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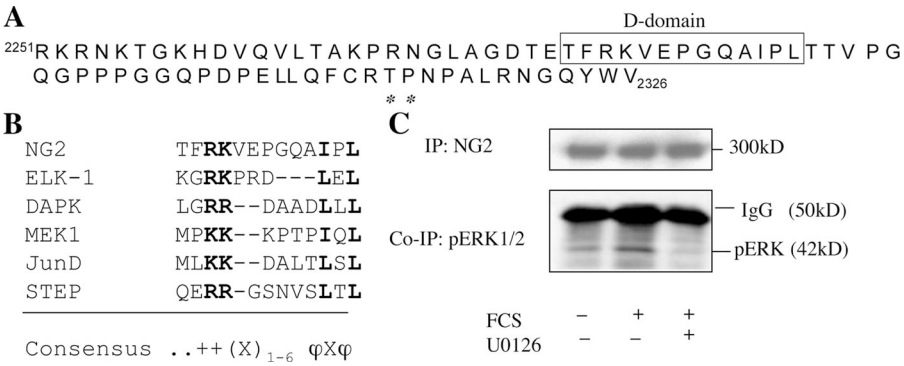

Figure 1. **Interaction of NG2 with ERK.** (A) Scansite analysis of the cytoplasmic sequence of rat NG2 identifies the amino acids T2278-L2290 (box) as a putative D domain–docking site for ERK. The asterisks denote the putative ERK phosphorylation motif x(S/T)P. (B) Alignment of T2278-L2290 with known ERK-docking sites (D domains) present in Elk-1 (Yang et al., 1998), death-associated protein kinase (DAPK; Chen et al., 2005), MEK1 (Xu et al., 1999), JunD (Vinciguerra et al., 2004), and striatal-enriched protein tyrosine phosphatase (STEP; Paul et al., 2000). Conserved residues are indicated in bold font. Consensus designations: +, basic amino acid; φ, hydrophobic amino acid; and X, any amino acid. (C) Serum-starved U251/NG2 cells were treated with 10% FCS for 15 min in the presence or absence of 20 μM U0126. Immunoprecipitated samples were analyzed by Western blotting using antiphospho-ERK1/2 or anti-NG2 antibodies.

important means for regulating its interaction with extracellular and cytoplasmic binding partners. We have reported that PKCα-mediated phosphorylation of Thr^{2256} in the NG2 cytoplasmic domain triggers the redistribution of NG2 from apical microprocesses to lamellipodia on the leading edge of the cell. This molecular redistribution is accompanied by enhanced cell motility (Makagiansar et al., 2004). This has led to our current exploration of the extracellular signal–regulated kinase (ERK)–mediated phosphorylation of NG2 at Thr^{2314} and the contrasting consequences of the two respective phosphorylation events on proliferation and motility.

Results

Evaluation of phosphorylation sites in the NG2 cytoplasmic domain

The Thr^{2256} residue phosphorylated by PKCα is one of several Thr residues in the NG2 cytoplasmic domain (Nishiyama et al., 1991; Makagiansar et al., 2004). Analysis of the cytoplasmic domain of rat NG2 for sequences involved in protein–protein interaction (Scansite 2.0) identified the amino acid residues 2,278–2,290 as a D domain, which is postulated to be a docking site for ERK (Fig. 1 A; Fantz et al., 2001). Fig. 1 B shows the alignment of the NG2 D-domain sequence with that of other proteins with known ERK-docking motifs.

Notably, NG2 also possesses a potential proline-dependent phosphorylation site (^{2313}RTPNP^{2317}; Fig. 1 A) that conforms to the minimal recognition sequence (S/T)P required for phosphorylation by ERK (Fantz et al., 2001). The residues surrounding Thr^{2314} are conserved in the human (Pluschke et al., 1996), chimpanzee (GenBank/EMBL/DDBJ accession no. XP_510685), mouse (Schneider et al., 2001), and dog (GenBank/EMBL/DDBJ accession no. XP_544783) homologues of NG2, which is suggestive of the functional importance of this region.

The NG2 cytoplasmic domain is a substrate for ERK-mediated phosphorylation

To detect the association between NG2 and ERK, the proteoglycan was immunoprecipitated from NG2-transfected U251 cells (U251/NG2). Immunoblotting for phosphorylated ERK (P-ERK) reveals that serum-starved U251/NG2 cells contain low levels of P-ERK associated with NG2 (Fig. 1 C). When serum-starved cells are treated with 10% serum, increased co-immunoprecipitation of P-ERK with NG2 is observed. Pretreatment of cells with the MAPK kinase (MEK) 1/2 inhibitor U0126 largely blocks the P-ERK–NG2 interaction, suggesting that the activation of ERK is required for its association with NG2.

To directly test the ability of ERK to phosphorylate NG2, we used active recombinant ERK in conjunction with γ-[^{32}P]ATP to carry out in vitro phosphorylation of GST fusion proteins containing the cytoplasmic regions of wild-type NG2 (GST-NG2c) and the Thr point mutants GST-NG2c-T2256E, -NG2c-T2265E, -NG2c-T2278E (Makagiansar et al., 2004), and -NG2c-T2314E. Results of the phosphorylation reactions were analyzed by two-dimensional mapping of tryptic phosphopeptides. The phosphopeptide maps of the GST-NG2c-T2265E and -NG2c-T2314E fusion proteins differ from those of the other species (Fig. 2). GST-NG2c-T2265E lacks the more highly charged of two major phosphopeptides derived from the wild-type fusion protein (spot 1), whereas the more neutral phosphopeptide (spot 2) is missing from GST-NG2c-T2314E. Simultaneous mutation of both of these Thr sites leads to the disappearance of all phosphopeptides from the GST-NG2c-T2265E/T2314E map, confirming the ability of recombinant ERK to phosphorylate the NG2 cytoplasmic domain at positions Thr^{2265} and Thr^{2314} in vitro.

ERK contributes to the phosphorylation of full-length cell surface NG2

In light of artifacts that may occur in vitro, caution must be used when interpreting the phosphorylation data in Fig. 2 (Manning and Cantley, 2002; Wingate et al., 2006). Therefore, we examined ERK phosphorylation of full-length NG2 in the context of living cells using two different motif-specific antibodies to detect Thr phosphorylation. The first antibody recognizes the sequence xTpx(K/R) found at Thr^{2265}, whereas the second recognizes the xTpP motif found at Thr^{2314}. The specificity of these antibodies in the context of the NG2 cytoplasmic domain was confirmed via immunoblotting of the GST-NG2c fusion protein that had

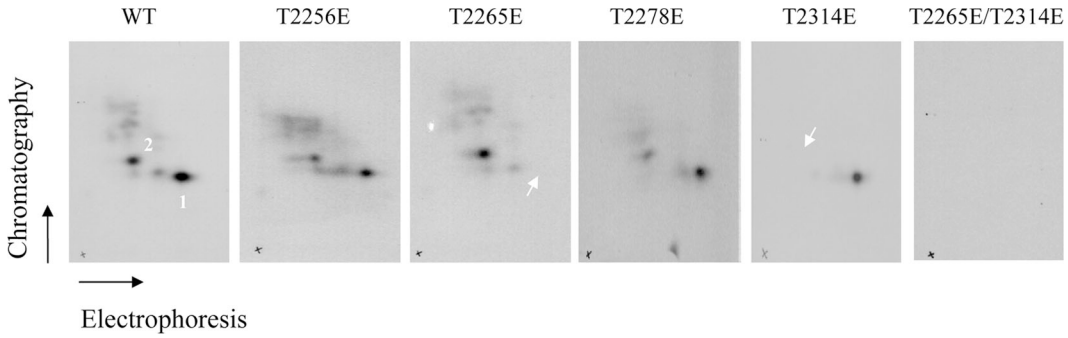

Figure 2. **In vitro phosphorylation of NG2 fusion proteins by ERK.** Two-dimensional analysis reveals two major ERK-phosphorylated peptides derived from the wild-type (WT) NG2 cytoplasmic domain and from the T2256E and T2278E mutants. The most highly charged of the two phosphopeptides (spot 1) is missing from the T2265E map (arrow), whereas the more neutral phosphopeptide (spot 2) is absent from the T2314E map (arrow). Both phosphopeptides are absent from the map of the double mutant (T2265E/T2314E).

been phosphorylated in vitro by nonradioactive ERK or PKCα-catalyzed reactions (unpublished data).

To stimulate the ERK-mediated phosphorylation of full-length NG2 in living cells, we treated U251/NG2 cells with PDGF-BB. This ligand activates both the α and β isoforms of the PDGF receptor, triggering the downstream activation of numerous intracellular signaling cascades, including both the PKC and MAPK pathways. After PDGF-BB stimulation, total cell extracts were examined to confirm the activation of PKCα and ERK. Levels of P-ERK and PKCα were found to increase within 15 to 30 min (Fig. 3 A). In parallel, NG2 was immunoprecipitated and immunoblotted with the respective phospho-Thr antibodies, revealing large increases in the quantity of phosphorylated NG2 detected by both the anti-xTpP and -xTpx(K/R) antibodies (Fig. 3 A). Similar patterns of NG2 phosphorylation were detected by these same phosphospecific antibodies in A375 human melanoma cells (Fig. 3 B). Therefore, phosphorylation of the proteoglycan is not an artifact of its overexpression but a normal physiological consequence of PDGF-BB treatment.

It was initially unclear whether NG2 phosphorylation detected by the xTpx(K/R) antibody was caused by the ERK-mediated phosphorylation of Thr^{2265} observed in vitro or by the reported PKCα-mediated phosphorylation of Thr^{2256} (Makagiansar et al., 2004). To resolve this issue, U251/NG2 cells were pretreated either with the MEK1/2 inhibitor U0126 or the PKCα inhibitor Gö6976 before the addition of PDGF-BB. U0126 inhibition of ERK activation did not alter the level of xTpx(K/R) phosphorylation (Fig. 3 C). In contrast, this phosphorylation was blocked by treatment with Gö6976. These data suggest that the PDGF-BB–induced xTpx(K/R) phosphorylation of NG2 is not caused by ERK phosphorylation at Thr^{2265} but instead by PKCα-mediated phosphorylation at Thr^{2256}. Thus, Thr^{2265} is unlikely to be an ERK phosphorylation site in NG2 expressed in living cells.

The substantial increase in PDGF-BB–induced xTpP motif phosphorylation implicates Thr^{2314} as an in vivo NG2 phosphorylation site (Fig. 3 A). This phosphorylation is not observed in the presence of U0126 (Fig. 3 C), establishing ERK as the likely mediator of Thr^{2314} phosphorylation. Interestingly, PDGF-BB–dependent ERK-mediated Thr^{2314} phosphorylation is not observed when PKCα activation is blocked by Gö6976 (Fig. 3 C), suggesting that PKCα is a key upstream effector of ERK activation in these cells.

To further establish that Thr^{2314} is the sole site of ERK-mediated NG2 phosphorylation in living cells, cells transfected with full-length NG2 constructs containing individual T→V substitutions (NG2-T2256V, -T2265V, and -T2314V) were stimulated with PDGF-BB in the absence or presence of the U0126 and Gö6976 inhibitors followed by immunoprecipitation of the mutant NG2 proteins. With the exception of the NG2-T2314V mutant, phosphorylation was detected in all NG2 species by blotting with the anti-xTpP antibody (Fig. 3 D), further establishing Thr^{2314} as the site of ERK phosphorylation. In addition, PDGF-BB treatment induced heavy xTpx(R/K) phosphorylation of the NG2-T2265 and -T2314 mutants but failed to phosphorylate an xTpx(K/R) site in the NG2-T2256V mutant. This identifies Thr^{2256} as the sole PDGF-BB–dependent xTpx(K/R) phosphorylation site in NG2. Thus, by activating both PKCα and ERK downstream of PDGF receptors, PDGF-BB stimulation results in the phosphorylation of NG2 at both Thr^{2256} and Thr^{2314}.

As a final demonstration that ERK phosphorylates only Thr^{2314} in cells, we transfected U251/NG2 cells with a constitutively active MEK mutant (MEK-DD) (Saxena et al., 1999; Delmas et al., 2003). As expected, an increase in ERK phosphorylation was observed in U251/NG2/MEK-DD cells compared with U251/NG2 cells, whereas PKCα phosphorylation was unchanged (Fig. 3 E). Correspondingly, when NG2 was immunoprecipitated from the two sets of cells and immunoblotted with the respective phospho-Thr antibodies, the phosphorylation of NG2 at Thr^{2314} was increased in U251/NG2/MEK-DD cells relative to that seen in U251/NG2 cells, whereas Thr^{2256} phosphorylation was unaffected.

Differential cell surface localization of NG2-T2256E and -T2314E

In U251/NG2 cells, the proteoglycan is present on microprotrusions from the apical cell surface (Fig. 4, a–c). It is noteworthy that these microprotrusions are infrequently seen on parental U251 cells (not depicted) and on NG2-negative cells in the U251/NG2 population (Fig. 4, a–c; arrows). In contrast, the mutant NG2-T2256E species that mimics Thr^{2256} phosphorylation (Makagiansar et al., 2004) is localized to broad lamellipodia

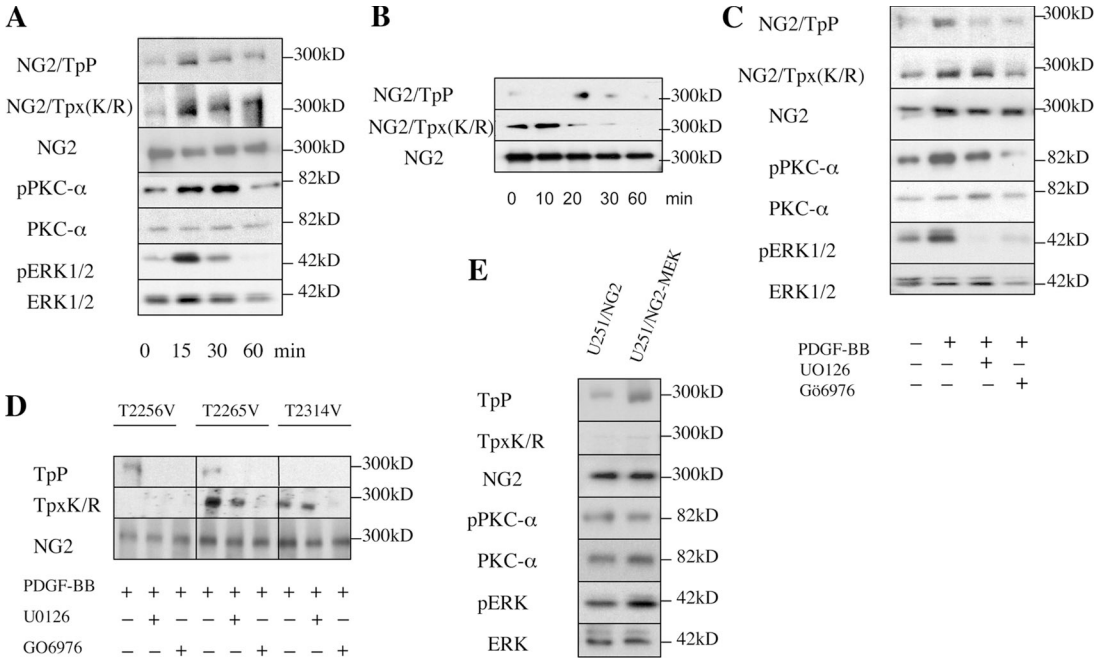

Figure 3. **ERK-mediated phosphorylation of full-length NG2.** (A) NG2 was immunoprecipitated after the treatment of U251/NG2 cells with 20 ng/ml PDGF-BB. Immunoblotting of immunoprecipitates with the xTpP and xTpx(K/R) antibodies reveals PDGF-BB–induced increases in NG2 phosphorylation at both types of Thr sites. Immunoblotting of total cell extracts for pPKC-α and pERK1/2 as well as for total PKC-α and ERK confirm the activation of both kinases by PDGF-BB treatment. (B) A375M melanoma cells were stimulated with PDGF-BB and processed as described in A. NG2 immunoprecipitates were immunoblotted with the xTpP and xTpx(K/R) antibodies to reveal phosphorylation at both types of motifs. (C) U251/NG2 cells pretreated with either 10 μM of the MEK inhibitor U0126 or 100 nM of the PKC-α inhibitor Gö6976 were stimulated with 20 ng/ml PDGF-BB for 20 min. NG2 immunoprecipitates were immunoblotted with the two phospho-Thr antibodies. The U0126 reagent blocks NG2 phosphorylation at the xTpP site but not at the xTpx(K/R) site, indicating that Thr^{2314} is a target for ERK-mediated phosphorylation of full-length NG2, whereas Thr^{2265} is not. The additional ability of Gö6976 treatment to block phosphorylation at the xTpP site (Thr^{2314}) suggests that ERK activation is dependent on the action of PKCα. In contrast, U0126 inhibits ERK phosphorylation without affecting PKC-α phosphorylation. (D) U251 cells transfected with NG2 variants were analyzed according to the same scheme outlined in B. Phosphorylation at Thr^{2314} (xTpP blots) is blocked in the NG2-T2314V mutant but occurs normally in both NG2-T2256V and -T2265V. Inhibition by U0126 confirms these two phosphorylation events as being ERK dependent. Phosphorylation at Thr^{2256} (xTpx(K/R) blots) is absent in the NG2-T2256V mutant but occurs normally in both NG2-T2265V and -T23124V. Inhibition of these phosphorylations by Gö6976 demonstrates their dependence on PKC-α. (E) Unstimulated U251/NG2 and U251/NG2/MEK-DD cells were analyzed for the phosphorylation of ERK, PKC-α, and NG2. Elevated ERK phosphorylation by the activated MEK-DD mutant leads to the increased phosphorylation of NG2 at Thr^{2314} but not at Thr^{2256}.

(Fig. 4, d–f). As suggested by its coimmunoprecipitation with NG2 (Makagiansar et al., 2004), phospho-PKC is also localized with NG2-T2256E in lamellipodia (Fig. 4, g–i). Furthermore, in both microprotrusions and lamellipodia, NG2 labeling is colocalized with β1-integrin labeling (Fig. 4, a–f). The α3-integrin subunit has an identical localization in these cell types (unpublished data), which is consistent with our observation that α3β1 is a predominant integrin heterodimer in U251 cells (Paulus et al., 1993).

Unlike NG2-T2256E, an NG2-T2314E species designed to mimic Thr^{2314} phosphorylation is largely present in β1-integrin–positive arrays of apical microprotrusions reminiscent of those seen on U251/NG2 cells. However, in U251/NG2-T2314E transfectants, these structures are more highly clustered or bundled than in U251/NG2 cells (Fig. 4, j–l). Cells expressing the NG2-T2314V variant resemble parental U251 cells in that they rarely display apical protrusions (Fig. 4, m–o). Collectively, these data suggest that Thr^{2314} phosphorylation not only directs NG2 localization to apical microspikes but may actually stimulate the formation of these protrusions. We propose that the level of Thr^{2314} phosphorylation found in U251/NG2 cells under basal conditions (Fig. 3) is sufficient to induce detectable microspike formation. Phosphorylation of additional NG2 molecules at Thr^{2314}, as mimicked by the NG2-T2314E mutant, appears to result in aggregation of the microprotrusions. We were unable to detect a consistent signal with phospho-ERK antibody in any of these cell types and, therefore, were not able to determine whether phospho-ERK localizes with NG2 or NG2-T2314E in apical microspikes.

Distinct effects of Thr^{2314} and Thr^{2256} phosphorylation on cell motility

NG2 expression has been shown to have a stimulatory effect on cell motility (Burg et al., 1997; Fang et al., 1999; Grako et al., 1999). We used transwell migration assays to study the effect of PDGF-BB on the motility of U251 cells expressing a variety of NG2 species (Fig. 5 A). Under basal conditions (i.e., no PDGF-BB), no reproducible differences in cell migration are observed between parental U251 cells and any of the NG2 transfectants except for the NG2-T2256E transfectant, which exhibits strongly enhanced migration. This supports our previous finding that actual or mimicked phosphorylation at Thr^{2256} promotes cell motility (Makagiansar et al., 2004). Notably, glutamic acid substitution at Thr^{2314} does not have such an effect on basal cell motility.

Although PDGF-BB treatment has a small stimulatory effect on the migration of parental U251 cells, the magnitude of this stimulation is enhanced in U251/NG2 cells, which migrate

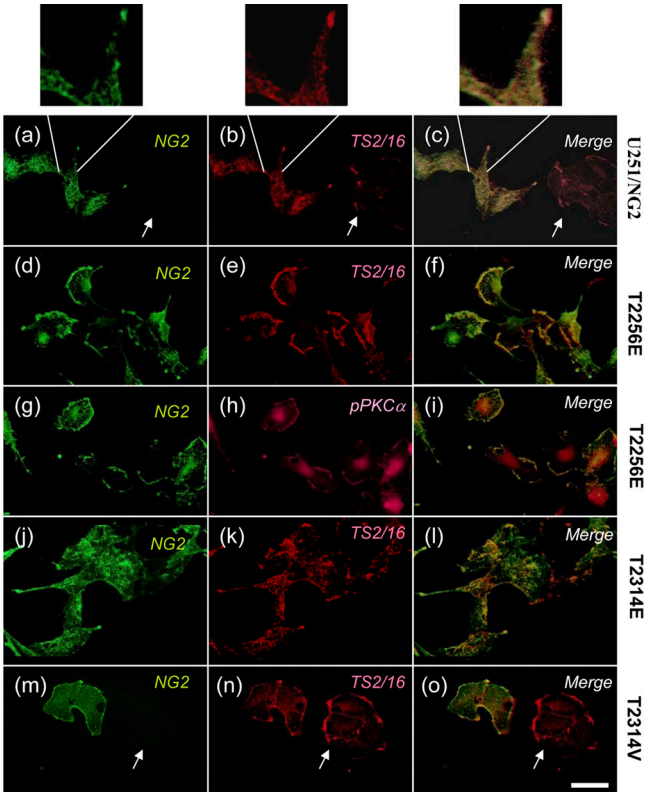

Figure 4. **Cell surface localization of NG2 species.** U251 cells expressing NG2 and the mutant species NG2-T2256E, -T2314E, and -T2314V were grown overnight under basal conditions. Cells were fixed with 4% PFA and labeled using a mix of rabbit anti-NG2 antibody (green) and mouse monoclonal TS2/16 anti–β1-integrin antibody (red). (a–f) Wild-type NG2 and the NG2-T2256E mutant are colocalized with β1-integrin on apical microprotrusions (a–c) and leading edge lamellipodia (d–f), respectively. Insets associated with a–c represent higher magnifications to highlight the colocalization of NG2 and β1-integrin in microprotrusions. (g–i) Phospho-PKC is also prominent in U251/NG2-T2256E lamellipodia. (j–l) The NG2-T2314E mutant resembles wild-type NG2 in its expression on microprotrusions, although these structures are more highly clustered than in U251/NG2 cells. (m–o) Microprotrusions are largely absent in U251/NG2-T2314V cells. Arrows in a–c and m–o identify cells that have lost NG2 expression. Bar, 10 μm.

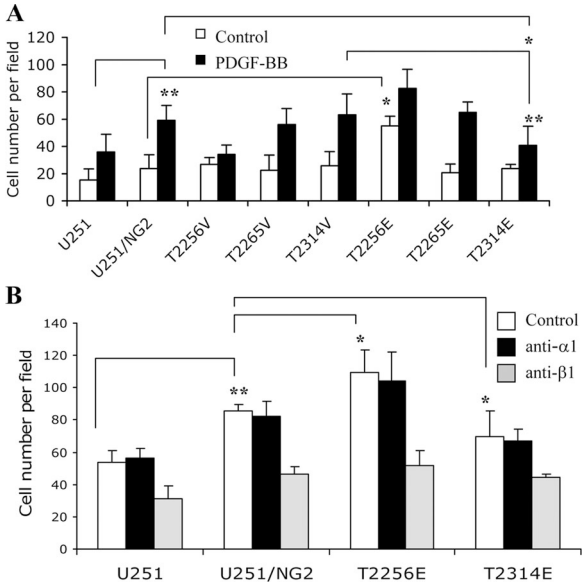

Figure 5. **Phosphorylations of NG2 at Thr^{2314} and Thr^{2256} have opposing effects on cell motility.** (A) Several U251/NG2 variants were assayed in transwell chambers for basal (open bars) and PDGF-BB–induced (closed bars) motility. Each bar represents the mean ± SD (error bars) for triplicate wells. These results are representative of three separate experiments. NG2 expression causes enhancement of the basal effect of PDGF-BB seen in parental U251 cells. The stimulatory effect of NG2 phosphorylation at Thr^{2256} is seen in the enhanced basal motility of the NG2-T2256E variant and in the inability of the NG2-T2256V variant to respond to PDGF-BB. An inhibitory effect of Thr^{2314} phosphorylation is seen in the reduced PDGF-BB response of the NG2-T2314E variant. *, $P < 0.02$; **, $P < 0.01$. (B) Effects of integrin-blocking antibodies on the PDGF-BB–induced motility of various U251 transfectants. Anti–β1-integrin antibody reduces the increased motility that results from the actual or mimicked phosphorylation of NG2 at Thr^{2256}. Control anti–α1 antibody has no effect on motility. Each bar represents the mean ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

at a level comparable with the high basal rate seen with the NG2-T2256E transfectants (Fig. 5 A). Thus, although PDGF-BB treatment is capable of stimulating the motility of U251 cells via NG2-independent mechanisms, the expression of NG2 permits an additional increase in PDGF-BB–induced motility via PKCα-mediated phosphorylation at Thr^{2256}. This NG2-dependent enhancement of PDGF-BB stimulation is also seen in the other mutant transfectants, with the exception of NG2-T2256V and -T2314E, which respond to PDGF-BB at the same modest level as parental U251 cells. For the T2256V transfectant, this loss of responsiveness is explained by the inability of PKCα to phosphorylate the mutated site. Regarding the T2314E transfectant, it is critical to note that the T2314V mutation does not exhibit this same depressed response to PDGF-BB. Therefore, the inhibitory effect of the T→E substitution is not the result of a block of Thr^{2314} phosphorylation. Instead, the ability of the mutation to mimic ERK-mediated phosphorylation at Thr^{2314} must counteract, to some degree, the stimulatory effect on motility caused by NG2 phosphorylation at Thr^{2256}.

There are several reports of interactions between NG2 and β1-integrins, including α4β1 and α3β1 (Iida et al., 1995; Doane et al., 1998; Midwood and Salter, 2001; Yang et al., 2004). We have documented the ability of exogenous NG2 to activate α3β1-integrin signaling on the endothelial cell surface (Fukushi et al., 2004). Because membrane-bound NG2 might also be able to activate β1-integrin signaling in a cis manner in U251/NG2 cells, we repeated the cell motility studies in the presence of the β1-integrin–blocking antibody P4C10 (Fig. 5 B). This antibody substantially inhibits both the PDGF-BB–enhanced motility of U251/NG2 cells and the spontaneous motility of NG2-T2256E transfectants. An antibody against the α1-integrin subunit, which is present at very low levels on these cells, provides a negative control for possible nonspecific antibody effects. These results suggest that the enhanced motility of U251/NG2 cells in response to PDGF-BB is a β1-integrin–dependent process whose activation is at least partially dependent on the phosphorylation of NG2 at Thr^{2256}.

Distinct effects of Thr^{2314} and Thr^{2256} phosphorylation on cell proliferation

Because previous in vitro and in vivo studies have demonstrated the involvement of NG2 in cell proliferation (Nishiyama et al., 1996; Grako et al., 1999; Ozerdem and Stallcup, 2004),

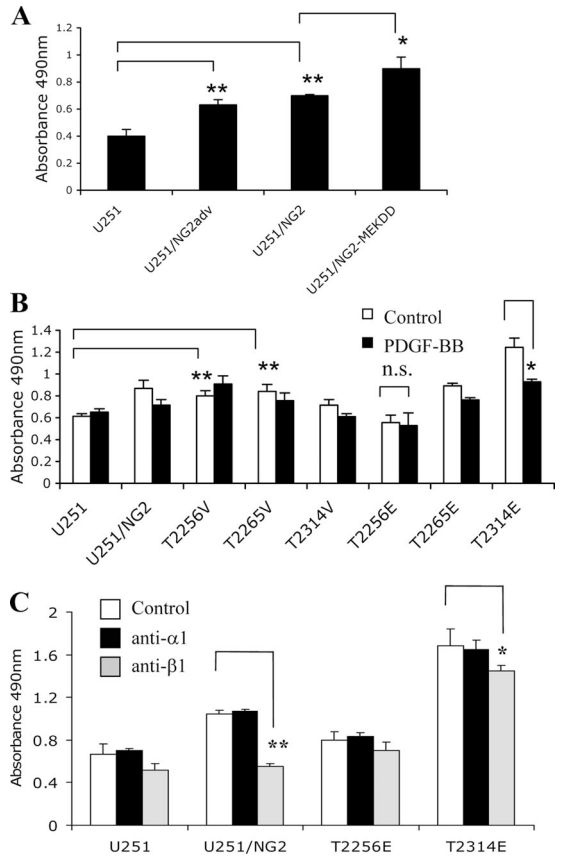

Figure 6. **The effects of NG2 phosphorylation at Thr^{2314} and Thr^{2256} are reversed in cell proliferation.** (A) Cell mass was determined after 48 h for serum-starved U251 cells, U251 cells expressing NG2 after adenovirus infection (U251/NG2adv), conventional U251/NG2 transfectants, and U251 cells doubly transfected for NG2 and MEK-DD. Both sets of U251/NG2 cells exhibit higher basal levels of proliferation than parental cells. The presence of constitutively active MEK-DD further enhances basal proliferation. Bars represent means ± SD (error bars) in triplicate wells from four independent experiments. *, $P < 0.005$; **, $P < 0.001$. (B) The protocol outlined in A was used to compare the proliferation of wild-type NG2 transfectants and a variety of NG2 mutants in the absence (open bars) or presence (closed bars) of PDGF-BB. PDGF-BB treatment does not affect proliferation of the U251 cells but has a negative effect on that of the NG2 transfectants. Mimicking Thr^{2314} phosphorylation in the NG2-T2314E variant results in greatly enhanced cell proliferation. Reduction of this elevated proliferation by PDGF-BB treatment suggests the possibility of inhibition by phosphorylation at Thr^{2256}. This is supported by the observation that basal proliferation of the NG2-T2256E variant is reduced to the level of U251 parental cells. Moreover, as a result of blocking the Thr^{2256} site, proliferation of the NG2-T2256V variant is enhanced by PDGF-BB treatment rather than reduced as seen in every other case. *, $P < 0.002$; **, $P < 0.001$. (C) Antibody against β1-integrin blocks the stimulatory effect of NG2 expression on the basal rate of proliferation in U251/NG2 cells and, to some extent, also in NG2-T2314E transfectants. The α1-integrin antibody provides a negative control. *, $P < 0.05$; **, $P < 0.0005$. (B and C) Each bar represents the mean ± SD ($n = 3$).

we investigated the involvement of ERK-mediated NG2 phosphorylation in this process. Under basal conditions in the absence of exogenous PDGF-BB over the course of 48 h, the number of NG2-expressing U251 cells is ∼50% greater than that of parental U251 cells (Fig. 6 A). This is true for both conventional U251/NG2 transfectants and U251 cells with adenovirally expressed NG2 (Yang et al., 2006). To determine whether the increased cell number is caused by increased proliferation or improved survival in the NG2-expressing cells, apoptosis levels were assessed via flow cytometry (see Materials and methods). The low apoptosis levels we observed (1.2% for U251 cells and 2.3% for U251/NG2 cells) cannot explain the difference in cell expansion seen in Fig. 6 A, indicating that NG2 expression enhances cell proliferation rather than cell survival under low serum conditions.

Interestingly, the constitutively active MEK-DD construct further enhances the proliferation of U251/NG2 cells (Fig. 6 A), suggesting the involvement of the ERK-dependent phosphorylation of NG2 at Thr^{2314} (Fig. 3 E). To further evaluate this possibility, we studied the effect of PDGF-BB treatment on cell proliferation (Fig. 6 B). PDGF-BB has little effect on NG2-negative U251 cells and actually decreases the proliferation of U251/NG2 cells. To determine the respective effects on proliferation of NG2 phosphorylation at Thr^{2256} versus Thr^{2314}, we performed proliferation studies on our panel of mutant NG2 transfectants. Under basal conditions, the T2256V and T2265V transfectants exhibit enhanced proliferation (relative to U251 cells) similar to that of the wild-type NG2 transfectant. In contrast, T2314V transfectants have the nonenhanced proliferation characteristics of NG2-negative U251 cells. Together with the enhancement produced by MEK-DD, these findings suggest that basal levels of ERK-mediated phosphorylation at Thr^{2314} are responsible for NG2-dependent proliferation under nonstimulatory conditions. After treatment with PDGF-BB, decreased cell proliferation is observed for all valine-substituted NG2 transfectants except for the T2256V variant, in which PDGF-BB enhances proliferation. This behavior may be explained by an inhibitory effect of phosphorylation at Thr^{2256} by PKCα, which is relieved by the NG2-T2256V mutation.

These possibilities were further assessed by examination of the T→E substitution variants. By mimicking phosphorylation at Thr^{2256}, the NG2-T2256E transfectant reduces both basal and PDGF-BB–stimulated cell proliferation to the level of parental cells, which is in agreement with the hypothesis that the Thr^{2256} phosphorylation event is inhibitory to cell proliferation. In contrast, by mimicking phosphorylation at Thr^{2314}, the NG2-T2314E transfectant exhibits increased proliferation beyond that seen with any other species, confirming the important role of Thr^{2314} phosphorylation in promoting cell proliferation. Treatment of U251/NG2-T2314E cells with PDGF-BB partially overcomes this stimulatory effect, most likely as a result of the negative effect of Thr^{2256} phosphorylation.

In light of the effect of β1-integrin–blocking antibody on U251/NG2 cell motility, we repeated the proliferation studies in the presence of this same antibody (Fig. 6 C). This antibody completely inhibits the increased proliferation seen in U251/NG2 cells under basal conditions. The effect of the β1 antibody is somewhat less striking in the case of NG2-T2314E transfectants but is still significant ($P < 0.05$) compared with the lack of effect of the control α1 antibody. These results indicate that the NG2-dependent increase in proliferation seen in U251/NG2 cells is caused by enhanced β1-integrin signaling in response to NG2 phosphorylation at Thr^{2314}.

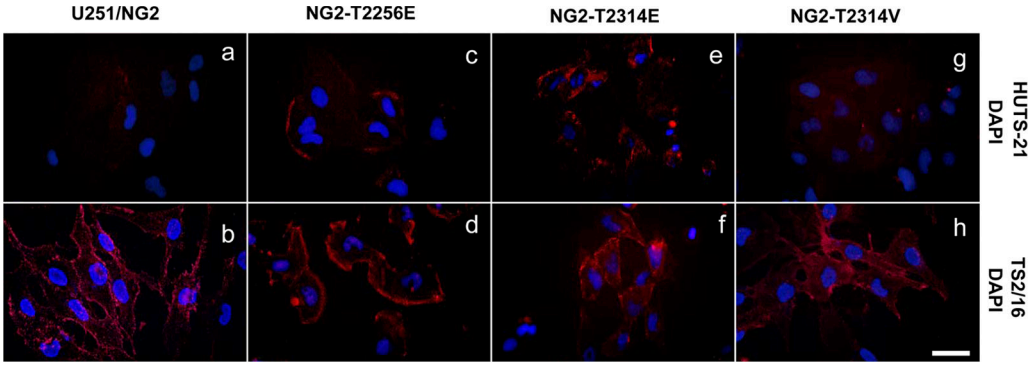

Figure 7. **Sites of β1-integrin activation detected by HUTS-21 and phosphotyrosine antibodies.** Living cells were labeled under basal conditions with the HUTS-21 antibody (a, c, e, and g) to detect β1-integrin activation and with the TS2/16 antibody (b, d, f, and h) to assess total β1-integrin levels. Compared with U251/NG2 cells (a) and U251/NG2-T2314V transfectants (g), U251/NG2-T2256E (c) and U251/NG2-T2314E (e) cells exhibit increased labeling with HUTS-21. Cell nuclei were labeled with DAPI. Bar, 10 μm.

NG2-dependent activation of β1-integrins

We sought additional evidence for NG2-dependent β1-integrin signaling through use of the HUTS-21 monoclonal antibody, which binds to the activated form of the β1 subunit (Luque et al., 1996; Fukushi et al., 2004). Because we were unable to detect HUTS-21 labeling on fixed cells, we used living cells for these experiments. Although this resulted in the loss of much of the morphological detail seen in Fig. 4, several interesting patterns of β1-integrin activation were nevertheless revealed. Whereas HUTS-21 binding is detectable only at very low levels in U251/NG2 cells (Fig. 7 a), labeling is greatly increased along lamellipodial edges in NG2-T2256E transfectants (Fig. 7 c), corresponding to the site of NG2 and β1-integrin localization in these cells (Fig. 4, d–f). HUTS-21 labeling of NG2-T2314E transfectants is also increased (Fig. 7 e) but is more evenly distributed across the cell surface, which is consistent with the localization of NG2 and β1-integrin to apical microprotrusions (Fig. 4, j–l). In contrast, HUTS-21 binds to the NG2-T2314V (Fig. 7 g) and -T2256V (not depicted) transfectants at the basal level seen in U251/NG2 cells, supporting the idea that phosphorylations at Thr^{2314} and Thr^{2256} trigger NG2-dependent β1-integrin activation. As shown by labeling with the β1 antibody TS2/16 (Fig. 7, b, d, f, and h), each of the cell types examined express comparable levels of total β1-integrin, although integrin distribution is skewed toward lamellipodia in the case of U251/NG2-T2256E cells.

Integrin activation is known to trigger the assembly of signaling complexes containing nonreceptor protein tyrosine kinases such as focal adhesion kinase (FAK; for review see Cox et al., 2006). Accordingly, we found high levels of tyrosine phosphorylation colocalized with NG2 in the lamellipodia of U251/NG2-T2256E cells (Fig. 8, a–c) and in the microprotrusions of U251/NG2-T2314E cells (Fig. 8, d–f). Consistent with the scarcity of microprotrusions in the absence of NG2 (Fig. 4), this type of phosphotyrosine labeling is not seen in cells in the U251/NG2-T2314E culture that have lost NG2 expression (Fig. 8, d–f; arrows). In these cells, phosphotyrosine labeling is faintly visible in focal adhesion plaques, which are completely obscured by the labeling of microprotrusions in NG2-positive cells.

Discussion

Phosphorylation and dephosphorylation have proved to be among the most versatile and functionally important types of posttranslational modifications (Manning et al., 2002; Alonso et al., 2004). In the case of NG2-dependent mechanisms, PKCα-mediated phosphorylation of NG2 at Thr^{2256} triggers increased cell motility via a mechanism that involves the translocation of NG2 to sites in leading edge lamellipodia of motile cells (Makagiansar et al., 2004). This profound effect of phosphorylation on NG2 localization/function prompted us to investigate the existence of additional NG2

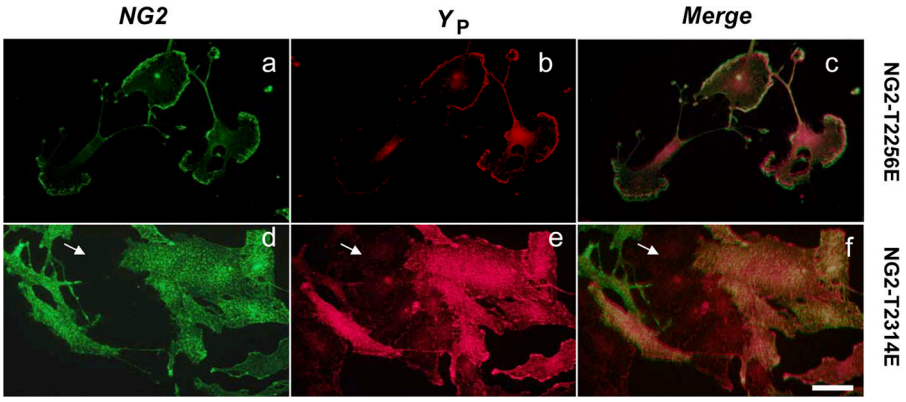

Figure 8. **Enhanced phosphotyrosine labeling at sites of NG2 expression.** After overnight growth under basal conditions, PFA-fixed U251/NG2-T2256E (a–c) and U251/NG2-T2314E (d–f) cells were used to demonstrate the colocalization of NG2 (green) and phosphotyrosine (red) labeling in lamellipodia (a–c) and microprotrusions (d–f). Arrows in d–f indicate NG2-negative cells in which phosphotyrosine labeling is confined to focal adhesions. Bar, 10 μm.

phosphorylation sites capable of influencing other aspects of cell biology.

Sequence analysis allows us to identify two previously unrecognized features of the NG2 cytoplasmic domain: (1) a putative D domain–docking site for ERK (residues 2,278–2,290) and (2) a potential site for ERK-mediated phosphorylation at Thr^{2314}. Immunoprecipitation/immunoblotting results confirm the existence of a physical interaction between NG2 and activated ERK. In addition, purified recombinant ERK is able to phosphorylate the isolated NG2 cytoplasmic domain at Thr^{2265} and Thr^{2314}.

In extending these studies to full-length NG2 expressed endogenously by A375 melanoma cells or by transfection in U251 glioma cells, we used PDGF-BB stimulation to activate both PKCα and ERK. This provides a more physiologically relevant means of stimulating phosphorylation than the PMA treatment we previously used to identify PKCα-mediated modification at Thr^{2256} (Makagiansar et al., 2004). We evaluated PDGF-BB–induced phosphorylation of NG2 by immunoblotting with phosphospecific antibodies against the xTpx(K/R) motif present at Thr^{2256} and Thr^{2265} and against the xTpP motif present at Thr^{2314}. The use of specific inhibitors of PKCα and ERK coupled with analysis of NG2 species with valine substitutions at the putative phosphorylation sites reveals that PKCα-mediated phosphorylation occurs at Thr^{2256}, whereas ERK-mediated phosphorylation occurs only at Thr^{2314}. Contrary to the results obtained in vitro with recombinant ERK treatment of the isolated cytoplasmic domain, Thr^{2265} is not used as a site for ERK phosphorylation in full-length NG2 expressed by living cells. This is more in keeping with predictions based on NG2 sequence analysis because the residues in the immediate vicinity of Thr^{2265} do not represent a canonical ERK phosphorylation motif.

An important mechanistic observation from the experiments with NG2-transfected U251 cells is that ERK activation in response to PDGF-BB is highly dependent on the activity of PKCα. This seems somewhat surprising because conventional wisdom would suggest that PDGF receptors should also be able to activate ERK via a Son of sevenless–Ras–Raf–MEK-dependent pathway (for review see Porter and Vaillancourt, 1998). However, the ability of the PKCα inhibitor Gö6976 to inhibit both the PKCα-catalyzed phosphorylation of Thr^{2256} and the ERK-catalyzed phosphorylation of Thr^{2314} does not provide evidence for an important contribution of the growth factor–activated Son of sevenless–Ras pathway to ERK activation in U251 cells. A similar dependence of ERK on PKC activity has been observed in PDGF stimulation of smooth muscle cells (Robin et al., 2004; Ginnan and Singer, 2005). The consequences of these findings in U251 cells are twofold: (1) PDGF-BB treatment inevitably activates both PKCα and ERK, thus leading to NG2 phosphorylation at both Thr^{2256} and Thr^{2314}, and (2) we cannot inhibit PKCα without also affecting ERK activity. In this regard, the constitutively active MEK-DD construct has provided an effective means for activating ERK independent of PKCα activation, enabling us to restrict NG2 phosphorylation to Thr^{2314}.

Having established the existence of two distinct types of Thr phosphorylation sites in the NG2 cytoplasmic domain, we addressed the effects of these two phosphorylation events on cell behavior. Use of the transwell cell motility assay allows us to confirm our previous report (Makagiansar et al., 2004) that both the actual and mimicked phosphorylation of NG2 at Thr^{2256} leads to enhanced cell migration. Not surprisingly, PDGF-BB increases the motility of parental U251 cells via mechanisms that are independent of NG2. Nevertheless, the presence of NG2 further enhances the response to PDGF-BB. The fact that this is caused by NG2 phosphorylation at Thr^{2256} is demonstrated by the loss of enhanced PDGF-BB responsiveness in NG2-T2256V transfectants and by the increased motility of NG2-T2256E variants even in the absence of growth factor. Notably, the other NG2 transfectant that fails to exhibit enhanced NG2-dependent motility in response to PDGF-BB is the NG2-T2314E variant. Because the NG2-T2314V variant does not exhibit this loss of function, the depressed motility of NG2-T2314E transfectants cannot be caused by the blockage of Thr^{2314} phosphorylation. Instead, the defect must be the result of mimicked phosphorylation at this site, indicating that phosphorylation at Thr^{2314} counteracts the stimulation of motility produced by the phosphorylation of Thr^{2256}.

We were also able to identify effects of NG2 phosphorylation on cell proliferation. In the absence of growth factor stimulation, U251 cells expressing NG2 exhibit a greater rate of cell proliferation than parental U251 cells. This phenomenon is observed in both conventionally transfected and adenovirally transformed cells, demonstrating that the behavior is independent of the means of NG2 expression. Elevated basal rates of proliferation are also observed in each of the valine-substituted NG2 variants with the exception of NG2-T2314V, suggesting that basal levels of phosphorylation at Thr^{2314} in nonstimulated cells (Fig. 3 A) may be responsible for the increased proliferation of NG2-expressing U251 cells relative to parental cells. Support for this idea is provided by the behavior of U251/NG2/MEK-DD transfectants and NG2-T2314E transfectants, both of which proliferate faster under basal conditions than any other species examined because of phosphorylation and mimicked phosphorylation at Thr^{2314}, respectively.

Interestingly, although the addition of PDGF-BB has no effect on the proliferation of parental U251 cells, it negatively affects the proliferation of U251/NG2 transfectants. This negative effect of PDGF is also seen in the valine-substituted NG2 variants with the exception of NG2-T2256V, in which proliferation is enhanced. The possibility that PDGF-induced phosphorylation at Thr^{2256} is capable of reversing the stimulatory effect on proliferation caused by Thr^{2314} phosphorylation is supported by the low rates of proliferation observed in NG2-T2256E transfectants, which mimic Thr^{2256} phosphorylation.

Our findings suggest the existence of an intriguing balance between cell proliferation and migration that can be regulated, in part, by phosphorylation of the NG2 cytoplasmic domain at two different sites. PKCα-mediated phosphorylation at Thr^{2256} appears to stimulate cell motility while inhibiting cell proliferation. Conversely, ERK-mediated phosphorylation at Thr^{2314} tends to block cell motility while promoting cell proliferation. In unstimulated cells, it appears that low levels of Thr^{2314} phosphorylation are responsible for the increased rate of proliferation of U251/NG2 cells compared with parental U251 cells. When cells are treated with PDGF-BB, increased phosphorylation

occurs at both Thr^{2314} and Thr^{2256}. Under these conditions, our data suggest that signals generated via Thr^{2256} phosphorylation may be dominant over those resulting from Thr^{2314} phosphorylation.

In this context, and considering that both PDGF-induced phosphorylation events seem to depend on PKCα activation, it is logical to ask under what circumstances Thr^{2314} phosphorylation would lead to increased cell proliferation. An answer may lie in the ability of non–growth factor–driven mechanisms to activate ERK independently of PKCα activation. An artificial example of such a mechanism is provided by our use of the MEK-DD construct to drive ERK activation independently of PKCα. Under these circumstances, Thr^{2314} phosphorylation is achieved in the absence of Thr^{2256} phosphorylation, resulting in the enhancement of cell proliferation. A more biologically relevant means of achieving Thr^{2314} phosphorylation independent of Thr^{2256} phosphorylation might involve the activation of ERK via a G protein–coupled receptor-dependent pathway (Pierce et al., 2001). Alternatively, integrin-mediated activation of the FAK–src–p130cas pathway could serve to stimulate ERK independently of PKCα (Defillippi et al., 2006).

With respect to integrins, it is noteworthy that we and others have reported the ability of NG2 to activate β1-integrin signaling (Iida et al., 1995; Fukushi et al., 2004; Yang et al., 2004). NG2 is able to form a signaling complex with α3β1-integrin when the proteoglycan is present in soluble exogenous form or when it is expressed in cis fashion on the same cells as the integrin (Fukushi et al., 2004). The relationship between NG2 and β1-integrins may be especially relevant to the behavior of U251/NG2 cells. Labeling with the activation-dependent HUTS-21 antibody reveals β1-integrin activation in U251 cells that express the NG2-T2256E and NG2-T2314E variants that mimic phosphorylation at Thr^{2256} and Thr^{2314}, respectively. In addition, a β1-blocking antibody has inhibitory effects on both the proliferation and motility induced by NG2 phosphorylation at Thr^{2314} and Thr^{2256}, respectively.

These results are initially paradoxical because it is not immediately clear how integrin activation by NG2 might be able to stimulate proliferation in one case (response to Thr^{2314} phosphorylation) and motility in the other (response to Thr^{2256} phosphorylation). We suggest two possible resolutions to this paradox. First, the two phosphorylated NG2 species might differentially influence integrin signaling by interacting with different β1-integrin heterodimers or by recruiting additional distinct cytoplasmic binding partners to the NG2–β1-integrin complex. Second, differential localization of the NG2–integrin complexes may be determined by the NG2 phosphorylation pattern with the result that integrin signaling occurs in distinctly different microdomains of the cell. We have presented evidence consistent with this second alternative. Specifically, the NG2-T2256E species is localized along with β1-integrin in broad lamellipodia so that integrin activation and the resulting tyrosine phosphorylation of downstream signaling intermediates are localized to a microdomain that is critical to cell motility. In contrast, the NG2-T2314E species is colocalized with β1-integrin and elevated tyrosine phosphorylation on apical microprotrusions that appear to be dependent on NG2 phosphorylation at Thr^{2314} for their formation. These observations are consistent with the concept that integrin-mediated signal transduction can activate both motility and proliferation via intermediates such as FAK and Crk-associated substrate, whose specific localization patterns determine the outcome of signaling (for review see Cox et al., 2006).

These proliferation and motility results obtained with NG2-transfected U251 cells contradict, to some extent, our previous results with aortic smooth muscle cells from wild-type and NG2-null mice (Grako et al., 1999). Wild-type smooth muscle cells proliferated and migrated in response to both PDGF-AA and -BB. NG2-null cells failed to respond well to PDGF-AA but had normal responses to PDGF-BB. Thus, unlike our results with U251 glioma cells, the presence or absence of NG2 did not affect smooth muscle cell responses to PDGF-BB. It seems likely to us that these apparent discrepancies reflect the primitive status of our understanding of differences in the details of signaling mechanisms that occur from one cell type to another. Several examples will serve to illustrate the complexity of the situation. (1) We have no information about the phosphorylation of NG2 in smooth muscle cells. If PKCα and ERK are not activated by PDGF-BB in these cells in the same way we have seen in U251 and A375 cells, the phosphorylation of NG2 at Thr^{2256} and Thr^{2314} will not occur, and NG2 will have no influence on motility or proliferation. (2) As a result of the proposed link between integrin signaling and PDGF receptor activation (Borges et al., 2000), the NG2-dependent integrin activation described in this study could have an effect on PDGF receptor signaling. Because we have not determined how the spectrum of integrins in smooth muscle cells compares with that of U251 cells, we cannot say whether integrin–PDGF receptor or NG2–integrin interactions would be comparable between the two cell types. (3) PDGF-BB activates signaling through both α and β receptors. We have presented evidence for an interaction between NG2 and PDGFα receptor that potentiates signaling via this receptor (Nishiyama et al., 1996; Grako et al., 1999). The relative abundance of α and β receptors has been found to differ between smooth muscle and glioma cells (Sachinidis et al., 1990; Lokker et al., 2002), and we have not determined the α/β ratio in the specific cases of our two cell types. Therefore, we cannot predict the extent to which NG2 itself affects PDGF receptor signaling in either cell type. These uncertainties make it clear that our understanding of the functional role of NG2 is at an early stage and that much additional work will be required to elucidate details of the mechanisms by which NG2–integrin–PDGF receptor interactions regulate cell proliferation and motility.

Materials and methods

Reagents

Gö6976 and U0126 were purchased from Calbiochem. PDGF-BB was purchased from Chemicon International, and type I collagen was obtained from Cohesion.

Antibodies

Affinity-purified rabbit antibody against rat NG2 has been described previously (Makagiansar et al., 2004). The 9.2.27 monoclonal anti-NG2 antibody was a gift from R. Reisfeld (The Scripps Research Institute, La Jolla, CA). Rabbit or mouse antibodies specific for the phospho-Thr motifs

xTpx(K/R) and xTpP were purchased from Cell Signaling Technology, as were polyclonal anti-ERK1/2 antibody and phosphospecific ERK1/2 and PKCα antibodies. Rabbit antibody against phosphotyrosine was provided by E. Pasquale (Burnham Institute for Medical Research, La Jolla, CA). Monoclonal antibody against PKCα and monoclonal HUTS-21 antibody against activated β1-integrin subunit were obtained from BD Biosciences. Monoclonal antibodies against human β1-, α3-, and α1-integrin subunits were purchased from Chemicon. Fluorescein and rhodamine-coupled second antibodies were obtained from Biosource International.

Cell culture and stable transfections

NG2-negative U251MG human astrocytoma cells were maintained in DME containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate. U251 cells transfected with cDNA for rat NG2 (U251/NG2) have been described previously (Makagiansar et al., 2004). NG2-positive A375 human melanoma cells and the B5 (anti-NG2) hybridoma were obtained from American Type Culture Collection. An adenoviral vector containing rat NG2 cDNA (provided by A. Nishiyama, University of Connecticut, Storrs, CT) was also used to express NG2 in U251 cells (Yang et al., 2006). For this purpose, viral stocks of 10^8 plaque-forming units/ml were added to semiconfluent U251 cultures.

Stable transfection with cDNA coding for mutant NG2 species was performed as described previously (Stallcup and Dahlin-Huppe, 2001; Makagiansar et al., 2004). Mutant NG2 cDNA species were made using the QuikChange mutagenesis kit (Stratagene). For some experiments, U251/NG2 cells were further transfected with a pUSE vector containing the constitutively active MEK-DD mutant (Saxena et al., 1999).

Cell lysis, immunoprecipitation, and Western blotting

Immunoprecipitation and immunoblotting were performed as previously described (Makagiansar et al., 2004). In the case of human NG2 from A375 cells, immunoprecipitation and immunoblotting were performed with the 9.2.27 and B5 monoclonal antibodies, respectively.

Immunofluorescence

Cells in culture were fixed and labeled as previously described (Stallcup and Dahlin-Huppe, 2001; Makagiansar et al., 2004). In the case of HUTS-21 labeling, living cells were stained at 37°C for 15 min with both first and second antibodies. For phospho-PKC, phospho-ERK, and phosphotyrosine labeling, 0.1% Triton X-100 was included in the incubation mix. In double-labeling studies, controls were included to rule out the cross-reactivity of second antibodies with inappropriate Ig species. Specimens were examined at room temperature using a microscope (Optiphot; Nikon) equipped for epifluorescence. Images were captured on color slide film (Fujichrome 400 ASA; Fuji) using a plan Apo 40× 1.0 NA oil immersion objective (Nikon) and scanned at 300 dots per inch resolution using a scanner (UY-S77; Sony). Images were saved as Photoshop tif files (3,072 × 2,048 pixels) and subsequently processed in Photoshop 5.0 (Adobe).

In vitro phosphorylation of NG2 cytoplasmic domain

GST fusion proteins containing the wild-type or mutant NG2 cytoplasmic domain were prepared as described previously (Makagiansar et al., 2004). 10 μg of fusion protein bound to glutathione–Sepharose beads were used for phosphorylation reactions. Protein-laden beads were suspended in 30 μl of kinase buffer (50 mM MES, pH 6.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 100 μM ATP, 10 μCi γ-[P^{32}]ATP; PerkinElmer) and incubated with 100 ng of recombinant ERK2 (Calbiochem) for 30 min at 30°C. In vitro phosphorylation catalyzed by recombinant PKCα was performed as described previously (Makagiansar et al., 2004). Samples were fractionated on 4–20% SDS-PAGE gels and transferred to Immobilon P. Labeled components were detected by autoradiography. In some cases, in vitro phosphorylation reactions were performed using unlabeled ATP. In these cases, phosphorylated bands were recognized by immunoblotting with specific phospho-Thr antibodies.

Phosphopeptide mapping

After SDS-PAGE fractionation, ^{32}P-radiolabeled fusion proteins were cut from dried gels and digested with TPCK trypsin (Cooper et al., 1983; Makagiansar et al., 2004). Phosphopeptide mapping was performed in two dimensions on cellulose TLC plates (Makagiansar et al., 2004).

Cell proliferation assay

After overnight incubation in DME containing 0.5% FCS, cells were harvested using enzyme-free cell dissociation buffer and seeded in 0.5% FCS medium in 96-well plates (7×10^3 cells/well) in the presence or absence of 20 ng/ml PDGF-BB. After a 48-h growth period at 37°C, the viable cell mass was determined by the addition of MTS (3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium) phenozine methosulfate solution (Promega) to each well. Incubation was continued for 2 h at 37°C in the dark. Absorbance (490 nm) was then measured on a microplate reader (model 550; Bio-Rad Laboratories).

Apoptosis assay

5×10^5 cells were seeded in six-well plates and maintained in DME/0.5 FCS% for 2 d. Both adherent and floating cells were collected and stained with annexin V and propidium iodide using the annexin V–FITC apoptosis detection kit (Invitrogen). 10^4 cells of each type were analyzed using a flow cytometer (FACSort; Becton Dickinson). Parental U251 and U251/NG2 cell populations were found to contain 1.2% and 2.3% apoptotic cells, respectively.

Cell migration assay

Cell migration was examined in transwell cell culture chambers (Costar). Polycarbonate membrane inserts (6.5-mm diameter and 8-μM pores) were coated overnight by immersion in 30 μg/ml of type I collagen at 4°C. Cells grown overnight in DME containing 0.5% FCS were harvested using enzyme-free cell dissociation buffer and were resuspended in medium containing 0.5% FCS. Cells were added to the upper chamber of each well (5×10^4 cells/well) and incubated at 37°C for 16 h. Where indicated, medium in the lower chamber of the well contained 20 ng/ml PDGF-BB. After incubation, cells on the upper side of the membrane were removed with a cotton swab. The membrane was fixed, stained with DAPI, and coverslipped with Vectashield (Vector Laboratories). DAPI-positive nuclei were then counted under 200× magnification in four contiguous fields of the membrane, excluding the edges.

Statistical analyses

Statistical analyses were performed using the two-tailed *t* test.

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