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Mapping interactions between p27 and RhoA that stimulate cell migration

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Abstract

p27 mediates cell cycle arrest by binding to and inhibiting cyclin-dependent kinase (Cdk)/cyclin complexes, but p27 can also contribute to pro-oncogenic signaling upon mislocalization to the cytoplasm. Cytoplasmic p27 stimulates cell migration by associating with RhoA and interfering with the exchange of GDP from RhoA stimulated by guanine nucleotide exchange factors (GEFs). We used biophysical methods to show that the N-terminus of p27 directly interacts with RhoA *in vitro*. The affinity of p27 for RhoA is low, with an equilibrium dissociation constant of hundreds of micromolar; however, at high concentrations, p27 interfered with GEF-mediated nucleotide exchange from RhoA. We also show that promotion of cell migration in scratch wound cell healing assays requires full-length p27 despite the C-terminus being dispensable for the direct interaction between p27 and RhoA *in vitro*. These results suggest that there may be an unidentified factor(s) that associates with the C-terminus of p27 to enhance its interactions with RhoA and promote cell migration.

Graphical Abstract

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Keywords

Protein Interactions; Cell migration; p27^{Kip1}; Rho; IDPs

Misregulation of the mammalian cell cycle is one of the classic hallmarks of cancer [1]. Progression through the cell cycle is regulated by the activity of cyclin-dependent kinase (Cdk)/cyclin complexes which, in turn, are regulated by the Cip/Kip family of Cdk inhibitors that includes p27^{Kip1} (p27) [2]. p27 functions as a tumor suppressor in that loss of a single gene copy increases the likelihood of tumorigenesis [3]. However, p27 levels are known to be elevated in many tumor types, a paradox that was resolved by independent reports that in tumors with elevated levels of p27, pro-growth signaling results in activation of Akt, that phosphorylates p27 at threonine 157 (T157) (Fig. 1A), thereby interfering with its import into the nucleus [4–6]. p27 that is mislocalized to the cytoplasm, largely driven by association with 14-3-3 [7, 8], is unable to mediate cell cycle arrest by inhibiting nuclear Cdk/cyclin complexes, resulting in uncontrolled proliferation [4–6]. In addition to interfering with p27's cell cycle inhibitory role, cytoplasmic mislocalization of p27 is also associated with increased migration of cultured cells [9] and increased metastatic potential *in vivo* [10], linking p27 to another hallmark of cancer—tissue invasion and metastasis. Indeed, elevated levels of cytoplasmic p27 are associated with poor prognoses in several types of cancer [10–15].

The effect of p27 on cell motility is mediated through RhoA, a small GTPase involved in cytoskeletal remodeling that occurs during cell migration [16]. p27 co-immunoprecipitates with both GTP-bound, active RhoA and GDP-bound, inactive RhoA and interferes with nucleotide exchange from GDP-bound RhoA induced by its cognate guanine nucleotide exchange factors (GEFs), including p115 and Lbc [16]. This link between cell motility and Cdk inhibitors extends to other Cip/Kip family proteins. For example, p21^{Cip1} and p57^{Kip2} have been shown to interfere with the downstream effectors of RhoA, ROCK and LIMK [17–19].

As pro-migratory signaling stimulated by cytoplasmic mislocalization of p27 increases the metastatic potential of affected tumors, we sought to understand the mechanism of the interaction between p27 and RhoA using biophysical methods. Surprisingly, we found that the direct interaction between p27 and RhoA is quite weak, with an apparent equilibrium dissociation constant of hundreds of micromolar. Despite this low affinity, we demonstrated that p27 directly interferes with p115-mediated GDP exchange from RhoA. By performing titrations using isotopically-labeled p27 and NMR spectroscopy, we identified residues 55–95 as the region of p27 that binds to RhoA. Considering literature reports, namely that the C-terminus of p27 is necessary for interactions with RhoA in cells, our observations are surprising. Although it is known that the C-terminus of p27 is sufficient for co-immunoprecipitation with RhoA in co-transfected cells [16], to our knowledge the effect of p27's C-terminus on cell migration has not been reported. Here we show that neither the N-terminus nor C-terminus of p27 recapitulate the cell motility phenotype of full-length p27. Together with the observation that phosphorylation of p27's C-terminus at threonine 198 (pT198) strongly potentiates the interaction between p27 and RhoA [20], our findings that

the direct binding and inhibition of RhoA by p27 is remarkably weak and is mediated by a region of p27's N-terminus encompassing residues 55–95 suggest that in cells p27 may be presented to RhoA by an additional factor whose affinity for p27 can be modulated by phosphorylation at T198. These results underscore the complex and diverse functions of p27 of which additional layers of control are still being discovered [21, 22].

NMR shows that p27 binds weakly to RhoA

We probed for a direct interaction between RhoA and p27 using NMR spectroscopy to monitor the binding of unlabeled p27 to isotopically-labeled RhoA. Our RhoA construct contains residues 1–180 and lacks the disordered ten residue-long C-terminus which is lipidated to anchor RhoA to the cell membrane [23]. During purification, we noticed the presence of a proteolytic fragment of RhoA. By mass spectrometry we determined that proteolysis occurred after arginine at position 5 (R5). Mutation of R5 to alanine prevented proteolysis and dramatically increased the stability of RhoA samples for NMR experiments. All RhoA proteins used in this study bear this mutation.

We used 2D ^1H - ^{15}N HSQC NMR spectroscopy to map the sites of interaction of unlabeled p27 with ^{15}N -RhoA (Fig. 1B). Many resonances of ^{15}N -RhoA exhibited small chemical shift perturbations (CSPs) and/or broadening upon titration of p27 up to a five-fold molar excess. The concentration dependence of CSPs for shifted resonances were fit to 1:1 binding isotherms (Fig. 1C) and yielded an average apparent equilibrium dissociation constant (K_D value) of $280 \pm 170 \mu\text{M}$, indicating that p27 binds weakly to RhoA. The residues whose resonances were most strongly shifted or broadened by p27 binding map to the Switch I and II regions of RhoA (Fig. 1D and 1E), which are known to be involved in nucleotide exchange [24].

Phosphorylation of T198 by RSK1 has been shown to potentiate the effect of p27 on RhoA signaling [20]. To determine whether phosphorylation of T198 has a direct effect on the weak interaction between RhoA and p27, we introduced the phosphomimetic T198 to glutamate (T198E) mutation into p27 (termed p27^{T198E}). Surprisingly, 2D ^1H - ^{15}N HSQC spectra of ^{15}N -RhoA upon titration of p27^{T198E} (Supplemental Fig. 1) were similar to those obtained with wild-type p27, suggesting strongly that phosphorylation of T198 does not contribute directly to the binding of p27 to RhoA. Given that the C-terminus of p27 is sufficient to observe co-immunoprecipitation of p27 with RhoA from co-transfected lysates [16], we titrated a C-terminal construct (p27-C; containing residues 96–198) as well as a phospho-mimetic mutant (p27-C^{T198E}) into ^{15}N -RhoA and found that neither p27-C nor p27-C^{T198E} detectably altered 2D ^1H - ^{15}N HSQC spectra of ^{15}N -RhoA (Supplemental Fig. 2), indicating that neither form of the p27 C-terminus associated with RhoA. Mutation of a phosphorylation site to glutamate can be a poor mimic of *bona fide* phosphorylation [25, 26]. To evaluate this possibility, we titrated a synthetic peptide corresponding to the final ten residues of p27 with a phosphorylated threonine at position 198 (p27^{189–198}-pT198) into ^{15}N -RhoA and recorded 2D ^1H - ^{15}N HSQC spectra (Supplemental Fig. 3) but again did not observe binding to RhoA. These results suggest strongly that the p27 C-terminus with pT198 does not directly bind to soluble (non-membrane anchored RhoA) *in vitro*.

The N-terminus of p27 mediates interactions with RhoA

We next sought to resolve the apparent discordance between the results of our NMR titration experiments and published results showing that the C-terminus of p27 contributes to interactions with RhoA in cells by performing additional NMR experiments with isotopically-labeled p27 and unlabeled RhoA. The 2D ^1H - ^{15}N HSQC spectrum of p27 exhibits limited chemical shift dispersion because the protein is highly disordered in solution [27]. Consequently, we used ^{13}C -detected 2D $^{13}\text{C}'$ - $^{15}\text{N}_\text{H}$ correlation spectra (CoN), which offer improved resonance dispersion for intrinsically disordered proteins (IDPs) [28], to map the sites of interaction between p27 and RhoA. Titration of RhoA into $^{13}\text{C}/^{15}\text{N}$ -labeled p27 resulted in 2D CoN spectra exhibiting extensive resonance broadening for a region in the N-terminus encompassing residues 55–95 (Fig. 2). The finding that this region of p27 mediates interactions with RhoA is consistent with our observations that p27-C does not detectably associate with RhoA (Supplemental Fig. 2). Mislocalization of p27 to the cytoplasm in cancer is often driven by phosphorylation at T157 by Akt [4–6]. Although it remains a formal possibility that a double phospho-mimetic mutant (p27^{T157E/T198E}) may associate more strongly with RhoA, given that pharmacological inhibition of RSK1 only affects levels of T198 phosphorylation [20] and the region of p27 containing T157 does not detectably associate with RhoA in our NMR titrations we have chosen not to pursue experiments with the double phospho-mimetic mutant.

p27 interferes with nucleotide exchange from RhoA

Our observation from NMR experiments that p27 directly binds RhoA with relatively low affinity was surprising because several reports demonstrated that cytosolic p27 alters cell migration through interactions with RhoA [16, 20, 29]. To further explore the p27/RhoA interaction, we determined the effect of p27 on GEF-dependent GDP exchange from RhoA. We monitored the kinetics of exchange of MantGDP, a fluorescent GDP analogue, from RhoA mediated by the GEF, p115, in the presence of an excess of free GDP. Titration of high concentrations of p27 modestly inhibited nucleotide exchange from RhoA, consistent with the K_D values we measured for direct binding using NMR (Fig. 3A). These effects were dependent on the presence of p115 (Supplemental Fig. 4). p27-C had no effect on nucleotide exchange from RhoA (Fig. 3B), consistent with our NMR results (Supplemental Fig. 3). To validate our findings that the region of p27 responsible for direct binding to RhoA encompasses residues 55–95, we prepared a construct of p27 spanning residues 50–105 (p27^{50–105}). p27^{50–105} also interfered with nucleotide exchange from RhoA (Fig. 3C), albeit with less potency than full-length p27, confirming that this central region of p27 is important in mediating direct p27/RhoA interactions.

Full-length p27 is required to promote cell migration

Using NMR spectroscopy and GDP exchange assays, we have shown that a region in the N-terminal region of p27 mediates binding to RhoA and interferes with nucleotide exchange. However, results from multiple, independent experiments demonstrated that the C-terminus of p27 is crucial for promoting cell migration upon mislocalization of p27 to the cytoplasm. For example, the C-terminus of p27 co-immunoprecipitates with RhoA from lysates of co-

transfected cells [16], truncation of the extreme C-terminal 28 residues of p27 interferes with signaling through the Rho pathway [30], and p27 phosphorylation at T198 by RSK1 inhibits the RhoA pathway [20]. We probed whether either the N- or C-terminal regions of p27 could promote cell migration in scratch wound migration assays using p27^{-/-} mouse embryonic fibroblasts (MEFs). While expression of full-length p27 clearly promoted cell migration in these assays, as previously demonstrated [16], neither p27-N nor p27-C stimulated cell migration with respect to the empty vector control (Fig. 4) despite similar levels of expression (Supplemental Fig. 5). The absence of an effect of either p27-N or p27-C on cell migration cannot be ascribed to nuclear restriction of the p27 fragments as both constructs were localized in both the nucleus and the cytoplasm (Supplemental Fig. 6). In addition to the constructs discussed above, we reproduced the finding that a Cdk binding deficient mutant (p27CK-) also stimulated migration, while truncation of only the final eight residues from this construct interfered with the ability of p27 to stimulate cell migration (Supplemental Fig. 5) [16, 31, 32]. The initial observation that the C-terminus of p27 is sufficient to co-immunoprecipitate with RhoA was made with a construct that was slightly longer than our p27-C construct [16]; however, extension of our construct to residue 88 (p27⁸⁸⁻¹⁹⁸) did not restore the migratory phenotype. For completeness, we also tested the complementary N-terminal construct, p27¹⁻⁸⁷, which also did not exhibit a migratory phenotype. In summary, promotion of RhoA-dependent cell migration by p27 requires both the previously identified C-terminal region as well as the N-terminal region of p27 identified here.

Discussion

Unlike canonical tumor suppressors, p27 is rarely mutated in cancer. Rather, cancer cells subvert the mechanism of p27-mediated cell cycle arrest by either upregulating its proteosomal degradation or by sequestering it in the cytoplasm where it can no longer bind to and inhibit Cdk/cyclin complexes [33, 34]. Upon mislocalization to the cytoplasm, p27 promotes migration and invasion by various mechanisms including modulating the dynamic cycles of RhoA activation that control remodeling of the actin cytoskeleton during cell migration [16], promoting the epithelial-mesenchymal transition through activation of the JAK2/STAT3 signaling pathway [35], and facilitating the recruitment of PAK1 to its substrate Cortactin to stimulate the turnover of invadopodia [31]. All of these effects promote metastasis and may provide a selective advantage to cancer cells that retain unmutated, yet mislocalized p27.

The earliest indications that mislocalized p27 could stimulate cell migration came from studies showing that the migration of cells stimulated by hepatocyte growth factor (HGF) was dependent on p27 and that HGF treatment induced nuclear export of p27 [9, 36]. The migration defect of p27-null MEFs can be rescued by the inhibition of ROCK, a RhoA effector, and co-immunoprecipitation experiments indicated that p27 can bind to RhoA and interfere with RhoA binding to GEFs [16]. As the interaction between p27 and RhoA has been implicated in promoting tumor invasion and metastasis, we used NMR spectroscopy to map the sites of direct interaction between p27 and RhoA as a prelude to future small molecule studies to disrupt this interaction. We found that there is indeed a direct interaction between p27 and RhoA, although it is quite weak with a K_D value of ~300 μ M (Fig. 1).

Moreover, by monitoring the backbone resonances of isotopically-labeled p27 upon titration with RhoA, we have shown that the region of p27 that directly interacts with RhoA encompasses residues 55–95 (Fig. 2). Taking into account prior reports, namely that the C-terminus of p27 is required for the interaction between p27 and RhoA in cells, the observation that the region of p27 that directly binds RhoA *in vitro* lies outside p27's C-terminus is striking. Also striking is the observation that neither the isolated N- nor C-terminal regions of p27 promoted cell migration in scratch wound assays (Fig. 4). The interaction between p27 and RhoA maps to the switch I and II regions of RhoA, which are well conserved amongst Rho proteins, suggesting that p27 may be able to interact with other small GTPases of the Rho family. Indeed, we recently found that p27 also interacts with RhoB, interfering with its activation in a similar manner as with RhoA and that this interaction plays a role during tumorigenesis (Calvayrac et al. Submitted).

In summary, we have shown that the direct interaction between p27 and RhoA *in vitro* is mediated by a central stretch of residues within the N-terminal region of p27, and that this region of p27 is sufficient to interfere with GDP exchange from RhoA stimulated by its GEF, p115. Experiments in cells indicate that although the N-terminus of p27 is responsible for direct binding to RhoA, p27-N is insufficient to promote cell migration, likely because the affinity for RhoA is too weak to have an effect in cells. To reconcile our seemingly disparate *in vitro* observations with the literature reported requirement for the p27 C-terminus for promotion of cell migration, we hypothesize that there is an additional partner that binds to the C-terminus of p27 in a pT198-dependent manner that in turn promotes interactions between p27 and RhoA. In this scenario, p27 is proposed to serve as a scaffold for RhoA and an additional partner, a role well established for IDPs [37]. Our *in vitro* studies were conducted using a form of RhoA that lacks the C-terminal lipidation site and therefore do not address the role of membrane anchoring in interactions between RhoA and p27. Disorder within certain scaffold proteins is proposed to mediate the assembly of large plasma membrane-associated platforms [38] and it is possible that p27 plays a similar role in the context of spatial and temporal control of cell migration by RhoA, Rac1, and Cdc42 [39, 40]. Assuming this scenario, the discovery of chemical compounds that can disrupt p27-N/RhoA interactions, as identified here, may provide a means to alter the migratory phenotype associated with aggressive and invasive tumors that express p27 at high levels. Interestingly, small molecules that specifically but weakly bind to a portion of the region of p27 identified here to bind directly to RhoA (residues 55–95) have been identified [41], providing a starting point for future studies to therapeutically modulate an IDP in invasive cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- It is known that cytoplasmic mislocalization of p27 stimulates migration through RhoA
- A N-terminal region of p27 binds RhoA and interferes with GDP exchange from RhoA
- However, only full-length p27 can stimulate migration in cells

Figure 1. 2D NMR shows that p27 binds weakly to the Switch I & II regions of RhoA
(a) Domain architecture showing functional regions of p27 and important phosphorylation sites. The kinase interacting domain (KID) that binds to and inhibits Cdk/cyclin complexes is shown in red. The nuclear export signal (NES) and nuclear localization signal (NLS) are shown in salmon and grey, respectively. Phosphorylation by non-receptor tyrosine kinases (NRTKs) at Y74 and Y88 partially reactivate bound Cdk/cyclin complexes leading to intracomplex phosphorylation of p27 at T187, creating a phosphodegrom that recruits the Skp2 ubiquitin ligase. **(b)** 2D ^1H - ^{15}N HSQC spectra of 200 μM ^{15}N -RhoA upon titration of unlabeled, full-length p27 show chemical shift perturbations and resonance broadening consistent with weak binding. The spectra of 200 μM ^{15}N -RhoA in the presence of 0, 200, 400, 600, 800 and 1000 μM p27 are shown in blue, cyan, green, yellow, orange, and red, respectively. **(c)** Representative binding isotherms of several individual resonances. The resonances with chemical shift perturbations greater than one standard deviation above the average for a majority of titration points were fit individually to a 1:1 binding model. Individual fits for A61, E64, and D65 are illustrated. The average K_D is $280 \pm 170 \mu\text{M}$ where the error represents the standard deviation of the mean for individually fit K_D values. Ten resonances (V35, V38, A44, A61, E64, D65, S73, W99, H126, and R150) could be fit confidently to a 1:1 binding model. **(d)** The residues that fit the above criteria are depicted in

red on the GDP bound structure of RhoA (PDB code: 1FTN). The surface representation does not include Mg^{2+} -GDP, which is shown explicitly. Several of the most perturbed resonances are located in the Switch I and Switch II regions, which are known to be important for GDP exchange. Residues A61, E64, and D65 are located in the Switch II region. (e) Chemical shift perturbations of 200 μ M RhoA upon addition of 1000 μ M p27 plotted versus the residue position. Residues shifted by more than 1 standard deviation above the average (denoted by the dashed line) are shown in red.

Figure 2. NMR titration of $^{13}\text{C}/^{15}\text{N}$ p27 with RhoA localizes the binding region of p27 to approximately residues 55–95

(a) Overlay of ^{13}C -detected 2D CoN correlation spectra of $^{13}\text{C}/^{15}\text{N}$ -labeled p27 with increasing concentrations of RhoA. The spectra of 500 μM $^{13}\text{C}/^{15}\text{N}$ -labeled p27 in the presence of 0, 200, 400, 600, 800 and 1000 μM RhoA are shown in blue, cyan, green, yellow, orange, and red, respectively. Select resonance assignments are shown. Due to slight pH changes during the titration, the resonance of a serine residue in the non-native N-terminal segment of the RhoA construct (N-GSHM; S_{tag}) exhibited chemical shift changes.

(b) The extent of resonance broadening with respect to the apo spectrum is plotted as stacked columns along the primary sequence with residues that are broadened above one standard deviation in a majority of titration points and the position of histidine residues denoted in the box above as black and magenta bars, respectively. The color scheme is the same as in (a).

Figure 3. p27 interferes with GDP exchange from RhoA induced by p115

Nucleotide exchange from 1 μ M MantGDP-loaded RhoA (RhoA*) in the presence of 100 μ M unlabeled GDP and increasing concentrations of p27 was initiated by rapid addition of p115 to a final concentration of 1 μ M. The exchange of MantGDP for unlabeled GDP was monitored by the change in fluorescence intensity over time. Panels **a**, **b** and **c** show titrations with p27, p27-C and p27⁵⁰⁻¹⁰⁵, respectively. The maximum p27 concentration tested in (**a**) and (**b**) was 0.5 mM. The potency of p27⁵⁰⁻¹⁰⁵ is slightly lower than that of full-length p27. The maximum concentration in (**c**) is 1.5 mM. The lower concentrations of p27 are 2-fold dilutions from these maxima. GDP exchange assays in the presence of p27 are depicted as circles with colors changing from red to blue to represent the serial 2-fold dilutions from the maximum concentration shown in red. The squares and triangles depicted in black are exchange assays conducted in the absence of p27 with and without p115, respectively. Error bars represent standard deviations from triplicate measurements.

Figure 4. Rescue of the cell migration phenotype of p27^{-/-} MEFs requires intact p27
 Representative images and quantification of scratch wound cell migration assays performed with immortalized p27^{-/-} MEFs transfected with empty vector, p27, p27-N, or p27-C. The darker grey areas show initial wound masks and dotted lines outline the migration fronts. The scale bar is drawn to 300 μ m. The gray and black columns in the graph below depict quantification of the relative wound density of the 12 and 24 hour time points, respectively. Error bars represent the standard error of the mean for independent experiments (n=5 empty vector, n=4 for p27, n=7 for p27-N and p27-C). $p < 0.005$ for all comparisons between p27^{-/-} MEFs transfected with empty vector, p27-N, or p27-C with respect to full-length p27 transfections.