厚生労働科学研究費補助金（創薬基盤推進研究事業）
分担研究報告書
がん遺伝子陽性肺癌の臨床病理学的解析
分担研究者：池田徳彦　東京医科大学呼吸器外科・甲状腺外科　教授

研究要旨：我々はEGFRを標的とするmi(cro)RNAを単離し、肺癌細胞に使用することにより肺癌細胞の増殖を抑制することを発見した。EGFR遺伝子変異は肺癌に一定頻度で発現が見られ、その変異遺伝子に対する治療薬であるEGFRチロシンキナーゼ阻害薬はすでに臨床において広く使用されているが、耐性化や副作用など解決すべき問題も多い。本研究において我々はmiRNAに注目した。まずEGFRのメッセンジャーRNAを標的とする、候補となりうるmiRNAを選別し、さらに有用と思われるmiRNAを単離した。その後、その候補miRNAが肺癌細胞のmRNAの発現を抑制し、がん細胞の増殖を抑制するかを検討した結果、有用と考えられるmiRNAを選別することに成功した。今後本miRNAを用いたさらなる肺癌治療への応用を検討してゆく。

A　研究目的

肺癌、特に非小細胞肺癌は世界における癌関連死の主な要因となっている。また上皮成長因子受容体(EGFR)の伝達機構は非小細胞肺癌の発症や悪性化に重要と考えられており、多くの研究が盛んに行われ、EGFRチロシンキナーゼ阻害薬も複数の薬剤が世界で広く使用されている。しかしながらその効果はEGFRの遺伝子変異を有する症例に限られ、また、長期間使用による耐性化遺伝子の獲得や、重篤な副作用など、問題もある。

miRNAはタンパク質をコードしない20～25塩基長の小RNAで、標的となるmRNAの転写抑制や分解を行うことにより、細胞の分化、増殖、アポトーシスなど生理的な働きに関与していることが報告されている。しかし、mi(cro)RNAがどのようにEGFRの伝達機構に影響し腫瘍の発生に関与するかは殆ど知られていない。そこで我々はEGFRを標的とするmiRNAを同定し、その抗腫瘍効果について検討した。将来的には、癌を促進、維持するのに機能しているがん遺伝子を抑制するmicroRNA核酸医薬を投与することにより、癌を制御する治療方法を確立することへ応用したい。

B　研究方法

EGFRを標的とするmiRNAの単離には96 well plateに83種類のoligoの入ったmiScan(B-Bridge)にHeLa細胞を培養して、遺伝子を導入後、24時間でRNAを回収し、Real time PCR法を用いてEGFRのmRNAの発現の解析を行って、候補となるmiRNAを選択した。

候補となったmiRNAをさらに限定するために、EGFR蛋白質の発現抑制を誘導するもの、さらに、細胞の増殖抑制に影響を与えるものを選択した。

EGFR蛋白質の発現の解析には、抗EGFR抗体(Cell Signaling社)を用いたWestern blot法で、肺癌細胞株にmiRNAをHiperFect(Qiagen社)による遺伝子導入後60時間の細胞蛋白質を解析した。細胞の増殖の解析には、同様な方法で細胞株に遺伝し導入後、72時間の細胞にMTT assay(NakaraiTesq社)を用いて解析を行った。続いてこれらのmiRNAについて、EGFRの標的となり得るかAGOとの複合体形成の確認を抗AGO抗体ビーズ（WAKO社）に吸着させた複合体から分離したmRNAを定量することで調べた。さらに摘出肺癌検体を用いてin situ hybridization及び免疫染色を行いEGFRの発現とmiRNAの発現との関係を検討した。

C　研究結果

まず83種類の候補となるmiRNAを7つに絞り、さらに同様の実験を行った所、その候補miRNAの中で最もEGFRの発現抑制効果の高かったものはmiR-542-5pであった。近年miR-7がEGFRのmRNAの発現を抑制し、細胞周期を停止させ細胞死を導くことが報告されているが、両者の比較の結果miR-5425pはmiR-7と同様な効果が得られた。また、細胞増殖に関してはmiR-7よりも高い増殖抑制効果を示した。また、ヒト肺癌組織に対してEGFRの免疫染色及びmiRNAに対するin situ hybridizationを施行したところそれぞれの発現は相反関係にあった。以上の

結果からmiR-542-5pによりEGFRの発現抑制による腫瘍増殖が抑制されることが示唆された。

D&E. 考察及び結論

miR-542-5pはEGFRの発現を調節し、癌細胞において細胞増殖、生存能力を減弱させることが示された。さらに同様の報告のあるmiR-7と比較し、miR-542-5pはより優れた細胞増殖抑制効果を得た。これらのmiRNAはそれぞれ異なる機構でEGFRの発現調節を行なっている可能性が考えられる。また、EGFRチロシンキナーゼ阻害剤が肺癌細胞の増殖を抑制させるが、EGFRの遺伝子変異のある症例に限られるのに対しmiR-542-5pはEGFRの発現を制御しているため、遺伝子変異の有無にかかわらず抗腫瘍効果が期待できる。
さらにmiR542-5pの過剰発現はヒトの肺癌において細胞数を減少させることも証明された。以上のことから、microRNA核酸医薬を投与するなどにより、miR542-5pは肺癌において分子標的治療に応用可能なことが示唆された。

F. 健康危険情報

なし

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H. 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（創薬基盤推進研究事業）
分担研究報告書
「がん細胞浸潤におけるTGF-βファミリーシグナルの制御メカニズム」に関する研究

分担研究者：　鯉沼　代造　　東京大学大学院医学系研究科　特任准教授

研究要旨：我々は肺がん細胞をはじめとして上皮間葉転換の抑制によるがん細胞の運動・浸潤能の制御を目的として、その重要な誘導シグナルである Transforming growth factor-β (TGF-β)による上皮間葉転換の分子メカニズムについて検討を行ってきた。これまでに次世代シーケンサーを用いた TGF-β ファミリー下流の転写因子 Smad の網羅的な結合部位同定を行い、その分布ががん細胞など細胞種によって大きく異なることを見出し、その制御因子の同定を行っている。本検討の結果、数々の AP-1 結合蛋白ファミリーががん細胞において Smad と相互作用し、TGF-β によるがん細胞浸潤に役割を果たしていることを見出した。同様の手法を用いて今後がん細胞の上皮間葉転換の制御の鍵となる分子の同定と作用機構解析を行う。

A　研究目的

Transforming growth factor (TGF)-β ファミリーに属するサイトカインは、細胞分化、上皮細胞の増殖抑制、細胞外マトリックス産生など多彩な作用を有する。TGF-β はまた、肺がん細胞をはじめとして上皮間葉転換(Epithelial-mesenchymal transition, EMT)を誘導することで、細胞の運動・浸潤能を促進することも知られる。従って TGF-β シグナルはがん細胞に対して、増殖抑制と EMT の促進という、がんの進展に対して相反する作用を有している。グリオーマをはじめとして、一部の腫瘍において TGF-β シグナルを抑制する臨床試験が行われ、その効果が有望視される中で、このようなシグナル作用の二面性は、がんの治療法開発戦略上、解決しなければならない課題である。

分担研究者らは TGF-β シグナルの下流で EMT 促進作用のみを選択的に制御する方法を見出すことを目的として、肺がん細胞をはじめとして、そのシグナル伝達のメカニズムと制御機構につき詳細に解析してきた。本研究によりこれまでに TGF-β によるがん細胞の EMT における、上皮特異的選択的 RNA スプライシング因子 ESRP1/2 発現抑制の意義と、その発現とがん組織型との関連が明らかになった(Horiguchi et al, Oncogene, 2011)。またがん細胞パネルで見いだされた、ZEB1/2 の強発現が EMT を促進してがん細胞の運動・浸潤能に関わる重要な機序であることを明らかにした。さらに NFκB の選択的阻害剤が TGF-β と TNF-α による肺がん細胞の EMT を部分的に抑制し、分子標的としてのこれらシグナル伝達経路の可能性が示唆された(Kawata et al, J Biochem, 2012)。また分担研究者らの見出した細胞特異的な Smad ファミリー結合部位の違い(Mizutani et al, J Biol Chem, 2011)はその後の他の報告でも裏付けられ、本来がん抑制的に作用する TGF-β シグナルが如何に個々のがん種で異常な制御を受け利用されてしまうのか、今後同様のアプローチを含めた解析を行うことで EMT 選択的な制御を含めた治療戦略構築に結びつく可能性が示唆されている。

平成 24 年度はこれまでの本研究課題で得られた知見を踏まえ、TGF-β 下流のシグナル伝達分子・転写因子である Smad2/3 のゲノム結合部位に極めて高頻度に存在する AP-1 結合モチーフに着目し、このモチーフに結合する転写因子群ががん細胞の浸潤能に与える影響につき検討を行った。

B　研究方法

まず分担研究者らが報告してきた、種々の細胞における TGF-β ファミリー下流の網羅的 Smad 結合部位情報(Koinuma et al, Mol Cell Biol 2009; Koinuma et al, Cancer Sci 2009; Mizutani et al, J Biol Chem 2011 他)に加えて、これまでに論文報告されている Smad ファミリー結合部位の ChIP-seq/ChIP-chip に関する全ての文献の調査を行った。これらの報告をもとにがん細胞をはじめとして Smad 結合部位に濃縮してゲノム上に存在する転写因子結合 DNA モチーフをピックアップした。

こうして見出した転写因子結合モチーフに結合し得る転写因子群について、次にがん細胞浸潤に与える影響を検討した。本検討では単純な 2 次元培養ではなく、スフェロイド浸潤アッセイを行うことができる、前悪性化段階にあ

るヒト乳腺上皮細胞 MCF10A-MII の実験系を採用した。そしてこの細胞において発現している、或いは TGF-β 刺激によって誘導される AP-1 結合転写因子群を同定した。

次にこれらの転写因子群に対する特異的な siRNA を用意してそのノックダウン効率を確認した。その上で、これらの siRNA が細胞のスフェロイドの TGF-β 依存的なコラーゲンゲルへの浸潤能に与える影響を半定量的に評価した。さらにこれら siRNA が EMT および細胞浸潤能に関わる遺伝子発現に与える影響を定量的 RT-PCR により評価した。

続いて in situ proximity ligation assay (PLA) 法を用いて、Smad とこれら AP-1 結合蛋白間の相互作用につき検討を行うとともに、これらの siRNA が Smad の標的遺伝子制御ゲノム領域への結合に与える影響を ChIP-qPCR で検討した。

(倫理面への配慮)

本研究では臨床検体は用いない。

C 研究結果

網羅的な Smad 結合部位情報に関する全ての文献調査を行った(Morikawa et al, Oncogene, 2012, in press)。その結果 Smad 結合部位に濃縮してゲノム上に存在する転写因子結合 DNA モチーフが種々ピックアップされたが、このうち結合部位での濃縮度が 10 倍前後と他に比べてきわめて高い AP-1 結合モチーフに着目した。

AP-1 結合因子として、c-Jun, JunB, c-Fos, FosB, Fra1, Fra2 などが知られる。これら転写因子群について、前悪性化段階にあるヒト乳腺上皮細胞 MCF10A-MII での蛋白発現量と TGF-β 影響をウェスタンブロッティングで評価したところ、c-Jun, JunB, c-Fos, FosB の発現誘導が認められた。一方 Fra1 の発現は恒常的に認められた。

これらの転写因子群に対する siRNA を用意してそのノックダウン効率と特異性をウェスタンブロッティングで確認した。そしてこれらの siRNA が細胞のスフェロイドの TGF-β 依存的なコラーゲンゲルへの浸潤能に与える影響を半定量的に評価したところ、c-Fos, Fra1, c-Jun, JunB で有意な浸潤能の抑制が認められた。また Smad4 のノックダウンによっても同様に抑制が認められた。EMT および細胞浸潤能に関わる遺伝子発現に与える影響では c-Jun, JunB, Fra1 の siRNA は TGF-β 依存的な MMP-1, MMP-9, MMP-10, Slug などの発現を Smad4 と同様に有意に抑制した。

in situ proximity ligation assay (PLA)法での検討では、Smad2/3 と Fra1 の結合が TGF-β で増強される一方で、Smad3 と c-Jun や JunB の結合は刺激非依存性に検出された。最後にこれら因子が Smad2/3 の MMP-10 プロモーターへの結合に与える影響を ChIP-qPCR で検討したところ、Smad2/3 の結合は c-Jun, JunB, Fra1 siRNA によって抑制された (Sundqvist et al, Oncogene, 2012, in press)。

D&E 考察及び結論

Smad2/3 結合部位に濃縮する AP-1 結合モチーフに着目した本研究により、TGF-β によるがん細胞の浸潤能亢進作用の分子メカニズムに、AP-1 結合モチーフに結合する種々の転写因子の重要な役割があることが明らかになった。これらの AP-1 結合蛋白質のいくつかはこれまで non-Smad 経路と呼ばれる、Smad ファミリー非依存的な TGF-β シグナル伝達に関わっているとされる一方で、Smad ファミリーのシグナル伝達経路に影響を与えることも指摘されていた。スフェロイド浸潤アッセイを用いた本研究での評価により、これらの AP-1 結合蛋白質群が TGF-β シグナルのがん細胞の浸潤能にそれぞれ特徴的に与える影響につき明らかにできたこと、さらに Smad が標的遺伝子座に結合するにあたっての役割を明らかにできたことで、細胞の浸潤能に関して個別の遺伝子の発現制御でない、TGF-β シグナル下流での一般的な機構の一端が明確になったものと思われる。一般的に転写因子の DNA 結合を分子標的とすることはこれまで成功例に乏しいと考えられているが、今回のようにシグナル伝達分子としての Smad および AP-1 結合蛋白の相互作用阻害に着目したアプローチは、今後適切な化合物スクリーニング系構築などにもつながる、興味深い結果であると考えられる。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

がん臨床検体における診断法開発

所属　（公財）がん研究会 がん研究所 病理部
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研究要旨
IRF4 と免疫グロブリン遺伝子の融合は多発性骨髄腫，高悪性度 B 細胞性リンパ腫で報告されているが，低悪性度 B 細胞性リンパ腫においてはいまだ報告されていない．今回，*IRF4* split FISH 法により 3 例の *IRF4* 再構成陽性低悪性度 B 細胞性リンパ腫を同定した．形態像，免疫形質，染色体異常および臨床所見の点で既存のリンパ腫 subtype に合致せず，“prolymphocytic/paraimmunoblastic lymphoma (PPL)”などの名称で呼称したい．今後，他の低悪性度 B 細胞性腫瘍との関連を明らかにすべきである．

A. 研究目的

分担研究者は，ALK 肺癌の診断法を開発した知識と経験を用い，種々のがんにおける融合遺伝子探索プロジェクトを展開している．本分担研究の今年度の目的は，*IRF4* 再構成陽性腫瘍の診断・同定法開発の過程において発見された新たなリンパ腫の characterization である．*IRF4* と免疫グロブリン遺伝子の融合は多発性骨髄腫，高悪性度 B 細胞性リンパ腫で報告されているが，低悪性度 B 細胞性リンパ腫においてはいまだ報告されていない．

B. 研究方法

がん研究会におけるリンパ腫 784 例，コンサルテーション例 1 例のうち *IRF4* split FISH 法により *IRF4* 再構成が確認された 3 症例に *BCL2, BCL6, MYC, CCND1* および各種免疫グロブリン遺伝子に対する FISH 法，CD5, CD10, CD20, CD23, CD43, CD138, MUM1/IRF4, BCL2, BCL6, IgM, IgD, TdT, Cyclin D1, Ki67 に対する免疫染色法を施行，臨床データとともにその特徴をまとめた．

C. 研究結果

自ラボで調整した*IRF4* split FISHプローブの性能チェックをリンパ腫検体で施行した際，*IRF4* 再構成症例を発見した（症例1．再発時検体）．原診断はlow-grade B-cell lymphoma, unclassifiedとされていた．G分染法による染色体解析レポートによると，本症例には*IGK* と*IRF4* の融合に合致する相互転座t(2;6)(p11.2;p25)が認められていた．そこで*IRF4*と*IGK*のfusion FISHを施行したところ両者の融合が確認された．本症例の初発時検体（再発の8年前）にも*IRF4*再構成が確認された．

症例1発見の十日後に行われた東京リンパ腫研究会にて，ある参加施設から診断困難例が病理標本とともに提示された．施設診断はlow-grade B-cell lymphomaと思われるが詳細不明とのことであった．本例の腫瘍細胞像は若干paraimmunoblastが多いものの，症例1の細胞像に類似していた．そこで*IRF4*再構成の有無をFISHで検討したところ，*IGL-IRF4*転座が存在することが明らかとなり，これを症例2とした．

232例の低悪性度B細胞性リンパ腫を含む784例のリンパ腫検体に対し*IRF4* split FISHを施行したところ，もう一例陽性例が見つかり症例3とした．原診断はlow-grade B-cell lymphoma, unclassifiedとされており，記録によると*IGK-IRF4*に合致する相互転座t(2;6)(p12;p25)が検出されていた．症例1も同一コホートにあるので，*IRF4*再構成陽性症例は低悪性度B細胞性リンパ腫のうち0.86%程度（2/232）を占めると推計された．

組織学的には，リンパ節の基本構造は消失し，腫瘍細胞は基本的にびまん性に浸潤している．しかしながら，症例2では線維性結合織が結節状に腫瘍組織を囲繞していた．構成細胞はprolymphocyte, paraimmunoblast, 小リンパ球よりなっているが，病変ごとにその割合は若干異なっていた（3症例における4病変）．Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)に見られるproliferation centerは認められなかったが，症例1においてはprolymphocyteやparaimmunoblastよりなる浸潤巣が腫瘍性小リンパ球の浸潤を背景に互いに融合していた．症例1の再発検体では，時折，paraimmunoblastを含んだprolymphocyteのびまん性浸潤が見られた．症例2では，大半の腫瘍細胞はparaimmunoblastであり，ときおりprolymphocyteがみられた．線維性結合織に囲繞され結節状に見えることと合わせ，細胞・組織像のみからはgrade 3のfollicular lymphoma (FL)も鑑別診断に挙がってくるだろう．しかしながら，症例2の結節状構造には濾胞樹状細胞のネットワークは見られなかった．症例3はprolymphocyteおよび小さな核小体を小型から中型の不整核を有する細胞よりなっており，mantle cell lymphoma (MCL)様の印象であるが，検体が小さく詳細な形態観察は困難であった．

免疫組織学的にはCD5− (症例3はフローサイトメトリーでのみCD5dim), CD10−, CD20+, CD23+, CD43+, CD138−, MUM1/IRF4+, BCL2+, BCL6+, IgM+, IgD−, TdT−, Cyclin D1−であった．Ki67-labeling indexは約10%と低値で

あり低悪性度リンパ腫に合致する.

D. E. 考察&結論

本症例群の組織像の概観はprolymphocyteやparaimmunoblastの浸潤が目立つCLL/SLL (tumor-forming subtype of CLL/SLLあるいはparaimmunoblastic variant of CLL/SLLなどと呼ばれた), もしくはB-cell prolymphocytic leukemia (B-PLL)の組織浸潤を想起させる. そこで症例1と2において, CLL/SLLに特徴的である4種の染色体異常[del(11)(q22.3), +12, del(13)(q14.3),およびdel(17)(p13.1)]をFISHで, CLL/SLLに特徴的に発現がみられるとされるLEF1を免疫組織染色で解析した. 症例2ではすべて陰性であったが, 症例1ではdel(13)(q14.3)とLEF1発現が見られた. したがって, 症例1 はCLL/SLLとの関連があるかもしれない.

CLL/SLLでは, 腫瘍性小リンパ球においてMUM1/*IRF4*およびBCL6発現は無いか極めてよわく, prolymphocyteとparaimmunoblastsはMUM1/IRF4を中等度発現するがBCL6は陰性である. これに対して, 今回の症例群では大半の細胞がMUM1/IRF4とBCL6を共発現していた. これは, これまで知られている通常の低悪性度B細胞性リンパ腫には見られない特徴である.

Paraimmunoblastic variants of CLL/SLL として報告されてきた症例はCD5陽性で, aggressiveな臨床経過を呈し, 検索された8例中5例で*IGH-CCND1*融合が検出されている. すなわち, これらの症例の本態はMCLであったと思われる. 今回の症例はいずれもaggressiveな経過を示さず, *IRF4*再構成を有し, B細胞性腫瘍でよく見られるその他の染色体転座を見ない.

CD5が一部で陽性となったこと, IgM強陽性, IgD陰性という所見はCLL/SLLに合致せずB-PLLの免疫形質を想起させる. しかしながら, 今回の症例群では白血化はなく, *IRF4*再構成はB-PLLで報告されていない. また, B-PLLのリンパ節浸潤はまれで, これまでの報告例はMCLを含んでいたことが指摘されている.

したがって, 今回の症例群は形態像, 免疫形質, 染色体異常および臨床所見の点で既存のリンパ腫subtypeに合致せず, "prolymphocytic/paraimmunoblastic lymphoma (PPL)"などの名称で呼称したい. 他の低悪性度B細胞性腫瘍との関連を明らかにすべきである.

Salaverriaらにより, IRF4再構成陽性の高悪性度B細胞性リンパ腫が2011年に報告されている. 今回の症例群が, その前駆組織型すなわちtransformation前の組織型か否かについては興味が持たれる. 症例1の染色体所見は, 46XX,t(2;6)(p11.2;p25)[10]/46XX,t(1;11)(q21;q23)[1]/46XX[9], 症例3では46XY,t(2;6)(p12;p25)[1]/46,sl,-Y,-4,-8,-9,-9,add(11)(q23),+5mar[1]/46XY[11]であり, *BCL2*, *BCL6*, *MYC* および*CCND1*再構成はいずれも陰性であった. 一方, *IRF4*再構成陽性高悪性度B細胞性リンパ腫19例では, 7例および1例がそれぞれ*BCL6*, *MYC*再構成陽性であった. 免疫グロブリン重鎖遺伝子再構成のPCR解析は, 症例1および2で結果が得られたが, *IGH*可変領域はそれぞれV3-11とV1-8であり, CDR1-FWR3領域における胚細胞配列との一致度は100%と94.7%で, 後者ではintraclonal diversityが見られた. これに対して, *IRF4*再構成陽性高悪性度B細胞性リンパ腫では, 比較的高度の*IGH*可変領域somatic hypermutationが報告されている (93.9%から86.1%) . 今回の症例群では免疫グロブリン軽鎖遺伝子が*IRF4*転座のパートナーであったが, *IRF4*再構成陽性高悪性度B細胞性リンパ腫では19例中17例が重鎖遺伝子をパートナーとしている (*P*=0.0065, Fisher's exact test) . 大半の*IRF4*再構成陽性高悪性度B細胞性リンパ腫は小児・若年症例であり*de novo*高悪性度リンパ腫である可能性が高い. 一方, 興味深いことに, Salaverriaらは*IGK-IRF4*転座を有する高齢のtransformation症例も報告している. これらの知見を総合的に考えると, *IRF4* 再構成は低・高悪性度双方のリンパ腫の*de novo*発症に関与している一方で, 一部の*IRF4*再構成陽性低悪性度B細胞性リンパ腫は他の低悪性度リンパ腫同様, 高悪性度のものにtransformする可能性が考えられた.

これらの問題の解決のために, 多数の"PPL" 症例を集積し解析することが望まれる. 確定診断のために*IRF4*再構成に対するFISHが必要であるが, その解析をとくにすべき症例の病理組織学的指標として, prolymphocyticないしparaimmunoblasticな細胞像, BCL6とMUM1/*IRF4*の共発現が考えられた.

F. 健康危険情報

該当せず.

G. 研究発表

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H. 知的財産権の出願・登録状況

該当せず.

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Transforming mutations of RAC guanosine triphosphatases in human cancers

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Members of the RAS superfamily of small guanosine triphosphatases (GTPases) transition between GDP-bound, inactive and GTP-bound, active states and thereby function as binary switches in the regulation of various cellular activities. Whereas HRAS, NRAS, and KRAS frequently acquire transforming missense mutations in human cancer, little is known of the oncogenic roles of other small GTPases, including Ras-related C3 botulinum toxin substrate (RAC) proteins. We show that the human sarcoma cell line HT1080 harbors both NRAS(Q61K) and RAC1(N92I) mutant proteins. Whereas both of these mutants were able to transform fibroblasts, knockdown experiments indicated that RAC1(N92I) may be the essential growth driver for this cell line. Screening for RAC1, RAC2, or RAC3 mutations in cell lines and public databases identified several missense mutations for RAC1 and RAC2, with some of the mutant proteins, including RAC1(P29S), RAC1(C157Y), RAC2(P29L), and RAC2(P29Q), being found to be activated and transforming. P29S, N92I, and C157Y mutants of RAC1 were shown to exist preferentially in the GTP-bound state as a result of a rapid transition from the GDP-bound state, rather than as a result of a reduced intrinsic GTPase activity. Activating mutations of RAC GTPases were thus found in a wide variety of human cancers at a low frequency; however, given their marked transforming ability, the mutant proteins are potential targets for the development of new therapeutic agents.

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The identification of transforming proteins and the development of agents that target them have markedly influenced the treatment and improved the prognosis of individuals with cancer. Chronic myeloid leukemia (CML), for example, has been shown to result from the growth-promoting activity of the fusion tyrosine kinase breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 (BCR-ABL1), and treatment with a specific ABL1 inhibitor, imatinib mesylate, has increased the 5-y survival rate of individuals with CML to almost 90% (1). Similarly, the fusion of echinoderm microtubule associated protein like 4 gene (*EML4*) to anaplastic lymphoma receptor tyrosine kinase (*ALK*) is responsible for a subset of non–small-cell lung cancer cases (2), and therapy targeted to EML4-ALK kinase activity has greatly improved the progression-free survival of affected individuals compared with that achieved with conventional chemotherapies (3). Therapies that target essential growth drivers in human cancers are thus among the most effective treatments for these intractable disorders.

V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS), and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) are the founding members of the rat sarcoma (RAS) superfamily of small guanosine triphosphatases (GTPases) that is known to comprise >150 members in humans (4). Five subgroups of these small GTPases have been identified and designated as the RAS; ras homolog family member (RHO); RAB1A, member RAS oncogene family (RAB); RAN, member RAS oncogene family (RAN); and ADP-ribosylation factor (ARF) families. All small GTPases function as binary switches that transition between GDP-bound, inactive and GTP-bound, active forms and thereby contribute to intracellular signaling that underlies a wide array of cellular activities, including cell proliferation, differentiation, survival, motility, and transformation (5). Somatic point mutations that activate KRAS, HRAS, or NRAS have been identified in a variety of human tumors, with KRAS being the most frequently activated oncoprotein in humans. Somatic activating mutations of KRAS are thus present in >90% of pancreatic adenocarcinomas, for example (6). Surprisingly, however, mutational activation of small GTPases other than KRAS, HRAS, and NRAS has not been widely reported.

Ras-related C3 botulinum toxin substrate (RAC) 1, RAC2, and RAC3 belong to the RHO family of small GTPases (7). RAC proteins orchestrate actin polymerization, and their activation induces the formation of membrane ruffles and lamellipodia (8), which play essential roles in the maintenance of cell morphology and in cell migration. Accumulating evidence also indicates that RAC proteins function as key hubs of intracellular signaling that underlies cell transformation. RAC1, for example, serves as an essential downstream component of the signaling pathway by which oncogenic RAS induces cell transformation, and artificial introduction of an amino acid substitution (G12V) into RAC1 renders it oncogenic (9). Furthermore, suppression of RAC1 activity induces apoptosis in glioma cells (10), and loss of *RAC1* or *RAC2* results in a marked delay in the development of BCR-ABL1–driven myeloproliferative disorder (11). Despite such important roles of RAC proteins in cancer, somatic transforming mutations of these proteins have not been identified in cancer specimens.

We have now discovered a mutant form of RAC1 with the amino acid substitution N92I in a human sarcoma cell line, HT1080, and have found that this mutation renders RAC1 constitutively active and highly oncogenic. Even though HT1080 cells also harbor the NRAS(Q61K) oncoprotein, RAC1(N92I) is the essential growth driver in this cell line, given that RNA interference (RNAi)-

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mediated knockdown of RAC1(N92I) markedly suppressed cell growth. Further screening for *RAC1*, *RAC2*, and *RAC3* mutations among cancer cell lines as well as public databases identified additional transforming mutations of RAC1 and RAC2. Our data thus reveal oncogenic amino acid substitutions for the RAC subfamily of small GTPases in human cancer.

Results

Discovery of the RAC1(N92I) Oncoprotein. To identify transforming genes in the fibrosarcoma cell line HT1080 (12), we isolated cDNAs for cancer-related genes (n = 906) from HT1080 cells and subjected them to deep sequencing with the Genome Analyzer IIx (GAIIx) system. Quality filtering of the 92,025,739 reads obtained yielded 45,325,377 unique reads that mapped to 843 (93.0%) of the 906 target genes. The mean read coverage for the 843 genes was 495× per nucleotide, and ≥70% of the captured regions for 568 genes were read at ≥10× coverage.

Screening for nonsynonymous mutations in the data set with the use of our computational pipeline (13) revealed a total of five missense mutations with a threshold of ≥30× coverage and a ≥30% mutation ratio (Table S1). One of these mutations, a heterozygous missense mutation of *NRAS* that results in a Gln-to-Lys substitution at amino acid position 61 (Q61K), was described previously in this cell line (14) and is the most frequent transforming mutation of *NRAS* (5). We also discovered a missense mutation in another small GTPase, RAC1 (Fig. S1 and Table S1). An A-to-T transversion at position 516 of human *RAC1* cDNA (GenBank accession no. NM_006908.4), resulting in an Asn-to-Ile substitution at position 92 of the encoded protein, was thus identified in 11,525 (47.5%) of the 24,238 total reads covering this position.

To examine the transforming potential of RAC1(N92I), we infected mouse 3T3 fibroblasts and MCF10A human mammary epithelial cells (15) with a retrovirus encoding wild-type or N92I mutant form of human RAC1 and then seeded the cells in soft agar for evaluation of anchorage-independent growth. Neither 3T3 nor MCF10A cells expressing wild-type RAC1 grew in soft agar (Fig. 1*A*), indicating the lack of transforming potential of RAC1. In contrast, the cells expressing RAC1(N92I) readily grew in soft agar (Fig. 1*A*), showing that this RAC1 mutant confers the property of anchorage-independent growth on both 3T3 and MCF10A cells. We also confirmed the transforming potential of an artificial mutant of RAC1, RAC1(G12V) (8),

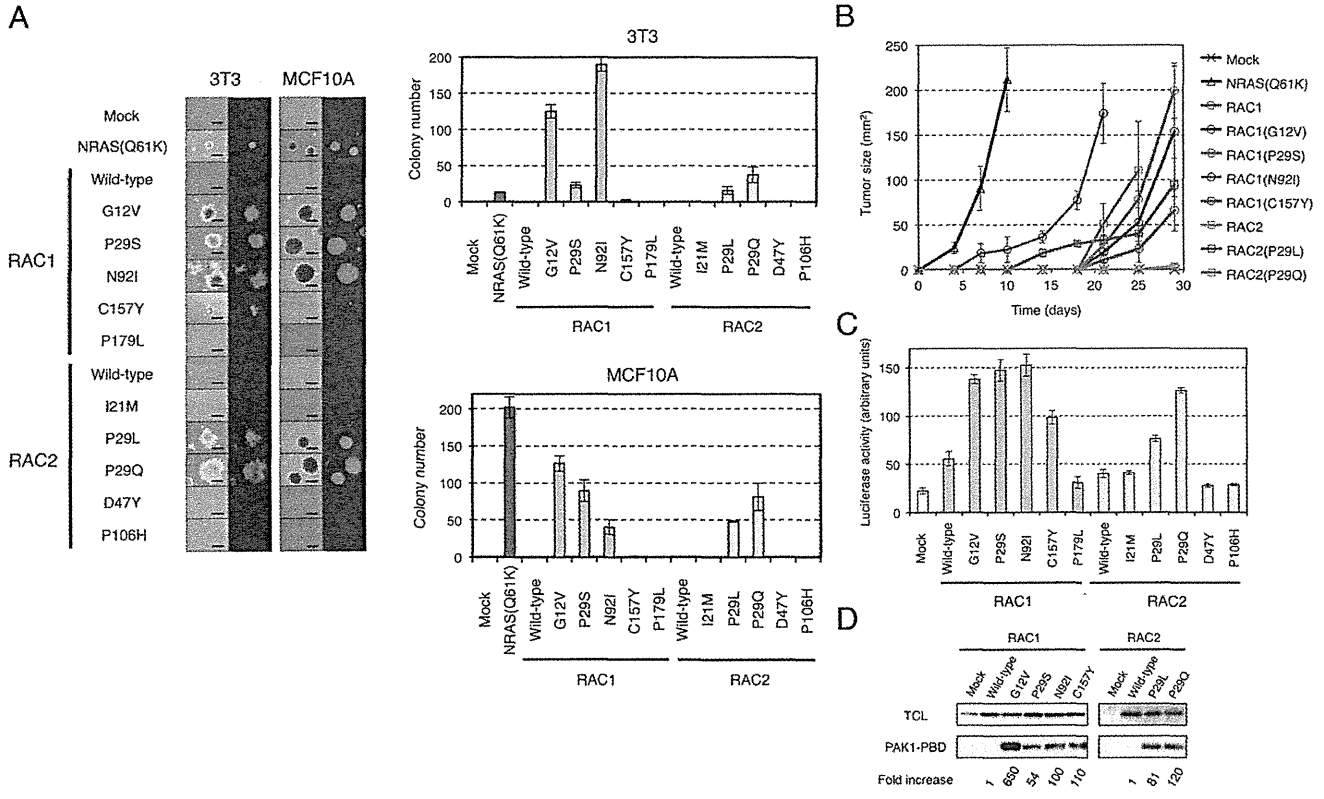

Fig. 1. Transforming potential of RAC1 and RAC2 mutants. (*A*) 3T3 or MCF10A cells were infected with recombinant retroviruses encoding enhanced green fluorescent protein (EGFP) as well as wild-type or mutant forms of RAC1 or RAC2 and were then assayed for anchorage-independent growth in vitro under the presence of 10% (vol/vol) FBS. After 14 d (3T3) or 20 d (MCF10A) of culture, the cells were stained with crystal violet and examined by conventional microscopy (*Left*: left image of each pair), and they were monitored for EGFP expression by fluorescence microscopy (*Left*: right image of each pair). (Scale bars, 0.5 mm.) The numbers of cell colonies were also determined as means ± SD from three independent experiments (*Right*). (*B*) 3T3 cells expressing wild-type or mutant forms of RAC1 or RAC2 were injected s.c. into the shoulder of nude mice, and the size of the resulting tumors [(length × width)/2] was determined at the indicated times thereafter. Tumor size for 3T3 expressing NRAS(Q61K) was similarly monitored. Data are means ± SD for tumors at four injection sites. (*C*) HEK293T cells were transfected with expression vectors for wild-type or mutant forms of RAC1 or RAC2 together with the SRE.L reporter plasmid and pGL-TK. The activity of firefly luciferase in cell lysates was then measured and normalized by that of *Renilla* luciferase. Data are means ± SD from three independent experiments. (*D*) Lysates of 3T3 cells expressing wild-type or mutant forms of RAC1 or RAC2 were subjected to a pull-down assay with PAK1-PBD. The precipitated proteins as well as the total cell lysates were then subjected to immunoblot analysis with antibodies to RAC1 or to RAC2. The relative amounts of pulled-down RAC proteins compared with their corresponding expression levels in total cell lysate were normalized to that of wild-type RAC1 (for the RAC1 mutants) or RAC2 (for the RAC2 mutants) and are shown at the bottom.

which harbors an amino acid substitution corresponding to that of the oncogenic G12V mutant form of RAS proteins.

Other Transforming Mutations of RAC1 and RAC2. We next searched for other transforming mutations of RAC proteins. Human RAC1, RAC2 (GenBank accession no. NM_002872.3), and RAC3 (GenBank accession no. NM_005052.2) cDNAs were isolated from 40 cancer cell lines (Table S2), and their nucleotide sequences were determined by Sanger sequencing, resulting in the discovery of RAC1(P29S), RAC2(P29Q), and RAC2(P29L) in the breast cancer cell line MDA-MB-157, the CML cell line KCL-22, and the breast cancer cell line HCC1143, respectively (Fig. S1 and Table S3). Further searching for *RAC1*, *RAC2*, and *RAC3* mutations in the COSMIC database of cancer genome mutations (Release V59; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic) revealed various amino acid substitutions detected in human tumors, namely RAC1(P29S), RAC1(C157Y), RAC1(P179L), RAC2(I21M), RAC2(P29L), RAC2(D47Y), and RAC2(P106H) (Table S3). Importantly, all of these *RAC1* and *RAC2* mutations identified in clinical specimens were confirmed to be somatic, given that the corresponding mutations were absent in the genome of paired normal cells.

To examine the transforming potential of these various RAC1 and RAC2 mutants, we expressed each protein in 3T3 and MCF10A cells and evaluated anchorage-independent growth. Whereas the wild-type form of RAC2 did not transform 3T3 or MCF10A cells, growth in soft agar was apparent for 3T3 cells expressing RAC1 (P29S), RAC1(C157Y), RAC2(P29L), or RAC2(P29Q), but not for those expressing RAC1(P179L), RAC2(I21M), RAC2(D47Y), or RAC2(P106H) (Fig. 1*A*). Of interest, colony number in the assay varied substantially in a manner dependent on the type of amino acid substitution as well as on cell type. RAC1(C157Y), for example, yielded fewer colonies in soft agar compared with the other transforming mutants. Furthermore, RAC1(P29S), which was identified in a breast cancer cell line, generated a larger number of colonies with MCF10A cells than with 3T3 cells. Conversely, RAC1(N92I), which was identified in a fibrosarcoma cell line, yielded a larger number of colonies with 3T3 cells than with MCF10A cells. The oncogenic activity of RAC1(P29S), RAC1 (N92I), RAC1(C157Y), RAC2(P29L), and RAC2(P29Q) mutants was further confirmed with a tumorigenicity assay in nude mice (Fig. 1*B*), with the activity of RAC1(N92I) being the most pronounced with regard to the transformation of 3T3 cells in this assay.

The colony number in soft agar for 3T3 cells expressing NRAS (Q61K) was fewer than that for the cells expressing oncogenic RAC1 or RAC2 mutants (Fig. 1*A*), whereas expression of these small GTPases was readily confirmed in 3T3 (Fig. S2). Interestingly, s.c. tumors from the same 3T3 cells expressing NRAS(Q61K) grew more rapidly than tumors expressing the RAC1/RAC2 mutants (Fig. 1*B*), indicating that the measured intensity of the transforming potential of GTPases may vary in a dependent manner on assay systems.

To examine whether such oncogenic potential is linked directly to the activation of RAC1 or RAC2, we investigated the activity of the mutant proteins with the use of a luciferase reporter plasmid that selectively responds to intracellular signaling evoked by RHO family GTPases (16). In concordance with the data from the soft agar and tumorigenicity assays, only the transforming mutants of RAC1 and RAC2 yielded a substantial level of luciferase activity in transfected HEK293T cells (Fig. 1*C*).

Activated RAC1 or RAC2 would be expected to be loaded with GTP. We therefore examined the GTP-binding status of the RAC1 and RAC2 oncoproteins with the use of a pull-down assay based on the p21-binding domain (PBD) of PAK1. All of the transforming RAC1 and RAC2 mutants were found to exist preferentially in the GTP-bound state (Fig. 1*D*), indicative of their constitutive activation. Furthermore, these RAC1 and RAC2 mutants induced marked reorganization of the actin cytoskeleton in 3T3 cells, resulting in the accumulation of polymerized actin in ruffles at the plasma membrane (Fig. 2).

RAC1 and RAC2 as Therapeutic Targets. Given that NRAS(Q61K) is also known to transform 3T3 cells (17) (Fig. 1*A*), our data show that HT1080 cells harbor two independent oncogenic GTPases. We therefore examined whether RAC1(N92I) or NRAS(Q61K) is the principal growth driver in this sarcoma cell line. Among several small interfering RNAs (siRNAs) designed to attenuate the expression of RAC1 or NRAS, we selected two independent siRNAs that specifically target each mRNA (Fig. 3*A*). Whereas transfection of HT1080 cells with either NRAS siRNA resulted in a moderate inhibition of cell proliferation under the presence of 10% (vol/vol) FBS, that with either RAC1 siRNA almost blocked cell growth (Fig. 3*B*). Transfection with an NRAS siRNA in addition to either RAC1 siRNA did not result in an additional effect on cell proliferation (Fig. 3*B*). Similar data were observed in a culture with 1% (vol/vol) FBS (Fig. S3*A*) or under FBS-free conditions (Fig. S3*B*). To further examine the effects of silencing RAC1/NRAS, we quantitated cell cycle distribution of HT1080 transfected with siRNAs against either RAC1 or NRAS. As shown in Fig. S4*A*, DNA synthesis was equally suppressed by the knockdown of RAC1 or NRAS. Interestingly, however, CASP3/CASP7 activity (a surrogate marker for apoptosis) was markedly induced only by RAC1 depletion (Fig. S4*B*). Therefore, RAC proteins are likely to provide RAS-independent cell survival

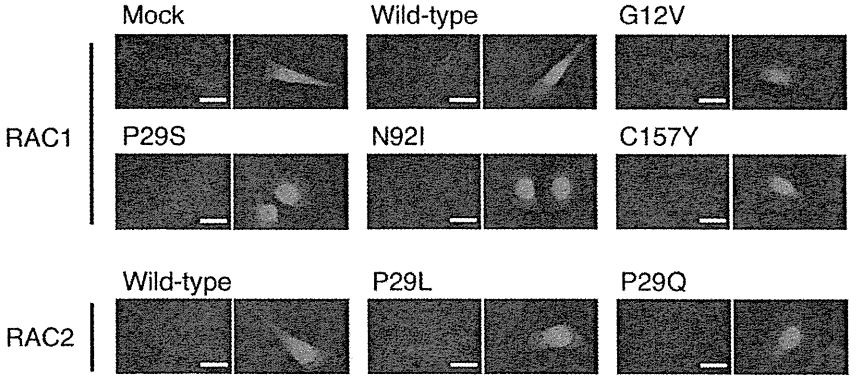

Fig. 2. Actin reorganization induced by the RAC1/RAC2 mutants. 3T3 cells infected with retroviruses encoding enhanced green fluorescent protein (EGFP) as well as wild-type or mutant forms of RAC1 or RAC2 were stained with Alexa Fluor 594-labeled phalloidin to visualize actin organization (*Left* image of each pair). The same cells were also examined for EGFP fluorescence (*Right* image of each pair). (Scale bars, 20 μm.)

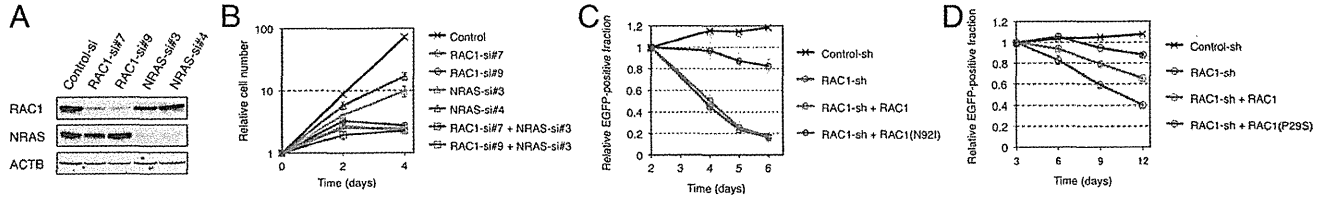

Fig. 3. Oncogenic RAC proteins as therapeutic targets. (*A*) HT1080 cells were transfected with control, RAC1, or NRAS siRNAs; lysed; and subjected to immunoblot analysis with antibodies to RAC1, NRAS, or ACTB (loading control). (*B*) HT1080 cells were transfected with control, RAC1, or NRAS siRNAs, as indicated, and cultured under the presence of 10% (vol/vol) FBS. Cell number was counted at the indicated times after the onset of transfection. Data are means ± SD from three independent experiments. (*C*) HT1080 cells were infected with a retrovirus encoding green fluorescent protein (EGFP) as well as a control or RAC1 shRNA. They were also infected with a retrovirus encoding shRNA-resistant wild-type RAC1 or RAC1(N92I), as indicated. The number of EGFP-positive cells was determined by flow cytometry after culture of the cells for the indicated times, and the size of the EGFP-positive fraction relative to that at 2 d was calculated. Data are means ± SD from three independent experiments. (*D*) MDA-MB-157 cells were infected with a retrovirus encoding EGFP as well as a control or RAC1 shRNA. They were also infected with a retrovirus encoding shRNA-resistant wild-type RAC1 or RAC1(P29S), as indicated. The number of EGFP-positive cells was determined by flow cytometry after culture of the cells for the indicated times, and the size of the EGFP-positive fraction relative to that at 3 d was calculated. Data are means ± SD from three independent experiments.

signals, which is supported by the fact that, even under FBS-free conditions, RAC1 depletion has more antiproliferative effects in HT1080 than NRAS depletion (Fig. S3*B*). These data show that active RAC1 may be the essential growth driver in HT1080 cells and is therefore a potential therapeutic target. Furthermore, our data suggest that oncogenic RAS proteins may require additional transforming hits to give rise to full-blown cancer.

We next infected HT1080 cells with a retrovirus expressing a short hairpin RNA (shRNA) targeted to RAC1 mRNA. Expression of the RAC1 shRNA markedly suppressed cell growth, whereas restoration of shRNA-resistant RAC1(N92I) expression reversed this effect (Fig. 3*C* and Fig. S5), showing that the effect of the RAC1 shRNA was not an off-target artifact. Forced expression of shRNA-resistant wild-type RAC1 failed to reverse the inhibitory effect of the RAC1 shRNA on cell growth, indicating that growth suppression by the shRNA was due to depletion of the N92I mutant, not to that of the wild-type protein. We performed similar experiments with the breast cancer cell line MDA-MB-157, which harbors RAC1(P29S). Again, the RAC1 shRNA inhibited cell growth, and this effect was reversed to a larger extent by restoration of the expression of shRNA-resistant RAC1(P29S) than by forced expression of the wild-type protein (Fig. 3*D* and Fig. S5).

RAC1(P29S), RAC1(N92I), and RAC1(C157Y) Are Rapid-Cycling Mutants. Oncogenic mutations at G12, G13, or Q61 of RAS proteins found in human tumors reduce the intrinsic GTPase activity of these

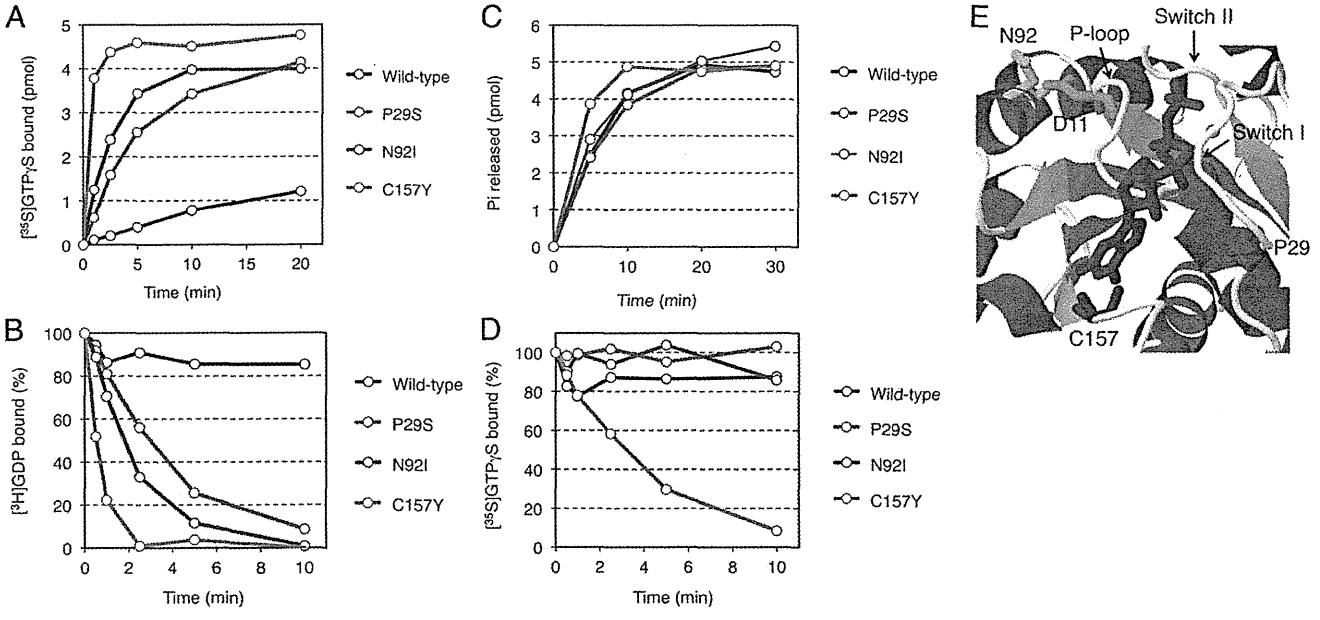

Fig. 4. Biochemical properties of RAC1 mutants. (*A*) Bacterially expressed and purified proteins of the wild-type, P29S, N92I, or C157Y mutant of RAC1 (5 pmol each) were incubated with [^{35}S]GTPγS in the presence of 0.8 mM Mg^{2+}, and the amounts of [^{35}S]GTPγS-bound proteins were determined at the indicated times. (*B*) [^{3}H]GDP dissociation from [^{3}H]GDP-bound RAC1 proteins was initiated by the addition of unlabeled GTPγS in the presence of 0.8 mM Mg^{2+}, and the amounts of [^{3}H]GDP-bound proteins were determined at the indicated times. (*C*) RAC1 proteins were preloaded with [γ^{32}P] GTP, and then GTP hydrolysis reactions were initiated by the addition of unlabeled GTP in the presence of 0.8 mM Mg^{2+}. P_i released from the proteins was isolated and measured at the indicated times. (*D*) [^{35}S]GTPγS dissociation from [^{35}S]GTPγS-bound RAC1 proteins was initiated by the addition of unlabeled GTPγS in the presence of 0.8 mM Mg^{2+}, and the amounts of [^{35}S]GTPγS-bound proteins were determined at the indicated times. (*E*) Schematic representation of the structure of the GTP-binding pocket of human RAC1 (ID 1mh1 in the Protein Data Bank; www.pdb.org) with α-helices and β-sheets shown in magenta and orange, respectively. The GTP analog guanosine 5′-(β,γ-imido)-triphosphate (GppNp) and Mg^{2+} are depicted in red and green, respectively. D11, P29, N92, and C157 amino acid residues are in orange, blue, yellow and purple, respectively. The positions of switch I and switch II regions and the P-loop are also indicated.

proteins and thereby maintain them in the GTP-bound state (18, 19). On the other hand, an artificial F28L substitution in HRAS or the RHO family protein Cdc42Hs was shown to confer constitutive activity by accelerating the transition from the GDP-bound to the GTP-bound state without the involvement of an exogenous guanine nucleotide exchange factor (GEF) (20, 21).

To determine how transforming mutations of RAC1 results in constitutive activation of these proteins, we examined their affinity for GTP and GDP. Compared with wild-type RAC1, all of RAC1(P29S), RAC1(N92I), and RAC1(C157Y) was found to bind GTPγS (nonhydrolyzable GTP analog) rapidly in vitro, even without the addition of a GEF protein (Fig. 4*A*). Likewise, the dissociation of GDP from the mutant forms of RAC1 was greatly accelerated (Fig. 4*B*). On the other hand, the intrinsic GTPase activity of these mutants was similar to (for P29S and N92I) or slightly higher (for C157Y) than that of the wild-type protein (Fig. 4*C*). These data thus indicated that, in contrast to transforming RAS mutants associated with human cancer, RAC1 (P29S), RAC1(N92I), and RAC1(C157Y) are fast-cycling mutants, for which the probability of being in the GTP-bound state is increased as the result of an increased rate of GDP dissociation, rather than as the result of a loss of GTPase activity.

Interestingly, dissociation of GTPγS was also accelerated only for RAC1(C157Y), but not for the wild-type, P29S, or N92I form of RAC1 (Fig. 4*D*). Thus, RAC1(C157Y) is a unique mutant in that both association and dissociation for GTP are accelerated, which may provide the molecular basis for its modest transforming potential compared with that of RAC1(P29S) or RAC1 (N92I) (Fig. 1).

In the 3D structure of RAC1 (Fig. 4*E*), P29 is located in the switch I region, whereas C157 is positioned adjacent to the guanine ring of bound GTP. Substitution of these residues would thus likely affect the affinity of the protein for GDP or GTP (Fig. S6), a phenomenon that has been demonstrated recently for RAC1 (P29S) (22). In contrast, N92 is located distant from the binding pocket for GDP/GTP, and so the structural mechanism by which the N92I substitution renders RAC1 constitutively active remains elusive (Fig. 4*E* and Fig. S6). Residue N92 is located close to D11 in the P-loop of RAC1, however (Fig. 4*E* and Fig. S7), and substitution with isoleucine at this position would abolish the interaction between the amino group of N92 and the carboxyl group of D11. It is thus possible that the N92I mutation affects the binding of GDP/GTP through an effect on the P-loop.

Discussion

We have here demonstrated the transforming potential of mutated RAC proteins. Our analysis of cell lines resulted in the identification of transforming mutants of RAC1 and RAC2, namely RAC1(N92I) and RAC2(P29Q), and we also revealed the transforming potential of the RAC1(P29S), RAC1(C157Y), and RAC2(P29L) mutants deposited the COSMIC database of cancer genome mutations (Release V59; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic) (Table S3). In contrast, the soft agar assay did not reveal a transforming potential of the RAC1(P179L), RAC2(I21M), RAC2(D47Y), or RAC2(P106H) mutants found in the database, suggesting a possibility that they are "passenger mutations." It may also be possible, however, that these mutants may still contribute to cancer development by modifying tumor properties (such as metastasis ability), given that they were somatically acquired and clonally selected in cancer.

An important finding of our study was that the oncogenic effects of RAC1(N92I) may be more pronounced than those of NRAS(Q61K), at least with regard to survival signals in HT1080 cells (Fig. 3*B*). It should be noted, however, that HT1080 expresses RAC1 almost exclusively among the RAC family proteins, whereas HRAS and KRAS are weakly expressed in addition to NRAS (Fig. S8). It is thus possible that the effects of NRAS knockdown in Fig. 3*B* may be partly complemented by the residual HRAS/KRAS proteins.

Paterson et al. previously isolated NRAS-attenuated subclones of HT1080 after treatment with an alkylating reagent (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) and a subsequent culture with 5-fluorodeoxyuridine and 1-β-D-arabinofuranosylcytosine (23). Such subclones had a flat cell shape and a reduced ability for anchorage-independent growth. Likewise, we noted that transfection with NRAS siRNAs renders HT1080 a flatter shape (Fig. S9). As demonstrated in Fig. 3*B* and by Paterson et al. (23), however, such NRAS-depleted HT1080 was still viable and kept proliferation in vitro, suggesting the presence of other oncogene(s) in addition to NRAS(Q61K). Therefore, NRAS(Q61K) and RAC1(N92I) are likely to cooperate to fully transform this fibrosarcoma.

Regarding the coexistence of mutations within RAC family proteins and RAS-RAF-MAPK proteins, two studies independently reported recurrent P29S mutation of RAC1 in melanoma during the preparation of this article (22, 24). Of note, BRAF (V600E) was also detected in four of six and in two of seven of the RAC mutation-positive melanomas, respectively. These observations, together with our findings with HT1080 cells, thus indicate that activating mutations of RAC1 and those of the RAS-RAF–signaling pathway are not mutually exclusive.

Members of the RAC subfamily of GTPases show a high level of sequence identity in humans. The amino acid sequence of RAC1 is thus 92% identical to that of RAC2 or RAC3. Furthermore, all of the amino acid residues of RAC1 or RAC2 found to be mutated in cancer (Table S3) are completely conserved among RAC1, RAC2, and RAC3. Thus, transforming RAC3 mutants with similar nonsynonymous mutations may also exist in human cancer, although such mutations were not detected in the current screening. Of interest, none of the frequent mutation sites in RAS family proteins (G12, G13, and Q61) were found to be affected in RAC1 or RAC2, although an artificial G12V mutant of RAC1 did manifest constitutive GTP loading and transforming potential. Given that RAC proteins perform intracellular functions (such as orchestration of the actin cytoskeleton) that are distinct from those of RAS family members, RAC-driven activation of specific intracellular pathways may be advantageous for cancer development in vivo.

Given that we detected activation mutations of RAC1 or RAC2 in cell lines from sarcoma (HT1080), triple-negative breast cancer (MDA-MB-157 and HCC1143), and the blast crisis stage of CML (KCL-22), we performed deep sequencing of *RAC1*, *RAC2*, and *RAC3* cDNAs with GAIIx for specimens of triple-negative breast cancer (n = 66), of *RAC1* and *RAC2* cDNAs for specimens of CML in blast crisis (n = 43), and of *BCR-ABL1*–positive acute lymphoblastic leukemia (n = 31), as well as of *RAC1* cDNAs for specimens of sarcoma (n = 53). We failed, however, to detect any nonsynonymous mutations among these RAC cDNAs.

Our results have shown that RAC proteins have the potential to become oncogenic through amino acid substitution in a wide array of cancers. Although such RAC mutations may occur at a low frequency, the recent studies of Krauthammer et al. (22) and Hodis et al. (24) suggest that they may be enriched in melanoma (~5%). Importantly, given that HT1080 cells are highly addicted to the increased activity of RAC1(N92I), the targeting of oncogenic RAC proteins or their downstream effectors with small compounds or RNAi may prove to be an effective approach to the treatment of cancer harboring such oncoproteins.

Materials and Methods

The human fibrosarcoma cell line HT1080 was obtained from American Type Culture Collection, and subjected to deep sequencing with GAIIx. Recombinant retrovirus expressing the wild-type or mutant forms of RAC1 or RAC2 was used to infect mouse 3T3 fibroblasts to examine its transforming potential. Detailed information for cDNA resequencing, transformation assays, biochemical analysis of RAC proteins, and RNAi are detailed in *SI Materials and Methods*.