The Nuclear Tyrosine Kinase Rak Associates with the Retinoblastoma Protein pRb

Rolf J. Craven, William G. Cance, and Edison T. Liu

Curriculum in Genetics and Molecular Biology [R. J. C., E. T. L.], Lineberger Comprehensive Cancer Center [R. J. C., W. G. C., E. T. L.], Departments of Surgery [R. J. C., W. G. C.] and Medicine, Biochemistry and Biophysics [E. T. L.], University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599

Abstract

Rak is a nuclear tyrosine kinase containing Src homology 2 and 3 domains at its NH₂ terminus. We report here that the retinoblastoma tumor susceptibility gene product pRb associates with Rak in vivo and in vitro. Rak binds to the A/B pocket region of pRb, a region that is frequently mutated in human cancer, during the G₁ and S phases of the cell cycle. Furthermore, Rak expression is elevated in G₁, and transfection of Rak into NIH 3T3 cells results in a significant decrease in the number of emerging colonies. Thus, Rak is a tyrosine kinase with growth suppressing activity that may function, in part, through its interaction with pRb.

Introduction

We originally identified the Rak tyrosine kinase in a screen for novel protein kinases expressed in human breast cancer (1). The primary structure of Rak is similar to the Src-related kinases, containing SH22 and SH3 domains at the NH₂ terminus and tyrosine residues within the kinase domain and near the COOH terminus (2). Unlike the Src-related kinases, however, Rak is expressed primarily in epithelial cells, particularly breast cancer cells, and localizes not to the plasma membrane but to the nucleus (2). Thus, Rak and the Src-related kinases have similar protein interaction domains, the role of Rak in the cell is likely to be distinct from Src because of its nuclear localization.

The two best characterized nuclear tyrosine kinases, Weel and c-Abl, may yield some insight into the potential role of Rak. Weel is a cell cycle regulatory protein that phosphorylates the cyclin-dependent kinase Cdc2 (3), whereas c-Abl associates with the tumor suppressor pRb in G₁ (4), and both kinases have growth-inhibitory activity (5, 6). Thus, the function of these nuclear tyrosine kinases includes interactions with cell cycle-regulatory proteins, and this may be reflected in their growth-inhibitory activity.

The tumor suppressor pRb is a key regulator of the cell cycle and is frequently inactivated in some human tumors (7, 8). pRb associates with a variety of proteins, including transcription factors and cyclin D (reviewed in Ref. 9), through a 400-amino acid region of pRb termed the “A/B pocket.” At the COOH-terminus, pRb contains a “C pocket,” which binds to the kinase domain of c-Abl. The binding of c-Abl to pRb and the presence of a putative pRb-binding site in Rak led us to investigate whether Rak may also interact with pRb. We report here that Rak associates with the pRb A/B pocket in breast cancer cells in a cell cycle-dependent manner and that overexpression of Rak causes a reduction in cell growth. This Rak-Rb association and growth-suppressive activity suggests that Rak may play a role in the regulation of cell growth in epithelial cells.

Materials and Methods

Cell Culture and Antibodies. BT474 and SK-BR-3 human breast cancer cells, SAOS-2 human osteosarcoma cells, and RD rhabdomyosarcoma cells were obtained from American Type Culture Collection and were maintained according to their specifications. Cells were grown in G₁, S, and M by serum starvation, 1 mM hydroxyurea (Sigma), and 40 ng/ml nocodazole (Sigma) essentially as described (10).

Early passage NIH 3T3 cells were transfected with Lipofectamine (GIBCO-BRL) according to the manufacturer’s instructions. After transfection, 1 × 10⁶ cells were plated and grown in 600 μg/ml G418 for 3 weeks, whereupon the colonies were stained and counted. Antibodies to cyclin A and Cdc2 were purchased from Santa Cruz, and the anti-pRb antibody G3-245 was purchased from Pharmingen.

Plasmid Construction. A 320-bp fragment encoding the NH₂-terminal 110 amino acids of Rak (2) was subcloned into the PGEX-4T plasmid (Pharmacia) using PCR with Pyrococcus furiosus DNA polymerase (Stratagene). This plasmid was used to produce a fusion protein called GST-RakNT, which consists of amino acids 1–110 of Rak and includes the unique NH₂-terminal and the SH3 domain. For subsequent binding experiments, Rab fusion proteins encoding amino acids 379–928 (RbABC, containing the A/B pocket region and the COOH-terminal domain) and 780–928 (RbC, for COOH-terminal domain only) were prepared similarly (see Fig. 2A). The pRak(152) construct used in Fig. 2B was prepared by digesting the pAl-Rak plasmid (2) with EcoRI and consisted of amino acids 1–152 of Rak. Deletion mutants of Rak diagrammed in Fig. 2C were prepared by PCR and encoded amino acids 1–505 (Rak), 47–505 (del46), and 111–505 (del110). In vitro transcription/translation reactions were performed using the TnT reticulocyte lysate system with T7 RNA polymerase (Promega). NIH 3T3 cells were transfected with the pcDNA3 vector (Invitrogen) containing the full-length Rak (pc3-Rak).

Affinity Precipitation. GST fusion proteins were induced and purified according to the manufacturer’s instructions, and were analyzed by SDS-PAGE to ensure that an equal amount of protein was used for the affinity precipitation procedure. For affinity precipitations, 250 μg of cell lysate was preclreated once with 25 μl of glutathione-Sepharose beads (Pharmacia), then once with 25 μl of the same beads containing 2 μg/ul GST. The preclreated lysate was incubated with 25 μl of GST-RakNT bound to glutathione-Sepharose in NP40 Lysis Buffer for 3 h at 4°C, then washed three times in NP40 lysis buffer. Bound proteins were eluted with SDS-PAGE sample loading buffer and analyzed by Western blot. The same procedure was followed for in vitro binding assays, except that the GST fusion proteins were incubated with in vitro translated forms of Rak in NP400 buffer containing 0.5% fraction V BSA (Boehringer Mannheim).

Immunological Techniques. Immunoprecipitations and in vitro kinase assays were performed as described (2) by immunoprecipitation in NP40 lysis buffer [50 mM Tris (pH 7.4)-1% NP40-150 mM NaCl-1 mM EDTA-1 mM Na₃VO₄ - 10 μg/ml of the protease inhibitors aprotinin and leupeptin], washing, and resuspending in kinase buffer [10 mM HEPES (pH 7.4)-5 mM MnCl₂ - 1 mM DTT]. Kinase reactions were initiated with 10 μCi of [γ-32P]ATP, incubated at 30°C for 15 min, and stopped with SDS-PAGE sample loading buffer and analyzed by SDS-PAGE. After electrophoresis, the gels were fixed for 15 min with 30% methanol/10% acetic acid, then soaked in 1 M KOH at 55°C for 1 h, then fixed again, dried, and autoradiographed.

Cdc2 activity was determined after immunoprecipitation by the addition of 1 μg of histone H1 (Boehringer Mannheim) and 15 μCi of [γ-32P]ATP in 10 mM

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2. To whom requests for reprints should be addressed, at University of North Carolina at Chapel Hill, Room 257, Lineberger Comprehensive Cancer Center, CB# 7295, Chapel Hill, NC 27599.

3. The abbreviations used are: SH2, Src homology 2; SH3, Src homology 3; pRb, retinoblastoma tumor suppressor protein; GST, glutathione S-transferase.
Results and Discussion

The possibility of a physical interaction between Rak and Rb was assessed because of the nuclear localization of Rak, and because Rak contains a sequence resembling a motif that is essential for pRb binding in a number of proteins: EPYLPCLSTE of Rak versus EHQLLCCEVE of Cyclin D1 (the LxCxE motif; Ref. 11). Furthermore, this sequence is located in the Rak NH2-terminal region preceding the SH3 domain, and the analogous region in the Src-related kinases directs several key protein-protein associations (12). In addition, the nuclear tyrosine kinase c-Abl, which is structurally similar to Rak, also associates with pRb.

To test whether Rak and pRb physically interact, a bacterial fusion protein, consisting of the GST gene fused to the first 110 amino acids of Rak, was prepared. This fusion protein, called GST-RakNT, included the Rak SH3 domain and the NH2-terminal sequence predicted to bind to pRb. GST-RakNT was incubated with cell lysates from SK-BR-3 breast cancer cells, precipitated with glutathione-Sepharose beads, and then washed extensively. The lysates were also incubated with GST alone as a control, and bound proteins were analyzed by Western blots probed for pRb. By this assay, pRb was found to bind the Rak NH2 terminus (Fig. 1A, Lane 3). In contrast, binding of Rak to signaling proteins such as Ras-GAP, GAP-associated p62, or Shc, which interact with the SH3 domains of other members of the Src family (13), was not detectable (data not shown). To assess whether the interaction between Rak and pRb occurs in vivo, Rak was immunoprecipitated from two cell lines, SK-BR-3 and SAOS-2, in which only the former expresses the pRb protein, and the precipitates were immunoblotted with antibodies to pRb. These data revealed that pRb coprecipitated with Rak in SK-BR-3 cells but was not detected in SAOS-2 cells (Fig. 1B). As a control, an immunogenic peptide for the anti-Rak antibody was added as a competitor, which effectively blocked the coprecipitation of pRb with Rak (Fig. 1C).

Next, an in vitro binding reaction was used to identify the essential components for the Rak-Rb interaction. Because many proteins bearing the LxCxE motif bind to the pRb A/B pocket region, in vitro translated Rak was incubated with two Rb-GST fusion proteins (diagrammed in Fig. 2A): RbABC, which spans the A/B and C domains; and RbC, which represents only the C pocket. Rak bound efficiently to the RbABC fusion protein and approximately 5-fold less to the RbC protein (Fig. 2B, upper panel), indicating that Rak binds primarily to the pRb A/B pocket. The weak binding of Rak to the RbC fusion protein could occur by a similar mechanism to that of c-Abl, which binds to the pRb C pocket domain through its kinase domain. Indeed, an NH2-terminal fragment of Rak lacking the kinase domain was found to bind to the RbABC fusion protein with approximately 30% less efficiency than the full-length Rak, and binding to the RbC protein was no longer detectable (Fig. 2B, lower panel). These results suggested that the Rak-pRb interaction is primarily between the Rak NH2 terminus and the pRb A/B region, although the kinase domain may weakly interact with the pRb C domain. As a control, the c-Src protein was translated in vitro and subjected to the same binding reaction, and no binding of c-Src to any portion of pRb was detected (data not shown).

To determine which sequences of Rak are required for the Rak-Rb association, amino acids 1–46 of Rak were deleted, removing the LPLCLSTE putative pRb-binding site but leaving the SH3 domain. This deletion mutant of Rak, "del46," was translated in vitro and

Fig. 1. In vitro and in vivo association of Rak with pRb. A. Western blot of an affinity precipitation reaction using the Rak NH2-terminal fusion protein. An SK-BR-3 lysate was probed for pRb (Lane 1) or was incubated with GST alone or GST-RakNT, and bound proteins were analyzed by Western blot for pRb (GST, Lane 2, and GST-RakNT, Lane 3). B. In vivo association of Rak with pRb. pRb (Lanes 1 and 3) or Rak (Lanes 2 and 4) were immunoprecipitated from SK-BR-3 (Rb+) or SAOS-2 (Rb−) cells and analyzed for pRb by Western blot. C. Rak was immunoprecipitated in the absence (Lane 2) or the presence (Lane 1) of immunogenic peptide and analyzed by Western blot for pRb.

HEPES (pH 7.4) with 10 mM MgCl2, then incubating and analyzing the reactions as described above but without KOH treatment.

Fig. 2. Rak binds to the pRb A/B pocket domain in vitro. A, pRb fusion proteins used in the binding reactions. B, in vitro translated [35S]methionine-labeled Rak (Lane 4, upper panel) or the NH2-terminal 152 amino acids of Rak (Lane 4, lower panel) were incubated with GST alone (Lane 1), GST-RbABC (Lane 2), or GST-RbC (Lane 3) and affinity precipitated glutathione-Sepharose, followed by extensive washes. Bound proteins were analyzed by SDS-PAGE and autoradiography, which confirmed the Rak-Rb interaction. C, Binding of deletion mutants of Rak to the RbABC fusion protein was assessed by the same in vitro binding reaction as in B, quantitated by densitometry, and assigned a "a" value based on the efficiency of binding. D, Top: in vitro kinase assay of immunoprecipitated pRb (Lanes 1–3) from different cell lines [RD (Lane 1), SAOS-2 (Lane 2), or SK-BR-3 (Lane 3)] and immunoprecipitated Rak from SK-BR-3 (Lane 4). Middle: western blot of pRb from the same three cell lines. Bottom: Western blot for Rak from the same three cell lines.

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tested for binding to the RbABC fusion protein. It was surprising that the deletion of the LxCxxxE sequence did not alter binding to RbABC (Fig. 2C). Furthermore, incubation of in vitro translated full-length Rak with an excess of peptide containing the SV40 large T antigen LxCxE pRb-binding site did not alter the binding of Rak to RbABC (data not shown). However, a Rak deletion mutant lacking the unique NH₂-terminal region and the SH3 domain, called "del10," now bound poorly to the RbABC fusion protein, suggesting that the Rak SH3 domain is important for the Rak-Rb association (Fig. 2C). The requirement of the Rak SH3 domain for binding to pRb suggested that there might be a proline-rich SH3-binding site in the pRb A/B pocket, but no such site was found. Thus, it is possible that the binding specificity of the Rak SH3 domain is novel. Indeed, Rak contains a seven-amino acid insert in its SH3 domain that is not present in any of the Src-related kinases or c-Abl (2), which may influence the binding specificity of the Rak SH3 domain.

Src-related kinases are frequently activated by binding to proteins via their NH₂-terminal region, making key regulatory tyrosine residues accessible to activating tyrosine kinases and/or phosphatases (14). Because Rak binds by its SH3 domain to sequences within the A/B pocket region of pRb, it is possible that Rak is active when bound to pRb. Indeed, pRb precipitates with a Mr 54,000 protein with kinase activity (Fig. 2D, upper panel, Lane 3), that comigrated with Rak (Fig. 2D, Lane 4). The phosphorylation of this band did not diminish after extensive alkaline treatment at 55°C, suggesting that the phosphorylation is on tyrosine. Furthermore, this coprecipitating p54 kinase was not detected in cell lines that do not express Rak (Fig. 2D, middle and lower panels). Thus, the electrophoretic mobility, activity, and expression pattern of this p54 kinase suggest that the p54 kinase is Rak. However, despite the association between Rak and pRb, immunoprecipitated Rak is not capable of phosphorylating the RbABC fusion protein or a recombinant pRb produced in Baculovirus (data not shown).

Cellular proteins that bind to the pRb A/B pocket are released and activated when pRb becomes phosphorylated at the G₁-S transition (9). Because Rak binds to the A/B pocket, it is possible that Rak is similarly released from pRb when pRb is phosphorylated. To test this, breast cancer cells were arrested in G₁, early S, and M by serum starvation, hydroxyurea, and nocodazole, respectively. This stage-specific arrest has been demonstrated previously in breast cancer cells (10) and was verified by three criteria: pRb mobility shift, expression of cyclin A, and Cdc2 activity. As expected, pRb was hyperphosphorylated in the S and M phase cells; cyclin A reached peak expression in S phase; and Cdc2 was active in the M phase (Fig. 3A). Western blot analysis for Rak expression revealed that Rak migrated as two bands of Mr 54,000 and 56,000 and was most highly expressed in G₁, with 5-fold lower expression in mitosis (Fig. 3B). This result has been found in other breast cancer cell lines. Immunoprecipitation results demonstrated that Rak coprecipitated with pRb not only in serum-starved G₁, cells but also in hydroxyurea-treated S-phase cells, and not in cells arrested in M phase (Fig. 3C). The disruption of the Rak-Rb association was confirmed in cells that were arrested in S phase by treatment with hydroxyurea and synchronized by refeeding complete media. The Mr 54,000 pRb-associated kinase, described in the previous section, disappeared during entry into mitosis, and pRb could no longer be detected in Rak immunoprecipitates in the mitotic fractions (data not shown). Thus, the association between Rak and pRb persisted through S phase, and was more apparent in S phase than in G₁ (compare Fig. 3B, Lanes 1 and 2, with Fig. 3C, Lanes 2 and 3).

The binding of Rak to pRb in S phase suggests that Rak is capable of binding pRb even when pRb is phosphorylated. To verify this, hydroxyurea-arrested cells were released by refeeding with complete media lacking drug, resulting in cell populations containing hypophosphorylated (at 24 h after release) and hyperphosphorylated pRb (at 5 h after release) as determined by Western blot (Fig. 3D). Rak bound to both phosphorylated and hypophosphorylated pRb (Fig. 3D), suggesting a model whereby the association between Rak and pRb is limited by the expression of Rak during the cell cycle and not by the phosphorylation of pRb.

Because Rak has elevated expression in G₁ and S-arrested cells and is an active kinase when bound to pRb, it is conceivable that constitutive overexpression of Rak might be inhibitory to cell growth. To test this, NIH 3T3 cells were transfected with Rak, grown in selective media, and monitored for colony formation efficiency. In three sep-

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*4 R. J. Craven and E. T. Liu, unpublished data.*
RAK NUCLEAR TYROSINE KINASE ASSOCIATES WITH pRb

Table 1 Growth reduction in cells transfected with Rak

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<th>Experiment No.</th>
<th>pcDNA3</th>
<th>pc3-Rak</th>
<th>Average</th>
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<tr>
<td>I</td>
<td>131</td>
<td>51</td>
<td>139 (100%)</td>
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<tr>
<td>II</td>
<td>173</td>
<td>81</td>
<td>149 (86%)</td>
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<tr>
<td>III</td>
<td>112</td>
<td>48</td>
<td>129 (70%)</td>
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In conclusion, we have shown that Rak associates with the tumor suppressor protein pRb and that overexpression of Rak causes a reduction in cell growth. Although it is possible that this activity reflects a toxicity of Rak expression rather than growth inhibition, the elevated expression of Rak in serum-starved cells suggests that Rak plays some specific role in growth arrest. Indeed, binding of Rak to pRb does not inactivate Rak kinase activity, so it is possible that the Rak-Rb interaction serves to bring Rak into the proximity of substrates, and that phosphorylation of substrate proteins by Rak may play some role in growth regulation. Rak-Rb interaction may contribute to epithelial carcinogenesis.

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