Ret-mediated Mitogenesis Requires Src Kinase Activity

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ABSTRACT

The proto-oncogene RET encodes a transmembrane growth neurotrophic receptor with tyrosine kinase (TK) activity. RET mutations are associated with several human neoplastic and nonneoplastic diseases, including thyroid papillary carcinoma, multiple endocrine neoplasia type 2 syndromes, and Hirschsprung’s disease. Activation of receptor TKs results in the binding and activation of downstream signaling proteins, among which are nonreceptor TKs of the Src family. To test the involvement of c-Src in Ret-mediated signaling, we measured the levels of c-Src activity in NIH3T3 cells coexpressing Ret and the accessory GFR α-1 receptor or an epidermal growth factor receptor/Ret chimERIC receptor when the cells were stimulated by glial cell line-derived neurotrophic factor or epidermal growth factor, respectively. Ret stimulation resulted in the activation of c-Src. We also measured the levels of Src kinase activity in cell lines expressing isoforms of the Ret receptor activated by different mutations. These cells showed higher Src kinase activity than the normal counterpart. Furthermore, we show that Ret is able to associate with the SH2 domain of Src in a phosphotyrosine-dependent fashion. Microinjection of a kinase inactive mutant of c-Src blocked Ret-mediated mitogenic effect. These experiments demonstrate that activated Ret is able to bind and stimulate c-Src kinase and that Src activation is essential for the mitogenic activity of Ret.

INTRODUCTION

The RET proto-oncogene encodes a TK1 transmembrane receptor, Ret (1). Inactivating mutations of RET are responsible for Hirschsprung’s disease (2–5). It has been demonstrated that transforming growth factor-β-related neurotrophic factors, including GDNF and neurturin, stimulate TK activity of Ret. A family of GPI-linked proteins, including GFR α-1, GFR α-2, GFR α-3, and GFR α-4, mediates high-affinity ligand binding and ligand-induced Ret phosphorylation (6–9). Oncogenic activation of RET can occur through multiple mechanisms. Gene rearrangements leading to the fusion of its terminal region with the 5′ terminal region of other genes, generate the RET/PTC oncoproteins in human thyroid papillary carcinomas (10). Specific point mutations of RET are responsible for the MEN syndrome types 2A and 2B and familial medullary thyroid carcinoma. MEN2A mutations, which involve cysteine residues of the extracellular domain of the protein, induce ligand-independent dimerization and constitutive activation of the Ret receptor. MEN2B mutation is a Met-918→Thr substitution, which likely activates Ret through a conformational change of its catalytic core and through altering its substrate specificity. Other mutations in the TK domain of Ret have been found that are responsible for a small number of MEN2B and familial medullary thyroid carcinoma cases (11). Activated RET isoforms can transform NIH3T3 and thyroid cells and induce the appearance of a neuronal-like phenotype in neuroectodermal cells (12–17).

Activation of growth factor receptors leads to the binding and activation of several molecules that are involved in the signal transduction cascade (18). Among the effector proteins that bind directly or indirectly to Ret are PLC-γ (19, 20), the Shc and Grb2 adaptor (21, 22), the Grb7 and Grb10 proteins (23, 24), and the Enigma protein (25, 26). Ret is able to activate the ras/mitogen-activated protein kinase pathway by phosphorylation and recruitment of the adaptors Grb2 and Shc to the plasma membrane (21, 22). Furthermore, activated Ret stimulates Jun kinases in certain cell lines (27).

The Src cytoplasmic TKs can bind to and are activated by membrane receptors. Most of the members of this family are expressed in a cell type-restricted fashion, whereas three of them (Src, Fyn, and Yes) are ubiquitous (28). Several studies show that Src family kinases are implicated in signal transduction mediated by transmembrane receptors, which are devoid of catalytic activity, such as T-cell receptor and FcgRII receptor (29). In addition, receptors with intrinsic TK activity (EGFR, c-erbB2/Neu, PDGF receptor, and colony-stimulating factor-1 receptor) have been shown to associate and/or activate Src, Fyn, and Yes through the SH2 domains of Src, Fyn, and Yes (30–33). Activation of Src kinase is required for PDGF- and EGF-mediated signaling (34–36).

Here, we show that different cell lines expressing oncogenic forms of Ret possess high Src kinase activity levels. Ligand stimulation of Ret resulted in activation of Src. Src kinase was found to coprecipitate in vivo with activated Ret. The binding between the two molecules is mediated by Src SH2 domain because an isolated Src SH2 was able to bind to Ret in vitro. Finally, a dominant negative form of c-Src was able to inhibit the mitogenic activity of Ret when it was microinjected in NIH3T3 and PCCl3 cells, indicating that Src (or Src-related) kinase activity is required for Ret-mediated mitogenic signaling.

MATERIALS AND METHODS

Cell Lines and Plasmids. NIH3T3, NIH RET7/MEN2A, NIH RET7/MEN2B (13), NIH RET/PTC1 ((37), EGFR/RET (19), NIH RET (38), and v-Src-expressing fibroblasts (39) have been described previously. They were grown in DMEM (Life Technologies, Inc.) supplemented with 10% calf serum. PCCl3 and PCCl3 RET/PTC1 (12) were maintained in Coon’s modified F12 medium supplemented with 5% calf serum (Life Technologies, Inc.) and six growth factors (thyrotropin, hydrocortisone, insulin, transferrin, somatostatin, and glycyel-histidyl-lysine; Sigma Chemical Co.). The long terminal repeat RET/MEN2A D100 was generated by recombinant PCR as described (5). A forward primer mapping on the RET sequence upstream the BglII site (position: 2690–2710) was used (5′-CCATGGGCGACCTCATCT-3′). The down-
stream primer was designed to delete the COOH-terminal residues of Ret, including Tyr-1015, Tyr-1029, and Tyr-1062 (5′-AGCCGTCTAGCTCTCTGCTCTCTAA-3′) and contained a MluI site for the cloning in the long terminal repeat vector. The cell line NIH3T3 RET/MEN2A D100 was obtained by calcium phosphate coprecipitation transfection technique. Transfected cells were subjected to selection by the addition of mycophenolic acid, as described elsewhere (19). The levels of Ret/MEN2A D100 protein in the mass population were similar to those of the wild-type Ret/MEN2A-expressing cells (data not shown). The cell lines F212 T1 and F212 T3 were obtained from two mammary tumors arising in RET/PTC1 transgenic mice (40). They were cultivated in Coon’s modified F12 medium supplemented with 5% calf serum (Life Technologies, Inc.). The HC11 is a normal mouse mammary epithelial cell line (41). The plasmids pSG5 Src and pSG5 Src K- were kind gifts of S. Courtneidge, Redwood City, CA.

**Src in Vitro Kinase Assay.** Confluent cells were serum starved for 12–24 h and, when indicated, stimulated with 100 ng of EGF or GDNF as described (19). After two washes with cold PBS, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) NP40, 1 mM EDTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml each aprotinin and leupeptin. Lysates were centrifuged at 10,000 × g for 30 min and immunoprecipitated by incubating 1 mg of total proteins with anti-Src (Ab-1; Oncogene Science) antibodies for 60 min at 4°C. Immunocomplexes were recovered by incubation with protein G-Sepharose beads (GammaBind G Sepharose; Pharmacia) on a rotating platform at 4°C for 60 min. After three washes with lysis buffer, the immunoprecipitates were washed with kinase buffer [20 mM Tris-HCl (pH 7.0)-5 mM MgCl2] and resuspended in 30 μl of kinase buffer, 10 μCi of [γ-32P]ATP (>10,000 Ci/mmol, Amersham), and 10 μl cold ATP. After 30 min of incubation at room temperature, the beads were washed twice with lysis buffer, and the reaction was terminated by adding an equal volume of SDS-gel loading buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 0.7 M 2-mercaptoethanol, and 0.25% bromphenol blue]. When indicated, 5 μg of acid-denatured enolase were added to the reaction (Boehringer Mannheim Biochemicals). The samples were electrophoresed on SDS-10% polyacrylamide gel. After the run, gels were incubated three times for 30 min in a fixing solution (20% methanol-10% acetic acid), dried, and processed for autoradiography with phosphor screens.

**Western Blot Analysis.** Total cellular proteins were quantitated by a modified Bradford assay (Bio-Rad). To evaluate Src expression, equal amounts of proteins were immunoprecipitated with the anti-Src antibodies (Ab-1; Oncogene Science). Antibodies purchased from Santa Cruz were used to evaluate p27kip1 (C-19), cyclin E (M-20), and D3 (C-16) expression.

**Complex Formation in Vivo and in Vitro.** In vivo association experiments were conducted by immunoprecipitating 1 mg of total cell lysate with the anti-Src antibody. The immunocomplexes were electrophoresed on a 7.5% SDS acrylamide gel and transferred onto a PVDF membrane (Millipore). After a 60-min incubation in 1% nonfat dry milk blocker (Bio-Rad), the membrane was probed with primary antibodies. Detection of the immunocomplexes was obtained by an enhanced chemiluminescence system (ECL; Amersham), following manufacturer’s instructions.

**Microinjection of Cells.** NIH EGFR/RET cells were seeded on glass coverslips in DMEM containing 10% calf serum and grown to 60% confluence. The medium was then replaced with DMEM supplemented with 0.5% calf serum, and the cells were incubated for another 30–48 h. The plasmids were injected into cell nuclei at a concentration of 25–50 ng/ml using an automated microinjection system (AIS, Zeiss). Six h later, cells were stimulated with EGF (Upstate Biotechnology) at 300 ng/ml to induce DNA synthesis. BrdUrd (Sigma) was added to the culture medium to a final concentration of 100 μM, and the labeling procedure was carried out for 18–20 h. Cells were then fixed for immunostaining. Codetection of Src and BrdUrd in the cells was essentially performed as described previously (42, 43). In brief, cells were fixed in parafomaldehyde, permeabilized with 0.1% Triton X-100, and incubated with a primary rabbit antiserum directed against c-Src. After several washes in PBS, rhodamine-conjugated anti-rabbit IgG was added to identify the Src expressing cells. A fluorescein-conjugated anti-BrdUrd mouse monoclonal antibody (Boehringer Mannheim Biochemicals) was used to detect the fraction of cells that were in S phase. All coverslips were finally washed in PBS containing Hoechst 33258 (final concentration: 1 mg/ml, Sigma), rinsed in water, and mounted in Moviol on glass slides. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2; Zeiss). BrdUrd incorporation was measured in injected and un.injected cells, stimulated or not with EGF. In each experiment, at least 60 Src K+ cells were counted and compared to 400 nonmicroinjected cells from the same coverslip. The results shown are the means of three independent experiments. Variations were <25% of the mean. Microinjection of RET/PTC1-expressing NIH3T3 and PCC1 RET/PTC1 was performed as described above with a few modifications. SrcK- and SrcK+ plasmids were microinjected in proliferating cells maintained in DMEM or in Coon’s modified F12 medium, respectively, supplemented with 5% calf serum (Life Technologies, Inc.). The day after, BrdUrd was added for 1 h. The cells were then fixed, permeablized, and stained both for Src expression and BrdUrd incorporation as described above. BrdUrd incorporation was evaluated in the injected and un injected cells. In each experiment, at least 100 Src K+ cells were counted and compared to 400 nonmicroinjected control cells from the same coverslip. The results shown are the mean of two independent experiments.

**RESULTS**

**Activation of Src in Cell Lines Expressing Inducible or Oncogenic RET Forms.** The GDNF is a functional ligand for Ret. GDNF binds to GFR α-1, a GPI-linked cell surface molecule, which, in turn, activates Ret. We have shown that NIH3T3 coexpressing Ret and GFR α-1 undergo DNA replication upon GDNF triggering (38). To evaluate whether stimulation of Ret with its physiological ligand was able to induce Src activation, Src kinase was immunoprecipitated using specific antibodies from unstimulated or GDNF-treated NIH-Ret cells and an in vitro kinase assay was performed. As shown in Fig. 1A, GDNF triggering resulted in Src activation. We have previously shown that an EGF/Ret chimeric receptor, when exogenously expressed in NIH3T3 cells, is able to transduce mitogenic and transforming signals upon EGF triggering (19). To determine whether c-Src was involved in Ret signaling, Src kinase was immunoprecipitated using specific antibodies from quiescent wild-type and EGF/Ret-expressing NIH3T3 cells (NIH EGFR/Ret), stimulated or not with EGF. Both Src autophosphorylation and its ability to phosphorylate an exogenous substrate, enolase, were measured. As shown in Fig. 1B, EGF stimulation was able to activate Src in NIH EGFR/Ret but not in the parental cells, which express negligible levels of endogenous EGFR (19). Immunoprecipitation of the same lysates with a preimmune serum gave negative results. Subsequently, time course evaluation of Ret and Src activation was performed in NIH EGFR/Ret cells (Fig. 1C). Cells were treated with EGF and harvested at different times during stimulation. Src (Fig. 1C, top) and Ret (Fig. 1C, bottom) activation were measured. Ret tyrosine phosphorylation reached a peak at 5 min and started decreasing at 15 min when Src reached its maximal activation. Furthermore, we studied molecular markers of S-phase entry upon EGF triggering of NIH EGFR/Ret cells. In particular, we quantitated the amounts of a cyclin-dependent kinase inhibitor, p27kip1, cyclin D3, and cyclin E. As shown in Fig. 1D, p27kip1 levels were high in quiescent cells, decreased after 6 h of stimulation with EGF, and remained low thereafter. Cyclin D3 accumulation peaked at 90 min, remained stable until 12 h, and then
ACTIVATION OF Src KINASE BY RET

Fig. 1. A, Src activation by GDNF. Serum-starved NIH3T3 cells coexpressing wild-type Ret and GFR α-1 were (NIH RET +) or were not (NIH RET –) stimulated with GDNF for 10 min, and Src kinase activity was measured. GDNF triggering of parental NIH3T3 cells gave negative results (data not shown). B, Src kinase assay in parental NIH3T3 and in NIH EGFR/Ret cells. Serum-starved cells were stimulated with EGF for 10 min, lysates were immunoprecipitated with anti-Src antibodies, and kinase assays were performed. The Src kinase (top arrow) and the substrate enolase (bottom arrow) are indicated. These results are typical and representative of at least three independent experiments. C, time course of Ret-mediated Src activation in NIH EGFR/Ret cells. Serum-starved cells were stimulated with EGF and harvested at the indicated time points. Top, Src kinase assays were performed as described above. Laser densitometry was used to quantitate Src autokinase activity after stimulation. Columns, relative inductions, with the levels of Src activation calculated as fold increases above the activity of unstimulated NIH EGFR/RET cells (×1), means of three experiments; bars, SD. Bottom, tyrosine phosphorylation of EGFR/Ret induced by EGF is shown. Five hundred μg of total lysates were immunoprecipitated with anti-Ret antibodies. After protein transfer, filters were immunoblotted with anti-pTyr antibodies. Equal amounts of Ret are present in all of the lanes (data not shown). D, EGF stimulation of NIH EGFR/RET cells induces molecular markers of S-phase entry. Serum-starved cells were stimulated with EGF and assayed at the indicated times for cyclin D3, cyclin E, and p27kip1 expression.

decreased. Cyclin E levels peaked at 12 h and were low after 24 h. These kinetics indicated that Ret-mediated activation of Src kinase precedes the early G1 events required for cell cycle progression.

To test whether different oncogenic forms of RET activate Src kinase, endogenous c-Src activity was measured in immunocomplex kinase assays in serum-starved NIH3T3 cells expressing RET/MEN2A(Cys634Tyr), RET/MEN2B(Met918Thr) (13), and RET/PTC1(H4-Ret) (37). Positive control fibroblasts expressing v-Src were used. Autophosphorylation of c-Src was higher in NIH3T3 cells expressing the activated Ret isoforms than in parental cells (×10-fold; Fig. 2A). The increase in Src autophosphorylation activity was paralleled by an analogous increase in the phosphorylation of the exogenous substrate enolase (data not shown). As shown by the immunoblot reported in Fig. 2A, bottom, this effect was not due to increased expression levels of the endogenous Src kinase, which were, indeed, comparable in all of the cell lines tested. We have reported previously that the exogenous expression of RET/PTC1 in a rat thyroid cell line, PCC13, has a mitogenic effect (12). To evaluate whether Src activation occurred also in RET/PTC1 transformed PCC13 (PCC13 RET/PTC1), Src kinase activity was measured. Src autophosphorylation was higher in PCC13 RET/PTC1 cells than in untransfected parental cells (Fig. 2B, top). The expression levels of endogenous Src were comparable in the cell lines tested, as shown by the immunoblot reported in Fig. 2B, bottom. PCC13 cells expressing a constitutively active v-Src oncogene were used as a positive control (39). Transgenic mice expressing the RET/PTC1 oncogene under the transcriptional control of the H4 gene promoter were generated in our laboratory. Despite the fact that the RET/PTC1 transgene was expressed in several tissues, the animals developed a restricted pattern of neoplasias: mammary adenocarcinomas and adnexial tumors (40). Two cell lines, F212T1 and F212T23, derived from two independent mammary adenocarcinomas that occurred in RET/PTC1 transgenics, were used to investigate if RET/PTC1 tumor induction in vivo correlated with an increase in endogenous c-Src activity. Levels of c-Src activity in the F212T1 and F212T23 cells were evaluated and compared to those of normal mammary cells and mammary tissue from nontransgenic littermates. As shown in Fig. 2C, c-Src autophosphorylation was higher in the tumor-derived cell lines than in normal mammary cells (HC11 cells) and in normal mammary tissue, and this event was not due to increased expression levels of endogenous c-Src, as demonstrated by Western blot analysis.

Association between c-Src and Activated Ret. Some TK receptors have been shown to directly interact with and activate members of the Src family (28). To test if this was also the case of Ret, equal amounts of proteins were immunoprecipitated with anti-c-Src antibodies from quiescent NIH EGFR/Ret cells stimulated or not with EGF. Immunocomplexes were then separated on a denaturing polyacrylamide gel, transferred to a PVDF membrane, and probed with antibodies directed against the COOH-terminal portion of the Ret protein (19). Association between c-Src and Ret was readily detectable (Fig. 3A). A preimmune serum was not able to coprecipitate the Ret protein from stimulated cells (Fig. 3A). Src associated only with tyrosine-phosphorylated Ret because unstimulated Ret was not coprecipitated by the anti-Src antibody (Fig. 3A). The requirement of Ret phosphorylation for association with Src, suggested that this interaction was mediated by the Src SH2 domain. To test this hypothesis, we used a GST-SH2-Src (GST-SH2-Src) recombinant protein. Fig. 3B shows that the GST-SH2-Src, but not the GST alone, was able to
The Ret COOH-terminal tail contains three of these autophosphorylation sites: Tyr-1062, Tyr-1029, and Tyr-1015. Tyr-1062 is the binding site for Shc (44–46), whereas Tyr-1015 is the docking site for PLC-γ (14, 20). On the basis of the optimal consensus sequence for Src SH2 binding (47), Tyr-1015 (YLDL) and, less likely, Tyr-1029 (YDDG) could represent candidates for Src binding. We then investigated whether these tyrosines were involved in Src binding and activation by using the mutant Ret/MEN2A Δ100. In this mutant, the last three tyrosine residues of Ret/MEN2A have been deleted. As expected, this mutant was unable to bind to Shc and PLC-γ (data not shown). Ret/MEN2A Δ100 was still able to coimmunoprecipitate Src (Fig. 4A), bind to GST-SH2-Src (Fig. 4B), and activate Src kinase (Fig. 4C).

**Fig. 2.** Src kinase activity in NIH3T3 cells expressing different activated forms of RET. Cell lysates from NIH3T3 cells and NIH3T3 cells expressing v-Src or activated RET oncogenes were prepared, and Src kinase was immunoprecipitated. Half of the immunoprecipitates were subjected to kinase assays (top), and half were subjected to Western blot analysis (bottom), as indicated (arrows). A, Src kinase activity in thyroid cells. Parental PCC1 cells were compared to RET/PTC1 and v-Src expressing cells for Src kinase activity. Src kinase assays and immunoblots were performed as described above. B, Src kinase activity in RET/PTC1-induced mammary tumors. Src kinase activity was evaluated in cell lines derived from two independent mammary adenocarcinomas explanted from RET/PTC1 transgenic animals, F212T1 and F212T23, in a normal mouse mammary epithelial cell line (HC11) and in a normal mouse mammary gland (N), derived from three independent nontransgenic litters (one is shown; the other two gave consistent results). Src kinase assays and immunoblots were performed as described above. Arrows, positions of Src kinase detected by either kinase assay (top arrow) or Western blot analysis (bottom arrow).

**Fig. 3.** A, in vivo association of activated Ret proteins and Src kinase. The cells were (NIH EGFR/RET +) or were not (NIH EGFR/RET −) stimulated with EGF and harvested. Their lysates were immunoprecipitated with a preimmune rabbit serum (Lane 3, left to right) or with anti-Src antibody (Lanes 1 and 2) and subjected to immunoblotting with anti-Ret antibody. Arrow, the migration of the EGFR/Ret protein. B, in vitro binding of activated Ret proteins to the SH2 domain of Src. Lysates from unstimulated (NIH EGFR/RET −) and EGF-stimulated (NIH EGFR/RET +) NIH EGFR/Ret cells were incubated either with GST-SH2-Src (Lanes 1 and 2, left to right) or with GST proteins immobilized on glutathione-agarose beads (Lanes 3 and 4). Proteins were separated on a SDS–7.5% polyacrylamide gel under reducing conditions and subjected to immunoblotting with anti-Ret antibodies. These results are typical and representative of at least three independent experiments.

**Fig. 4.** A, in vivo binding of Ret/MEN2A Δ100 mutant to Src kinase. NIH cells expressing RET/MEN2A and the mutant RET/MEN2A Δ100 were harvested. Proteins were immunoprecipitated with anti-Src antibody and immunoblotted with anti-Ret antibodies. B, in vitro binding of RET/MEN2A Δ100 mutant to Src kinase. Cell lysates of NIH3T3 cells expressing RET/MEN2A and RET/MEN2A Δ100 were incubated with purified GST-SH2-Src protein. Bound proteins were eluted with SDS sample buffer, subjected to electrophoresis in a SDS 7.5% polyacrylamide gel, and immunoblotted with anti-Ret antibody. Arrow, the migration of the receptor. C, Src kinase activity in NIH3T3 cells expressing the RET/MEN2A Δ100 mutant. Cells expressing RET/MEN2A and RET/MEN2A Δ100 were harvested, and cellular proteins were immunoprecipitated with the anti-Src antibody. The immunoprecipitates were subjected to either kinase assays (top) or Western blot analysis (bottom) as described in “Materials and Methods.”

**Dominant Negative Src Inhibits Ret-mediated S-Phase Entry.** To investigate whether c-Src activity was required for Ret-mediated mitogenesis, we applied a microinjection technique. Serum-starved NIH EGFR/Ret cells, when stimulated with EGF, enter S phase, as measured by thymidine incorporation (19). Serum-deprived NIH EGFR/Ret cells were stimulated or not with EGF in the presence of BrdUrd, and S-phase entry was measured by counting cells stained with anti-BrdUrd-specific antibodies. Arrested NIH EGFR/Ret cells showed only a very low level of BrdUrd incorporation. Upon stimu-
Fig. 5. A, quantitation of BrdUrd incorporation in NIH EGFR/Ret cells injected with Src K− and Src K+. Quiescent NIH EGFR/Ret cells seeded on coverslips were microinjected with an expression plasmid encoding Src K−. Six h later, cells were incubated in media containing EGF and BrdUrd. After 18–20 h, they were fixed, stained, and processed for immunofluorescence as described. As shown, a decreased fraction of the cells injected with Src K− but not with Src K+ incorporated BrdUrd after stimulation with EGF. In each experiment, at least 60 Src K− cells were counted and compared to at least 400 nonmicroinjected cells from the same coverslip. Columns, mean results of three experiments; bars, SD, B, a representative experiment is shown. Injected cells are visualized with anti-Src antibody (Src) and rhodamine-conjugated secondary antibodies. BrdUrd incorporation was visualized with a fluorescein-conjugated anti-BrdUrd monoclonal antibody (BrdU). Cell nuclei are stained with Hoechst dye (Hoechst).

DISCUSSION

Here, we showed that Ret triggering is able to stimulate the activity of pp60 c-Src. A 2–3-fold increase of c-Src activity following Ret triggering was reproducibly measured; this is consistent with the increases observed for activated PDGFR, colony-stimulating factor-1 receptor, and erbB2/Neu (30–33). We also show that, in NIH3T3, thyroid and mammary cell lines stably expressing activated forms of RET, levels of c-Src kinase activity are elevated in comparison to wild-type cells. The induction of c-Src activity correlated with the ability of Src to interact with tyrosine-phosphorylated Ret, as shown by coinmunoprecipitation experiments. Activated Ret was also able to bind c-Src in vitro, and the SH2 domain of c-Src alone was sufficient for this association.

Time-course experiments indicated that Src activation follows the peak of Ret activation. Moreover, Ret-dependent Src activation occurs before the first events of G0–G1 transition. This is consistent with the possibility that Src activation may be necessary for the induction of Ret-mediated cell cycle progression. Indeed, in PDGF-stimulated NIH3T3 fibroblasts, the block of Src activation inhibits cyclin E accumulation.

We used NIH3T3 and PCCl3 cells expressing the Ret/PTC1 oncoprotein. These experiments were carried on asynchronously growing cells: cells were microinjected with either Src K− or Src K+ plasmids, and BrdUrd incorporation was measured. As shown in Fig. 6, 50% of the cells entered S phase in normal growing conditions. Microinjection of Src K− but not Src K+ strongly inhibited BrdUrd incorporation of both cell types. Microinjection of Src K− in asynchronously growing parental NIH3T3 and PCCl3 did not affect the fraction of cells that entered S phase.

Fig. 6. Quantitation of BrdUrd incorporation in NIH RET/PTC1 and PC RET/PTC1 cells injected with Src K− and Src K+. Asynchronously growing cells were injected with Src K− and Src K+ plasmids and processed for immunofluorescence as described. In these conditions, Src K− but not Src K+ inhibited BrdUrd incorporation.

Accleration of EGF, almost 50% of the cells entered S phase (Fig. 5A). Arrested NIH EGFR/Ret cells were microinjected with a dominant negative form of Src (Src K−; Ref. (43)). After 6 h, they were stimulated with EGF for 18 h. The average results of three independent experiments, in which at least 60 Src K− microinjected cells were counted, are shown in Fig. 5A. The entrance into S phase was inhibited by Src K−; only 8% of injected cells incorporated BrdUrd. This effect was specific because serum-induced BrdUrd incorporation was not affected by the expression of Src K− (data not shown). Moreover, microinjection of a plasmid expressing wild-type Src (Src K+) did not affect the mitogenic response to EGF. An example of these assays is reported in Fig. 5B. Cells expressing Src K− were identified by immunostaining with anti-Src (Src). These cells did not incorporate BrdUrd, whereas surrounding cells did, as shown by the staining with anti-BrdUrd antibody (Fig. 4B, BrdU). These experiments strongly support the concept that Src kinase activity is required for Ret-induced mitogenic response.

To investigate the involvement of Src in mitogenic signaling mediated by oncogenic Ret proteins, we used NIH3T3 and PCCl3 cells expressing the Ret/PTC1 oncoprotein. These experiments were carried on asynchronously growing cells: cells were microinjected with either Src K− or Src K+ plasmids, and BrdUrd incorporation was measured. As shown in Fig. 6. 50% of the cells entered S phase in normal growing conditions. Microinjection of Src K− but not Src K+ strongly inhibited BrdUrd incorporation of both cell types. Microinjection of Src K− in asynchronously growing parental NIH3T3 and PCCl3 did not affect the fraction of cells that entered S phase.

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in EGF-stimulated NIH EGFR/Ret cells and of Ret/PTC1 in NIH3T3 and PCC13 cells.

The SrcK– mutant used in our study is probably also active on other members of Src kinase family because its SH2 domain could theoretically compete with the binding to the receptor mediated by SH2 domains of closely related Src-like kinases. For this reason, we cannot exclude that the activity of other Src-like kinases may be required for Ret-mediated biological activity. In support of this hypothesis, we have observed that the EGFR/Ret chimera is also an efficient inducer of the c-Fyn kinase. Similarly, in addition to Src, other Src-like kinases are required for Ret-mediated biological activity. In support of this hypothesis, we cannot exclude that the activity of other Src-like kinases may be required for Ret-mediated biological activity. In support of this hypothesis, we have observed that the EGFR/Ret chimera is also an efficient inducer of the c-Fyn kinase. Similarly, in addition to Src, other Src-like kinases are required for Ret-mediated biological activity. In support of this hypothesis, we cannot exclude that the activity of other Src-like kinases may be required for Ret-mediated biological activity.

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