Agonist-independent Activation of Src Tyrosine Kinase by a Cholecystokinin-2 (CCK₂) Receptor Splice Variant*

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Src activity is activated in a majority of colon and pancreatic cancers and is associated with late stage aggressive cancers. However, the mechanisms leading to its increased activity remain largely undefined. Agonist binding to the cholecystokinin-2 (CCK₂) receptor (CCK₂R), a G-protein-coupled receptor, increases Src activity in a variety of normal and neoplastic cell lines. Recently, we and others (Hellmich, M. R., Rui, X. L., Hellmich, H. L., Fleming, R. Y., Evers, B. M., and Townsend, C. M., Jr. (2000) J. Biol. Chem. 275, 32122–32128; Ding, W. Q., Kuntz, S. M., and Miller, L. J. (2002) Cancer Res. 62, 947–952; Smith, J. P., Verderame, M. F., McLaughlin, P., Martenis, M., Ballard, E., and Zagon, I. S. (2002) Int. J. Mol. Med. 10, 689–694) have identified a splice variant of CCK₂R, called CCK₂i4svR, that is expressed in a human colon cancer cell line but not by cells of the adjacent nonmalignant tissue. Compared with CCK₂R, CCK₂i4svR contains an additional 69 amino acids within its third intracellular loop (3il) domain. Because CCK₂i4svR is the only splice variant expressed in some human colon and pancreatic cancers, we questioned whether CCK₂i4svR could regulate Src activity. Stably transfected HEK293 cells were used because, unlike many cancer-derived cells, they have a low level of basal Src activity. We report that, in contrast to CCK₂R, CCK₂i4svR activates Src kinase in the absence of agonist stimulation. In vitro kinase assay of immunoprecipitated receptor protein showed a 6–8-fold increase in Src kinase activity associated with CCK₂i4svR compared with CCK₂R. Expression of the 3il domain of the CCK₂i4svR alone was sufficient to partially activate Src kinase. Together, these data support the hypothesis that the increased Src activity observed in some pancreatic and colorectal cancers is due, in part, to the co-expression of CCK₂i4svR.

Increased activity of the non-receptor tyrosine kinase, Src, has been implicated in the development and progression of a variety of human cancers including breast, brain, pancreas, and colon, where it is associated with increased growth, invasiveness, angiogenesis, and metastasis (1, 2). Src activity is increased 5–8-fold in a majority of human colon cancers (3, 4). Changes in the level of Src activity correlate with the grade of malignancy. Incremental increases in Src activity are observed in premalignant ulcerative colitis, benign polyposis, invasive cancer, and liver metastasis (5–7). Reduction of Src expression with antisense oligonucleotides inhibits the growth of subcutaneous HT29 colon cancer cell tumors in nude mice (8), while forced overexpression of Src in KM12C colon cancer cells stimulates tumor growth (9). Immunohistochemical analysis of 13 human pancreatic adenocarcinomas and 17 cancer-derived cell lines revealed elevated levels of Src protein in 100% of the tumor samples and 80% of the cell lines when compared with normal pancreatic cells (10). Furthermore, 12 of 17 (71%) pancreatic cancer cell lines also exhibited increased Src kinase activity. The mechanism(s) responsible for the increased Src activity in human colorectal and pancreatic malignancies remain largely undefined.

The gastrointestinal peptide hormone gastrin-1–17 (G17)1 stimulates Src activation in dog colon cells (15), rat intestinal epithelial cells (11, 12), and a rat pancreatic cancer cell line, AR4–2J (13). G17 also promotes tumor cell proliferation, motility, and invasion in a subset of stomach, pancreatic, and colon cancer cells in vitro and in vivo (14–16). The cholecystokinin-2 (CCK₂) receptor (CCK₂R), a member of the G-protein-coupled receptor (GPCR) superfamily, mediates the biological actions of G17, including the regulation of Src kinase activity. Recently, our laboratory has identified and cloned a splice variant of CCK₂R, called CCK₂i4svR (17). Generated by intron 4 retention, the mRNA for CCK₂i4svR encodes a receptor protein that contains an additional 69 amino acid residues in its third intracellular loop (3il) domain. Interestingly, this longer protein is the only detectable CCK₂R splice variant expressed by some human colorectal cancers (17) and pancreatic cancer cell lines (18, 19), and, in contrast, it is not detectable in normal cells from the same tissues. The co-expression of elevated Src activity and CCK₂i4svR in colon and pancreatic cancers, along with the demonstrated ability of the other variant, CCK₂R, to activate Src in intestinal and pancreatic cell lines from dog and rodents raised the question of whether CCK₂i4svR expression was also linked to Src kinase activation.

We report that, in contrast to CCK₂R, CCK₂i4svR activated Src kinase in an agonist-independent manner. In vitro kinase assay of immunoprecipitated receptor protein showed a 6–8-fold increase in Src kinase activity associated with CCK₂i4svR compared with CCK₂R in the absence of agonist stimulation. Furthermore, we show that expression of the 3il domain of the CCK₂i4svR alone was sufficient to partially activate Src kinase. The agonist-independent activation of Src kinase by CCK₂i4svR

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1 The abbreviations used are: G17, gastrointestinal peptide hormone gastrin-1–17; CCK₂R, cholecystokinin-2; CCK₂, CCK₂R, CCK₂/gastrin receptor; GPCR, G-protein-coupled receptor; MOPS, 4-morpholinepropanesulfonic acid; FAK, focal adhesion kinase; PP2, 4-amino-5-(4-chlorophenyl)-7- (trans-4-chloro-pyrrol-3,4-difluoropyrimidine; SH3, Src homology 3; GFP, green fluorescent protein; EGFP, enhanced GFP.

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suggests a possible mechanism for the increased Src activity observed in colon and pancreatic cancers.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Six different expression constructs were used for these studies. Receptor constructs containing a C-terminal enhanced green fluorescent protein (EGFP) tag, designated CCK$_{2a}^{-}$R-EGFP and CCK$_{2i4sv}^{-}$R-EGFP, were constructed by subcloning the full open reading frames of CCK$_{2a}^{-}$R and CCK$_{2i4sv}^{-}$R, in frame, to the N terminus of EGFP using the pEGFP-N1 vector (BD Biosciences). For receptors containing an N-terminal FLAG-epitope tag, designated FLAG-CCK$_{2a}^{-}$R and FLAG-CCK$_{2i4sv}^{-}$R, the FLAG tag and restriction endonuclease sites (EcoRI and NdeI) were added to the full-length receptor cDNAs by PCR. The PCR products were then subcloned into the EcoRI/NdeI sites of pCDNA3.1 (Invitrogen). The third intracellular loop (III) constructs, designated EGFP-III, and EGFP-III, were generated by PCR and subcloned in frame to the C-terminal end of EGFP using the pEGFP-C1 vector (BD Biosciences). The nucleotide sequences of all plasmid constructs were confirmed by automated sequencing in the University of Texas Medical Branch Recombinant DNA Core Facility (Galveston, TX).

**Transfected Cell Lines**—HEK293 cell cultures were routinely cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified 95% air, 5% CO$_2$ atmosphere. Stably transfected HEK293 cells were used in these studies because, unlike many colonic and pancreatic cancer-derived cell lines, they have a low level of basal Src activity. Cells were transfected with either receptor construct or empty control vectors using LipofectAMINE Plus Reagent (Life Technologies, Inc., Gaithersburg, MD) and initially selected using G418 (600 µg/ml). Cells expressing the EGFP-tagged receptors or control EGFP vectors were selected using a FACS-Vantage cell sorter in the University of Texas Medical Branch Flow Cytometry Core Laboratory (Galveston, TX). Clonal cell populations expressing the FLAG-tagged receptors were selected using an agonist-stimulated Ca$^{2+}$ mobilization assay as described previously (17). The level of receptor expression for each clonal line was quantified using $^{125}$I-labeled agonist binding (17). Receptor expression levels between clones ranged from ~0.43 to 1.8 pmol of receptor/mg of membrane protein. All experiments were repeated three times with at least two independently isolated subclones for each receptor construct.

**Immunoprecipitation and Western Blotting**—Immunoprecipitations were performed using a modification of the procedure described previously (20). Cells were solubilized in lysis buffer (LB) (150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EGTA, 5% glycerol, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) containing a protease inhibitor mixture (Complete, Roche Diagnostics) at 4°C for 10 min. After centrifugation, supernatants were collected and the protein concentrations were determined using Bio-Rad DC protein assay kit (Bio-Rad). Epitope-tagged receptor protein was immunoprecipitated from 1 to 2 mg of total cell lysate using either anti-FLAG antibody (Rockland, Gilbertsville, PA) or anti-FLAG antibody (Sigma) at 4°C overnight. The immunoprecipitated proteins were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with primary antibodies at the dilutions indicated in the figure legends. Immunoreactive proteins were visualized using ECL™ reagent (Amer sham Biosciences).

**In Vitro Src Kinase Assay**—For Src kinase assays, A/G-Sepharose beads containing the immunoprecipitated proteins were washed three times with LB and once with kinase buffer (20 mM MOPS, pH 7.2, 25 mM glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol). Reactions were carried out in 40 µl of kinase buffer supplemented with 100 µCi cold ATP, 5 µCi [γ-$^{32}$P]ATP, 20 mM MgCl$_2$, 0.1 mM MnCl$_2$, 0.1 mM CaCl$_2$, 1 µg of the Src kinase substrate, SAM68 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min at 20°C. Reactions were stopped with Laemmli sample buffer, and the proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylated protein was visualized by autoradiography and quantified using a densitometer and UN-SCAN-IT software (Silk Scientific, Orem, UT).

**Statistical Analysis**—Data were plotted and analyzed using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA).

**RESULTS AND DISCUSSION**

**Gastrin Stimulates Src and Focal Adhesion Kinase Phosphorylation in HEK293 Cells Expressing CCK$_{2a}^{-}$R-EGFP or CCK$_{2i4sv}^{-}$R-EGFP**—Amidated G17 induces a rapid activation of Src family kinases in animal cells expressing CCK$_{2a}^{-}$R (21). To determine whether CCK$_{2i4sv}^{-}$R is also coupled to agonist-dependent activation of Src kinase, we treated HEK293 cells expressing either CCK$_{2a}^{-}$R-EGFP or CCK$_{2i4sv}^{-}$R-EGFP with G17 over a time course. An important mechanism for regulation of Src kinase activity is through control of its phosphorylation state. Tyrosine residue 419 (Tyr$^{419}$) of human Src (Tyr$^{416}$ of chicken Src) is autophosphorylated upon Src activation (22). Immunoblot analysis of HEK293 cell lysates, using an antibody that recognizes phosphorylated Tyr$^{419/416}$ residue of Src (p-
**CCK2i4svR Regulation of Src**

Tyr\(^{419/416}\)Src, revealed a time-dependent increase in the level of Src phosphorylation in cells expressing either CCK\(_{2R}\)-EGFP or CCK\(_{2i4svR}\)-EGFP (Fig. 1A). Quantification of receptor expression using radiolabeled ligand binding indicated that the CCK\(_{2R}\)-EGFP cells expressed approximately twice the number of binding sites compared with cells expressing CCK\(_{2i4svR}\) (1.8 pmol/mg of membrane protein versus 0.8 pmol/mg of membrane protein, respectively). After normalization of the level of p-Tyr\(^{419/416}\)Src immunoreactivity to the amount of radiolabeled-agonist binding sites, from three independent experiments, G17 treatment induced an ~2.5-fold increase in the level of p-Tyr\(^{419/416}\)Src at 5 min in cells expressing either receptor compared with untreated control cultures (Fig. 1B). Most strikingly, in contrast to cells expressing CCK\(_{2R}\), cells expressing CCK\(_{2i4svR}\) exhibited a 2-fold higher level of basal Src phosphorylation (1.9 ± 0.2 for CCK\(_{2i4svR}\) versus 1.1 ± 0.1 for CCK\(_{2R}\)) (Fig. 1B), suggesting possible constitutive activation of Src in CCK\(_{2i4svR}\) expressing cells.

Agonist-dependent activation of Src kinase was confirmed by assessing the effects of G17 stimulation on the level of tyrosine phosphorylated focal adhesion kinase (FAK), a Src substrate. Src kinase phosphorylates tyrosine residue 576 of FAK (p-Tyr\(^{576}\)FAK) (21). Immunoblot analysis of HEK293 cell lysates revealed a G17-induced increase in the level of p-Tyr\(^{576}\)FAK in both CCK\(_{2R}\)-EGFP- and CCK\(_{2i4svR}\)-EGFP-expressing cells (Fig. 1C). Pretreating the cells with the Src kinase inhibitor, 4-amine-5-(4-chlorophenyl)-7-(4-buty1)pyrazolo[3,4-d]pyrimidine (PP2), blocked the G17-induced increases in p-Tyr\(^{576}\)FAK, demonstrating a requirement for Src kinase activity. Normalization of the level of p-Tyr\(^{576}\)FAK to receptor number showed that G17 stimulated a similar fold increase in p-Tyr\(^{576}\)FAK levels in both CCK\(_{2R}\) and CCK\(_{2i4svR}\) cells (Fig. 1D). Together, these data establish that, like CCK\(_{2R}\), CCK\(_{2i4svR}\) can mediate the agonist-dependent activation of Src kinase. However, the increased basal level of phosphorylated Src in cells expressing CCK\(_{2i4svR}\) suggests that, unlike CCK\(_{2R}\), the intron 4 variant may also stimulate Src activity in an agonist-independent manner.

**Src Co-immunoprecipitates with CCK\(_{2i4svR}\)—Activation of Src by members of the GPCR superfamity can occur either indirectly through a β-arrestin-mediated mechanism (23) or directly through the interaction of the SH3 domain of Src kinase with proline-rich motifs on the intracellular domains of the GPCR (24–26). To assess whether G17-induced activation of Src involves an interaction of the kinase with either receptor, we immunoprecipitated receptor proteins before and after treatment with G17 using a polyclonal anti-GFP antiserum. Immuno blotting of the GFP-immunoprecipitated proteins from cells expressing CCK\(_{2i4svR}\) with an anti-Src antibody revealed intense staining of two bands of ~60 kDa in both G17-treated and -untreated cells. Only faint Src immunoreactive bands were observed in immunoprecipitated proteins from cells expressing CCK\(_{2R}\) (Fig. 2A). There was no detectable effect of agonist treatment on the amount of immunoprecipitated Src protein associated with either receptor. Src immunoreactive bands were not detected in the GFP-immunoprecipitated proteins from cells transfected with the control EGFP expression vector (see Fig. 2A, V).

To assess the level of receptor expression in the immunoprecipitated cell lysates, the blots were probed with an anti-GFP monoclonal antibody. GFP-immunoreactive bands consistent with the predicted size of the receptor-EGFP fusion protein were detected in the immunoprecipitated proteins from both CCK\(_{2R}\)- and CCK\(_{2i4svR}\)-expressing cells (Fig. 2B). The relative difference in the amounts of receptor detected by immunoblotting and radiolabeled ligand binding were similar. Densitometric analyses of Western blots using revealed 2-fold less receptors in cells expressing CCK\(_{2i4svR}\) than with cells expressing CCK\(_{2R}\). Radiolabeled ligand binding experiments indicated 2.4-fold less receptor in cells expressing CCK\(_{2i4svR}\). Normalization of the amount of immunoprecipitated Src protein to the amount of immunoprecipitated receptor in untreated cells showed 9.8 ± 0.5-fold more Src protein co-immunoprecipitated with CCK\(_{2i4svR}\)-EGFP compared with CCK\(_{2R}\)-EGFP (Fig. 2C).

**CCK\(_{2i4svR}\) Co-immunoprecipitates with Activated Src Kinase—**To determine the activity state of the Src protein co-immunoprecipitated with CCK\(_{2i4svR}\), in vitro kinase assays were performed using the Src-specific substrate, SAM68 (27, 28). Because cells expressing EGFP-CCK\(_{2i4svR}\) expressed ~2-fold less receptor protein than cells expressing CCK\(_{2R}\), we adjusted the total amount of cellular proteins in the immunoprecipitation reactions to compensate for the difference in receptor number. Thus EGFP-tagged CCK\(_{2i4svR}\) was immunoprecipitated from 2 mg of lysis buffer-soluble proteins, whereas CCK\(_{2R}\) was immunoprecipitated from 1 mg of soluble proteins. Western blots confirmed a similar level of GFP immunoreactive receptor protein in immunoprecipitates from both EGFP-CCK\(_{2i4svR}\) and EGFP-CCK\(_{2R}\)-expressing cells. In vitro kinase assays revealed an increased level of SAM68 phosphorylation in immunoprecipitates from cells expressing EGFP-CCK\(_{2i4svR}\).
CCK<sub>2i4sv</sub>R Regulation of Src

**Fig. 3.** Activated Src kinase co-immunoprecipitates with CCK<sub>2i4sv</sub>R. **A,** autoradiogram showing phosphorylation of the Src substrate, SAM68. Cells expressing either CCK<sub>2i4sv</sub>R-EGFP or CCK<sub>R</sub>-EGFP were stimulated with G17 (10 nM) for the indicated times. Either 2 mg of LB-soluble proteins from CCK<sub>2i4sv</sub>R-EGFP cells or 1 mg from CCK<sub>R</sub>-EGFP cells was immunoprecipitated (IP) with anti-GFP antibody and assayed for Src kinase activity as described under "Experimental Procedures." The amount of immunoprecipitated receptor protein was determined by Western blotting (WB) with a monoclonal anti-GFP antibody. B, summary data from three independent experiments showing relative increase in Src kinase activity co-immunoprecipitated with CCK<sub>2i4sv</sub>R-EGFP (black bar) compared with CCK<sub>R</sub>-EGFP (white bar). Data are the mean fold change in Src kinase activity ± S.D. (*, p < 0.05, CCK<sub>R</sub> versus CCK<sub>2i4sv</sub>). C, in vitro Src kinase assay comparing two clonal cell lines expressing N-terminal FLAG-tagged CCK<sub>R</sub> receptor (lanes 1 and 2) to two lines expressing FLAG-CCK<sub>2i4sv</sub>R (lanes 3 and 4). Increased levels of Src kinase activity co-immunoprecipitated with FLAG-CCK<sub>2i4sv</sub>R. The differences in receptor expression between lanes 1 and 3 and lanes 2 and 4 were 2.5- and 3.5-fold, respectively. D, summary data from three independent experiments showing the relative Src kinase activity corrected for receptor number that co-immunoprecipitated with the FLAG-CCK<sub>R</sub> clone shown in lane 1 and the FLAG-CCK<sub>2i4sv</sub>R clone in lane 3 (*, p < 0.05, FLAG-CCK<sub>R</sub> versus FLTag-CCK<sub>2i4sv</sub>R). (Fig. 3A). Densitometric analyses of three independent experiments revealed a 7-fold increase in the level of SAM68 phosphorylation in immunoprecipitates of cells expressing CCK<sub>2i4sv</sub>R-EGFP when compared with cells expressing CCK<sub>R</sub>-EGFP (Fig. 3B). Consistent with previous data, there was no detectable effect of G17 treatment on the level of immunoprecipitated Src kinase activity associated with either receptor (Fig. 3A) and Src kinase activity was not detected in immunoprecipitated proteins from cells transfected with the control EGFP expression vector (Fig. 3A, V).

To exclude the possibility that the EGFP tag was affecting the interaction of CCK<sub>2i4sv</sub>R with Src, we repeated the immunoprecipitation experiments using cell lysates from HEK293 cells stably transfected with either N-terminal FLAG-tagged CCK<sub>2i4sv</sub>R (FLAG-CCK<sub>2i4sv</sub>R) or CCK<sub>R</sub> (FLAG-CCK<sub>R</sub>). A comparison of two clonal lines expressing FLAG-CCK<sub>R</sub> to two clonal lines expressing FLAG-CCK<sub>2i4sv</sub>R showed increased Src kinase activity co-immunoprecipitated with FLAG-CCK<sub>2i4sv</sub>R (Fig. 3C). Normalization of the level of SAM68 phosphorylation to receptor number showed about 10-fold more Src kinase activity co-immunoprecipitated with the FLAG-CCK<sub>2i4sv</sub>R compared with FLAG-CCK<sub>R</sub> (Fig. 3D), demonstrating that the increased Src kinase activity co-immunoprecipitated with CCK<sub>2i4sv</sub>R is not dependent on either the location (N-terminal versus C-terminal) or character (FLAG versus EGFP) of the epitope tag.

The Third Intracellular Loop Domain of CCK<sub>2i4sv</sub>R Co-immunoprecipitates with Activated Src Kinase—Retention of intron 4 during RNA processing results in a CCK<sub>2i4sv</sub>R mRNA that encodes a receptor protein containing an additional 69 amino acid residues within its 3il domain (17). The 3il domain of many GPCRs plays a critical role in receptor-mediated signal transduction (23, 24, 26, 29), including activation of Src family tyrosine kinases (30, 31). GPCRs can directly interact with the SH3 domain containing proteins through proline-rich motifs (P-X-X-P) or other consensus sequences (24). Src binds the β<sub>2</sub>-adrenergic receptor via a phosphorylated tyrosine residue (Tyr<sup>255</sup>), which creates a conditional SH2 binding site on the SH3 domain of CCK<sub>2i4sv</sub>R was sufficient to immunoprecipitate Src

**Fig. 4.** The 3il domain of CCK<sub>2i4sv</sub>R is sufficient to partially activate Src kinase. **A,** Western blot (WB) showing expression of EGFP-tagged 3il constructs EGFP-3il<sub>2</sub>, and EGFP-3il<sub>2i4sv</sub>. Blots were probed with either a rabbit polyclonal antiserum to the 69-amino acid insertion of CCK<sub>2i4sv</sub>R or an anti-EGFP antibody. An ~47-kDa protein band was detected by both anti-CCK<sub>2i4sv</sub>R and anti-EGFP in cells expressing EGFP-3il<sub>2i4sv</sub> whereas a 40-kDa protein band was detected with the anti-EGFP antibody, but not the anti-CCK<sub>2i4sv</sub>R antibody, in cells expressing EGFP-3il<sub>2</sub>. **B,** autoradiogram showing level of SAM68 phosphorylation in immunoprecipitated (IP) protein from cells expressing either the EGFP vector (V), EGFP-3il<sub>2</sub>, or EGFP-3il<sub>2i4sv</sub> constructs and the corresponding Western blot (WB) showing the amount of immunoprecipitated EGFP proteins. C, summary data comparing the relative Src kinase activity normalized to the level of EGFP immunoreactive protein detected by WB. The amount of Src activity in EGFP vector (V)-transfected cells equals one (open bar (*, p < 0.05 EGFP-3il<sub>2</sub> versus EGFP-3il<sub>2i4sv</sub>)). Blots were autoradiographed showing phosphorylation of the Src substrate, SAM68. Cells expressing either CCK<sub>2i4sv</sub>R-EGFP or CCK<sub>R</sub>-EGFP were stimulated with G17 (10 nM) for the indicated times. Either 2 mg of LB-soluble proteins from CCK<sub>2i4sv</sub>R-EGFP cells or 1 mg from CCK<sub>R</sub>-EGFP cells was immunoprecipitated (IP) with anti-GFP antibody and assayed for Src kinase activity as described under "Experimental Procedures." The amount of immunoprecipitated receptor protein was determined by Western blotting (WB) with a monoclonal anti-GFP antibody. An ~47-kDa protein band was detected by both anti-CCK<sub>2i4sv</sub>R and anti-EGFP in cells expressing EGFP-3il<sub>2i4sv</sub> whereas a 40-kDa protein band was detected with the anti-EGFP antibody, but not the anti-CCK<sub>2i4sv</sub>R antibody, in cells expressing EGFP-3il<sub>2</sub>. **B,** autoradiogram showing level of SAM68 phosphorylation in immunoprecipitated (IP) protein from cells expressing either the EGFP vector (V), EGFP-3il<sub>2</sub>, or EGFP-3il<sub>2i4sv</sub> constructs and the corresponding Western blot (WB) showing the amount of immunoprecipitated EGFP proteins. C, summary data comparing the relative Src kinase activity normalized to the level of EGFP immunoreactive protein detected by WB. The amount of Src activity in EGFP vector (V)-transfected cells equals one (open bar (*, p < 0.05 EGFP-3il<sub>2</sub> versus EGFP-3il<sub>2i4sv</sub>)). Blots were autoradiographed showing phosphorylation of the Src substrate, SAM68. Cells expressing either CCK<sub>2i4sv</sub>R-EGFP or CCK<sub>R</sub>-EGFP were stimulated with G17 (10 nM) for the indicated times. Either 2 mg of LB-soluble proteins from CCK<sub>2i4sv</sub>R-EGFP cells or 1 mg from CCK<sub>R</sub>-EGFP cells was immunoprecipitated (IP) with anti-GFP antibody and assayed for Src kinase activity as described under "Experimental Procedures." The amount of immunoprecipitated receptor protein was determined by Western blotting (WB) with a monoclonal anti-GFP antibody. An ~47-kDa protein band was detected by both anti-CCK<sub>2i4sv</sub>R and anti-EGFP in cells expressing EGFP-3il<sub>2i4sv</sub> whereas a 40-kDa protein band was detected with the anti-EGFP antibody, but not the anti-CCK<sub>2i4sv</sub>R antibody, in cells expressing EGFP-3il<sub>2</sub>.
kinase activity, we generated plasmid constructs that expressed only the 3i domain of either CCK2i4svR (amino acid residues Arg242–Arg389) or CCK2i4svR (amino acid residues Arg42–Arg130) fused in frame to the C terminus of EGFP. Western blot analysis with either an antibody to the 69-amino acid insertion of CCK2i4svR or GFP confirmed expression of the EGFP-3il2i4sv and EGFP-3il1 in whole cell lysates of transfected HEK293 cells (Fig. 4A). Western blot analysis also confirmed the presence of EGFP-3il2i4sv and EGFP-3il2 proteins in GFP-immunoprecipitated proteins (Fig. 4B), and in vitro Src kinase assays revealed an increased level of SAM68 phosphorylation in cells expressing EGFP-3il2i4sv compared with cells expressing EGFP-3il2 (Fig. 4B). A comparison of the ratio of the amount of phosphorylated SAM68 to the amount of 3il protein in the GFP immunoprecipitates showed a 7-fold more Src kinase activity associated with EGFP-3il2i4sv than with EGFP-3il2 (Fig. 4C). There was little detectable Src kinase activity in immunoprecipitates from EGFP vector transfected cells. Although more Src kinase activity co-immunoprecipitated with EGFP-3il2i4sv (Fig. 4C, filled bar) than with EGFP-3il2 (Fig. 4C, open bar), a direct comparison of the amount of Src kinase activity associated with the third intracellular loop of CCK2i4svR (filled bar) and full-length CCK2i4svR showed 10-fold more Src activity co-immunoprecipitated with the full-length receptor (Fig. 4C, hatched bar) than with the 3il construct, suggesting that other receptor structures, in addition to the 3rd intracellular loop domain, are required for maximal Src activation.

Previously, we have demonstrated constitutive (agonist-independent) signaling by CCK2i4svR when expressed in mouse fibroblasts (17). Expression of CCK2i4svR in Balb3T3 fibroblast cells causes spontaneous oscillatory changes in [Ca2+]i (Fig. 1) and verified by the immunoprecipitation experiments showing increased Src kinase activity associated with CCK2i4svR compared with CCK2i4svR. Additionally, we have demonstrated that the 3il domain of CCK2i4svR contains an element or elements capable of partially activating Src kinase. Future studies will determine whether the 3il domain structures involved in Src activation are contained within the primary sequence of the 69-amino acid insertion or are due to a unique secondary or tertiary folding of the protein.

Several mechanisms for the increased Src kinase activity in cancers have been identified, including activation by receptor tyrosine kinases such as the epidermal growth factor and hepatic growth factor receptors (32, 33). Some breast cancer cells exhibited increased phosphatase activity that enhance Src by dephosphorylating the negative regulatory tyrosine residue, Tyr530 (34). In hepatocarcinomas, increased Src kinase activity is associated with decreased expression of Cak, the protein kinase that phosphorylates residue Tyr530 of Src (35), and a rare activating mutation of Src kinase is present in a subset of advanced human colon cancers (36). Our data, showing a constitutive activation of Src in cells expressing CCK2i4svR, support the hypothesis that the increased Src activity observed in some pancreatic and colorectal cancers is due, in part, to the co-expression of CCK2i4svR.

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