Nature **382**, 177 (1996).
20. Surface biotinylation was performed according to the manufacturer's instructions with the use of NHS-LC-biotin (Pierce, Rockford, IL) at 40 nmol per 10^6 cells.
21. Cell extracts (1% Triton X-100) were immunoprecipitated [4 μg of immunoglobulin G (IgG) per 400 to 600 μg of lysate per 80 μl of immobilized Protein A/G or goat antibody to mouse IgG] and subjected to protein immunoblotting (1:2000 dilution of IgG) and ECL detection. Signals were quantitated with a Molecular Dynamics system.
22. Induced K44A and WT cells were serum-starved [0.4% serum in Dulbecco's modified Eagle's medium (DMEM)] for 20 hours and then cultured for 2 days in the presence or absence of 4 nM EGF. For the last 4 hours, MTT (Sigma) was added at 1 mg/ml and its reductive conversion was measured [F. Denizot and R. Lang, *J. Immunol. Methods* **89**, 271 (1986)]. For thymidine incorporation measurements, cells (~25% confluency) in DMEM + 10% fetal bovine serum (FBS) were incubated in serum-free DMEM, with or without tetracycline (1 μg/ml), for 40 hours. EGF (3.5 nM) was then added for 18 to 20 hours. Methyl-[^{3}H]thymidine (1 μCi/ml) (Amersham) was added for the last 4 hours. Cells were then processed as described [A. Obermeier, I. Tinhofer, H. H. Grunicke, A. Ullrich, *EMBO J.* **15**, 73 (1996)], and incorporated radioactivity was quantitated in the presence of ProteinPlus scintillant (Beckman).
23. Cells were seeded at ~20% confluency in DMEM + 10% FBS without tetracycline. After 36 hours, they were incubated in DMEM without serum for 16 to 20 hours. EGF (3.5 nM) was then added for 2 to 20 min, and the cells were rinsed three times with phosphate-buffered saline and lysed with MKAL buffer [0.5% Triton X-100, 20 mM Hepes, 100 mM NaCl, 200 μM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, aprotinin (1 μg/ml), leupeptin (2 μg/ml), and 1 mM dithiothreitol (pH 7.4)]. Lysate (400 μg) was immunoprecipitated with 4 μg of affinity-purified anti-ERK IgG (UBI, catalog number 06-182). To the washed immunoprecipitates was added 1/10 volume of 10× assay buffer stock [200 mM Hepes, 500 mM NaCl, 1 mM sodium orthovanadate, 200 mM $MgCl_2$, 200 μM adenosine triphosphate (ATP), and 200 mM β-glycero-phosphate (pH 7.4)]. To each tube was added 2 μCi of γ-[^{32}P]ATP and myelin basic protein (MBP; 0.25 mg/ml), and the reactants were incubated at 32°C for 15 min. [^{32}P]MBP was then quantitated as described in Fig. 3.
24. We thank G. Gill and D. Cadena for the antibodies to human EGFR and T. Hunter for critical reading of the manuscript. Supported by National Cancer Institute grants CA58689 and CA69099 to S.L.S. A.V.V. was supported by the Human Frontier of Science Programme (grant LT 461/95), and C.L. was supported by the U.S. AMRMC (grant DAM17-94-J-4031). S.L.S. is an American Heart Association Established Investigator. This is The Scripps Research Institute manuscript number 10219-CB.

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Association of Src Tyrosine Kinase with a Human Potassium Channel Mediated by SH3 Domain

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The human Kv1.5 potassium channel (hKv1.5) contains proline-rich sequences identical to those that bind to Src homology 3 (SH3) domains. Direct association of the Src tyrosine kinase with cloned hKv1.5 and native hKv1.5 in human myocardium was observed. This interaction was mediated by the proline-rich motif of hKv1.5 and the SH3 domain of Src. Furthermore, hKv1.5 was tyrosine phosphorylated, and the channel current was suppressed, in cells coexpressing v-Src. These results provide direct biochemical evidence for a signaling complex composed of a potassium channel and a protein tyrosine kinase.

Potassium channels are important for such cellular electrical properties as resting potential, excitability, and the repolarization of the action potential. Thus, modulation of these channels can profoundly affect physiological processes including neuronal integration, vesicle secretion, and muscle contraction. The modulation of potassium channel activity by serine-threonine kinases has been studied extensively (*1*). The recently discovered PYK2 tyrosine kinase (*2*), as well as endogenous tyrosine kinases in human embryonic kidney (HEK) 293 cells (*3*), can also phosphorylate and suppress the activity of potassium channels. In spite of emerging evidence concerning the functional effects of tyrosine phosphorylation of potassium channels, there is no information available about the mechanisms of targeting and association of these channels with tyrosine kinases. However, the existence of signaling complexes consisting of ion channels and closely associated protein kinases and phosphatases has been inferred from biochemical and functional electrophysiological studies (*4*).

Specific protein-protein interactions between signaling proteins are mediated by modular binding domains (*5*). Among the first of these to be characterized was a conserved sequence found in the Src tyrosine kinase, known as the Src homology 3 (SH3) domain. SH3 domains bind to proline-rich regions in partner proteins. We examined the sequences of mammalian voltage-dependent potassium channels, and noted that several species isoforms of Kv1.5—including those from human (hKv1.5), dog, and rabbit (*6*)—contain one to two copies of the preferred Src SH3 domain binding motif RPLPXXP (*7, 8*). In particular, hKv1.5 contains two repeats of the sequence RPLPPLP between amino acid residues 65 and 82 of the channel protein (*6, 8, 9*). To determine whether hKv1.5 and Src are associated in vivo, we coexpressed the channel and kinase in HEK 293 cells and tested for their interaction by immunoprecipitation followed by protein immunoblotting with specific antibodies to hKv1.5 and Src (*10*).

When hKv1.5 and associated proteins were immunoprecipitated from cell lysates with a specific antibody, Src was co-precipitated (Fig. 1A). Similarly, when Src and associated proteins were immunoprecipitated from HEK 293 cell lysates, hKv1.5 co-precipitated with endogenous and coexpressed Src (Fig. 1A). Expression of hKv1.5 protein was not altered by v-Src coexpression, as verified by protein immunoblot analysis of cell lysates with antibodies directed against tagged (Fig. 1A) and native (Fig. 2A) sequences of the channel. Furthermore, immunoblot (Fig. 1A) or protein silver stain (Fig. 3A) analysis of immunoprecipitates demonstrated that the efficiency of immunoprecipitation of hKv1.5 was not affected by v-Src coexpression. Enzymatic activity of Src also co-precipitated with hKv1.5, as detected by an in vitro kinase assay with hKv1.5 immunoprecipitates and an Src-specific substrate (*11*) (Fig. 1B).

The association between hKv1.5 and Src was also observed in human tissue. Native Src was detected in immunoprecipitates, prepared with a Kv1.5 antiserum, from human myocardium ventricle tissue lysates (Fig. 1C). The native Src that co-immunoprecipitated with native Kv1.5 co-migrated on protein immunoblots with native Src, immunoprecipitated directly with a polyclonal anti-Src antibody (Fig. 1C). Thus association of hKv1.5 and Src occurs under physiological conditions, and does not depend on expression in a heterologous system. This association may contribute to the co-localization of Kv1.5 and Src in cellular adhesion zones in myocardium (*12*). Although the stoichiometry of the association between hKv1.5 and Src is not known, only a fraction of the total myocardial Src co-immunoprecipitated with hKv1.5 (Fig. 1C), consistent with the fact that Src phosphorylates other substrates.

There are specific sequence requirements for the association of hKv1.5 and Src. For example the NH_2-terminal region of the rat Kv1.5 (rKv1.5) channel also contains a pro-

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line-rich motif (9, *13*), but this sequence (RPLPPMA) (8) does not appear to be favorable for binding to the Src SH3 domain, as shown by the absence of selection of this sequence with phage display libraries (*7*). In contrast to hKv1.5, rKv1.5 failed to co-immunoprecipitate with Src (Fig. 2A). Thus, the association between channel and Src is detected only for the hKv1.5 channel isoform, possibly because its proline-rich binding motif is preferred by the Src SH3 domain. In addition phospholipase C–γ and the p85 regulatory subunit of phosphatidylinositol 3–kinase, which contain SH3 domains with different binding sequence requirements than that of Src (*7*), do not co-immunoprecipitate with hKv1.5 (*14*).

We tested hKv1.5 binding to the Src SH3 domain itself expressed as a fusion protein with glutathione-S-transferase (GST) (*15*). Cell lysates prepared from vector control and hKv1.5 transfected cells were incubated with a GST fusion protein containing the Src SH3 domain (GST-Src-SH3) or no insert (GST). The hKv1.5 protein was effectively precipitated by GST-Src-SH3, but not by GST (Fig. 2B). The specificity of this interaction was tested by preabsorption of the fusion proteins with a peptide containing the sequence of the proline-rich region of hKv1.5 (peptide $hKv1.5_{62-83}$). Binding of hKv1.5 to GST-Src-SH3 was attenuated by preabsorption of the fusion protein with peptide $Kv1.5_{62-83}$ (Fig. 2B). The direct binding of the Src SH3 domain to hKv1.5 was demonstrated in a filter binding assay (far Western blot). GST-Src-SH3 bound to hKv1.5 on the filter, whereas no binding was detected with rKv1.5 (Fig. 2C). The role of the proline-rich motif in the channel in the binding of GST-Src-SH3 to hKv1.5 was demonstrated further by the absence of filter binding after preabsorption of the GST-Src-SH3 with peptide $hKv1.5_{62-83}$ (Fig. 2C).

The hKv1.5 protein was tyrosine phosphorylated when it was coexpressed with v-Src (Fig. 3A). To determine whether coexpression of v-Src influenced channel activity, we measured hKv1.5 macroscopic currents in cell-attached membrane patches, with and without v-Src coexpression (*3*, *16*). Current through hKv1.5 channels was suppressed when the channel was coexpressed with v-Src (Fig. 3B), even though channel protein expression was not altered (Fig. 3A; see also Figs. 1A and 2A). We do not yet know whether the suppression of hKv1.5 current (Fig. 3B) results from tyrosine phosphorylation of the channel protein (Fig. 3A), or whether direct binding of hKv1.5 to Src is required for either of these phenomena.

Many mammalian ion channels, including channels that are known to be modulated by tyrosine kinases, have proline-rich sequences that may bind to SH3 domains (*9*). Furthermore, signaling complexes containing multiple protein kinases, or ion channels together with scaffolding and regulatory proteins, may be common in cells (*4*, *17*). Potential consequences of a closely associated channel-kinase signaling complex include increased specificity of signaling pathways, faster coupling, and a higher probability of channel phosphorylation after kinase activation.

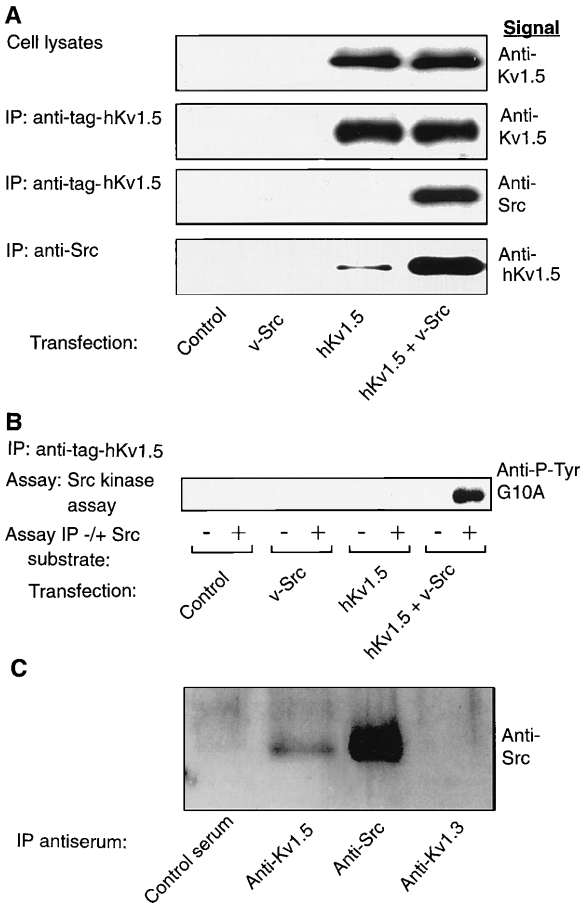

Fig. 1. Co-immunoprecipitation of hKv1.5 and Src. (**A**) HEK 293 cells were transfected with CMV vector with no insert (control), vector encoding v-Src, vector encoding hKv1.5, or two separate vectors, one encoding hKv1.5 and the other encoding v-Src (*10*). Expression of hKv1.5 was measured on protein immunoblots of cell lysates probed with anti-tag-hKv1.5 (top panel). Densitometry of autoradiograms was used to quantitate channel expression. For each experiment, the density (expression level) of hKv1.5 transfected alone was set to 1. When Src was cotransfected, the relative density of hKv1.5 was 0.98 ± 0.05 (mean ± SEM; $n = 9$; not significant by *t* test). Immunoblot analysis of anti-tag-hKv1.5 immunoprecipitates (IP) with anti-tag-hKv1.5 probe (second panel) confirmed that the efficiency of immunoprecipitation of hKv1.5 (density set to 1) was not affected by coexpression of v-Src (relative density 1.06 ± 0.10; $n = 8$; not significant by *t* test). Src that co-immunoprecipitated with hKv1.5 was detected by probing anti-tag-hKv1.5 IP with anti-Src (third panel). The hKv1.5 that co-immunoprecipitated with endogenous c-Src and expressed v-Src was detected by probing anti-Src IP with anti-tag-hKv1.5 (bottom panel). (**B**) HEK 293 cells were transfected as in (A). Anti-tag-hKv1.5 IP were assayed for Src activity (*21*) by incubation with or without the Src substrate fusion protein G10A. The reaction products were separated on protein immunoblots that were probed with antibody 4G10 to phosphotyrosine ($n = 4$). (**C**) Native Src that co-immunoprecipitated with native Kv1.5, or was immunoprecipitated directly with anti-Src, was detected by probing IP prepared from human myocardium tissue lysates (separated on immunoblots) with anti-Src ($n = 4$). IP with an antiserum (*3*) against Kv1.3, another potassium channel, was used as an additional control.

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8. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; L, Leu; M, Met; P, Pro; R, Arg; S, Ser; and V, Val. Unspecified amino acid residues are indicated by X.
9. The sequences of proline-rich motifs in 15 mammalian potassium channels and two *N*-methyl-D-aspartate receptor subunits, together with their GenBank/European Molecular Biology Laboratory accession numbers, can be retrieved from *Science*'s Beyond the Printed Page site on the World Wide Web: http://sciencemag.org/science/feature/beyond/#holmes.
10. All cDNA mammalian expression vectors contained the cytomegalovirus (CMV) promoter sequence upstream from the coding region for channels and kinases. A vector encoding hKv1.5 fused to an epitope tag (*18*) was provided by L. Philipson (University of Chicago), and one encoding v-Src was provided by R. Huganir (Johns Hopkins University, Baltimore, MD). HEK 293 cells were lipofectamine transfected and lysed as described (*3*). Tissue lysate was prepared from human myocardium with a glass–teflon homogenizer. Proteins were immunoprecipitated overnight at 4°C from lysates with 4 μg of primary antibody and 25 μl of protein A/G (Pierce, Rockford, IL) per 400 μg of lysate protein. The lysates and washed immunoprecipitated proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), and protein immunoblots were probed with

an antibody (*19*) that recognizes both rKv1.5 and hKv1.5 [anti-Kv1.5; provided by J. Trimmer (State University of New York, Stony Brook, NY)], an antibody (*18*) to an epitope tag sequence fused to hKv1.5 (anti-tag-hKv1.5; provided by L. Philipson), or antibody to Src (MAB 327; Oncogene Science, Cambridge, MA). Antibody binding was visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Silver stain (*20*) was used to detect immunoprecipitated proteins separated by SDS-PAGE. Src activity was measured under standard conditions (*21*) with the substrate G10A, a GST fusion protein that contains an Src SH3 domain binding motif and a tyrosine phosphorylation site (*11*). The reaction products were separated by SDS-PAGE, and protein immunoblots were probed with an antibody to phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) and visualized by ECL.

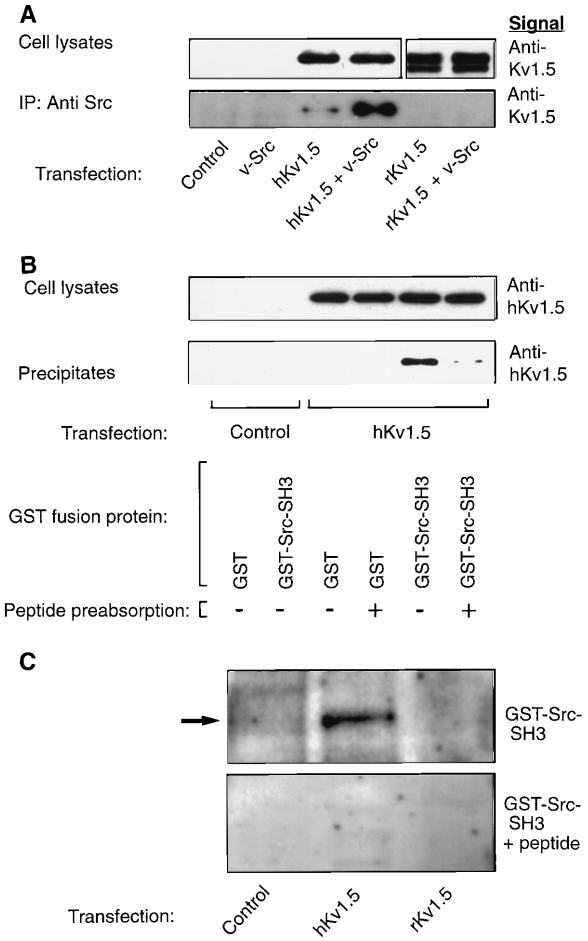

Fig. 2. Domains that mediate binding of hKv1.5 to Src. (**A**) HEK 293 cells were transfected with CMV vector with no insert (control), vector encoding v-Src, vector encoding hKv1.5 or rKv1.5, or two separate vectors, one encoding hKv1.5 or rKv1.5 and the other encoding v-Src (*10, 15*). Expression of hKv1.5 and rKv1.5 was detected in cell lysates by protein immunoblotting with anti-Kv1.5 (*19*), which recognizes both channels (top panel). Because this antibody recognizes rKv1.5 much better than hKv1.5, the two parts of this panel were exposed for different times. The apparent doublet in the rKv1.5 lanes may represent phosphorylated or glycosylated or both types of isoforms of the channel protein (*3*). Anti-Src IP, separated on immunoblots, were probed with anti-Kv1.5 (bottom panel) ($n = 4$). (**B**) HEK 293 cells were transfected with CMV vectors coding for vector with no insert (control) or hKv1.5. Expression of hKv1.5 was confirmed by immunoblotting the cell lysates with anti-tag-hKv1.5 (top panel). Cell lysates were incubated with GST alone or GST fusion protein containing the Src SH3 domain (GST-Src-SH3) (*22*), with or without fusion protein preabsorption with a peptide corresponding to the proline-rich sequence comprising amino acids 62 through 83 of hKv1.5 (peptide $hKv1.5_{62\text{-}83}$) (*15*). Proteins bound to GST fusion proteins were separated by SDS-PAGE, and immunoblots were probed with anti-tag-hKv1.5 (bottom panel) ($n = 4$). (**C**) Far western blots were prepared with anti-Kv1.5 IP from cells transfected with hKv1.5 or rKv1.5. The blots were probed with biotinylated GST-Src-SH3 (1 μg/ml) (*22*) (top panel; the arrow indicates position of the hKv1.5 band) or biotinylated GST-Src-SH3 preabsorbed with peptide $hKv1.5_{62\text{-}83}$ (bottom panel). The blots were then incubated with avidin-horseradish peroxidase, and bound fusion protein was visualized by ECL ($n = 4$).

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15. HEK 293 cells were transfected as described (*3*, *10*). The CMV vector encoding rKv1.5 was provided by J. Trimmer. For fusion protein precipitation experiments, cell lysates were incubated overnight at 4°C with GST fusion protein (1.0 μg/ml of cell lysate) (*22*). The GST fusion proteins and bound proteins were separated from unbound protein by incubation with glutathione-agarose beads. Where indicated, GST fusion proteins were preabsorbed by incubating with 100 times greater concentration of peptide NH_2-SGVRPLPPLP-DPGVRPLPPLPE-COOH (peptide $hKv1.5_{62\text{-}83}$; Bio-Synthesis, Lewisville, TX) (*8*). The proteins bound to the washed agarose beads were separated by SDS-PAGE, and immunoblots were probed with anti-tag-hKv1.5.
16. Patch-clamp recordings of macroscopic currents were made in the cell-attached patch configuration (*3*). Patches were held at −90 mV and stepped to depolarizing potentials in 5-mV increments to 0 mV. The pulse duration was 400 ms and the interpulse interval was 10 s.
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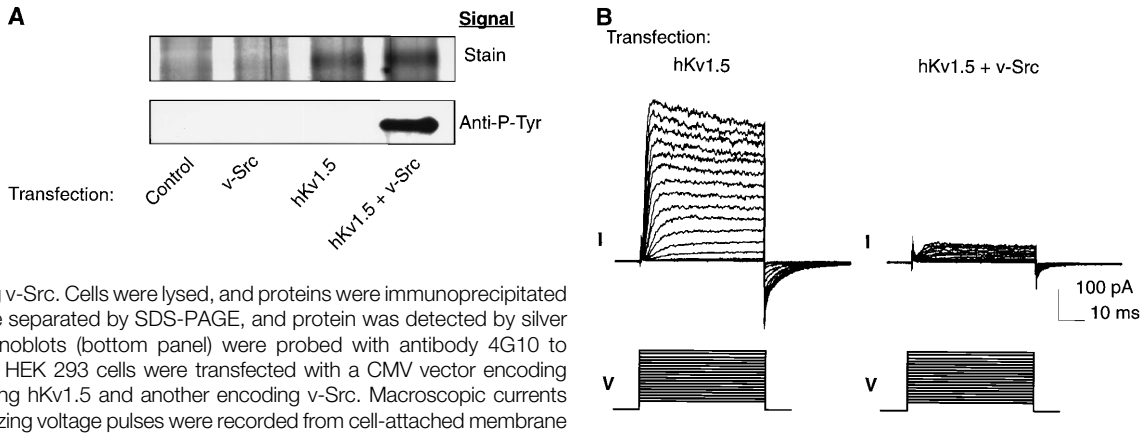

Fig. 3. Tyrosine phosphorylation of hKv1.5 and suppression of channel current by coexpression with v-Src. (**A**) HEK 293 cells were transfected with CMV vectors as indicated: vector with no insert (control); v-Src; hKv1.5; or one vector encoding hKv1.5 and another encoding v-Src. Cells were lysed, and proteins were immunoprecipitated with anti-tag-hKv1.5. IP were separated by SDS-PAGE, and protein was detected by silver stain (*20*) (top panel). Immunoblots (bottom panel) were probed with antibody 4G10 to phosphotyrosine ($n = 4$). (**B**) HEK 293 cells were transfected with a CMV vector encoding hKv1.5 or one vector encoding hKv1.5 and another encoding v-Src. Macroscopic currents evoked by a series of depolarizing voltage pulses were recorded from cell-attached membrane patches (*3, 16*) 2 days after transfection. The peak current at +40 mV was 592 ± 163 pA (mean ± SEM; $n = 8$) in patches from cells expressing hKv1.5 alone, and 27 ± 15 pA ($n = 9$) in patches from cells coexpressing v-Src (significantly different, $P \leq 0.02$, *t*-test).