Regulation of the Bovine Kidney Microsomal Chloride Channel p64 by p59\textsuperscript{fyn}, a Src Family Tyrosine Kinase*  

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p64 is a chloride channel of intracellular membranes which is present in regulated secretory vesicles. Mechanisms by which the p64 channel could be regulated are largely unknown. p59\textsuperscript{fyn} is a non-receptor tyrosine kinase of the Src family that has been implicated in a variety of intracellular signaling events. The N-terminal portion of p64 has several potential binding sites for Src family SH2 domains. In this paper, we demonstrate that p64 becomes tyrosine phosphorylated when co-expressed with p59\textsuperscript{fyn} in HeLa cells. We show that co-expression of p64 with p59\textsuperscript{fyn} renders p64 a ligand for the SH2 domain of p59\textsuperscript{fyn} and this SH2 binding is eliminated by treating p64 with alkaline phosphatase. Using site-directed mutagenesis, we find that tyrosine 33 in the p64 sequence is necessary for SH2 binding. We also characterized p64-p59\textsuperscript{fyn} interactions using native material from bovine kidney. We found that a small fraction of native kidney p64 can bind Fyn SH2 in vitro. Immunoprecipitation of p64 from solubilized kidney membranes yields a kinase activity with the same mobility by SDS-polyacrylamide gel electrophoresis as authentic bovine p59\textsuperscript{fyn}. Finally, we demonstrate that co-expression of p64 and p59\textsuperscript{fyn} in HeLa cells results in enhanced p64-associated chloride channel activity.

p64 is a chloride channel protein originally discovered by purification of chloride channel activity from bovine kidney microsomes (1). cDNA encoding p64 was subsequently cloned (2). p64 was found to be a 428-amino acid protein consisting of a hydrophilic N-terminal domain comprising about half the protein and a C-terminal domain containing two hydrophobic stretches. Expression of p64 in HeLa cells results in increased chloride permeability of membranes isolated from these cells and in the appearance of a novel chloride channel as detected in a planar lipid bilayer (3).

p64 is normally expressed at high levels in intracellular vesicles of renal proximal tubule cells (4). In cultured cells, p64 is expressed in the limiting membranes of intracellular dense core vesicles which redistribute to the periphery in response to phorbol ester and calcium ionophore but not forskolin. Thus, p64 is a candidate for the chloride channel activity known to be present in certain regulated secretory vesicles. The contents of the p64-associated regulated secretory vesicles are not known. P64 has subsequently been discovered to be a member of a family of related proteins which to date includes 7 members (5–13). This family has been named the CLIC family for chloride intracellular channel (9). Chloride channel activity has been attributed to several of these p64 homologs (5, 7, 10, 11, 13). Each of the CLIC family members shows high homology to the C-terminal half of p64 containing the hydrophobic domains. In contrast, none of the other CLIC family members have a region with significant homology to the long hydrophilic N-terminal domain of p64. The channel activity of CLIC family proteins must reside in the portion of the molecule which is shared among the CLIC family members, i.e. the C-terminal half. The non-conserved N-terminal domain of p64 may confer some specific regulation or targeting to the channel forming C-terminal domain (2, 4).

The N-terminal domain of p64 in fact contains sequences which are candidates for sites of protein-protein interaction. Within this domain lies a multiply repeated, acidic, proline-rich motif which contains the core PXXP consensus for binding to SH3 domains (14–16). Deletion of this motif was shown to have a dramatic effect on the subcellular distribution of p64 (4). A binding partner for this region has not yet been identified.

In addition, within the N-terminal domain of p64 are a number of tyrosine residues which lie within consensus sequences for binding by various families of SH2 domains (17). SH2-containing proteins carry out a variety of regulatory functions within cells and SH2 interactions have been shown to be important in the regulation of at least one other ion channel (18, 19).

p59\textsuperscript{fyn} is a member of the Src family of tyrosine kinases which have been implicated as key regulatory molecules in a variety of intracellular pathways (20–22). Like other members of the Src family, p59\textsuperscript{fyn} contains both SH2 and SH3 domains that are critical to the various regulatory functions of the protein. p59\textsuperscript{fyn} is expressed in kidney cells (20, 24) and is known to be present in membrane fractions in cells in which it is expressed (25).

In this paper, we show that co-expression of p64 and p59\textsuperscript{fyn} in HeLa cells leads to tyrosine phosphorylation of p64 and that tyrosine-phosphorylated p64 is a ligand for the SH2 domain of Fyn. We demonstrate that tyrosine 33 in the p64 sequence is necessary for binding to the SH2 domain of Fyn. We go on to demonstrate that native bovine kidney p64 is a ligand for the SH2 domain of Fyn and that immunoprecipitation of p64 from solubilized bovine kidney membranes brings down a tyrosine kinase activity indistinguishable from Fyn. Finally, we demonstrate that co-expression of Fyn with p64 in HeLa cells leads to increased chloride channel activity compared with expression of p64 alone.
MATERIALS AND METHODS

Cells, DNA, and General Methods—Plasmid pNHAP encoding p64 downstream of a T7 promoter has been previously described (1). HeLa cells, recombinant vaccinia encoding T7 RNA polymerase (26), and plasmids encoding mouse Fyn (pBS-Fyn) (27, 28), glutathione S-transferase (GST; pGEX-KG) (29), and GST-Fyn fusion proteins (30) were obtained from Dr. Andrey Shaw, Washington University, St. Louis, MO. Protein assays were performed with the BCA kit from Pierce Biochemical, Rockford IL.

Preparation of Bone Kidney Membranes—Fresh bovine kidney from a slaughterhouse was chilled on ice. Two grams of cortical slices were minced and suspended in 20 ml of 150 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.1 mM NaVO₄, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0. The suspension was homogenized with 20 strokes in a glass tissue grinder, then 20 strokes in a Teflon Dounce homogenizer on ice. The suspension was centrifuged at 1,000 rpm for 5 min at 4 °C in an SS34 rotor. The supernatant was collected and centrifuged at 40,000 rpm for 10 min at 4 °C in the same rotor. The supernatant again was collected and centrifuged at 40,000 rpm for 1 h in a Beckman Ti70.1 rotor. The pellet was solubilized in 5 ml of 150 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.1 mM NaVO₄, 1.0% Triton X-100, pH 8.0. After mixing for 10 min at 4 °C, the solution was again centrifuged at 40,000 rpm for 1 h at 4 °C. The supernatant was used as solubilized bovine kidney membrane protein.

Expression of Proteins in HeLa cells—p64 and Fyn were expressed in HeLa cells using a vaccinia-T7 system as described previously (3, 26). Cultures on 3.5-cm dishes were transfected with a 3.75 μg of pNHAP and 3.75 μg of pBS-Fyn. For those samples which did not require Fyn and p64 encoding plasmids, the missing DNA was replaced with the parent plasmid, pBluescript SK. After transfection, cultures were incubated for 10 h at 37 °C in a 5% CO₂ incubator. Cells lysates were prepared by solubilizing a culture in 500 μl of 150 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.1 mM NaVO₄, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0 (solvublibization buffer).

Preparation of Immobilized GST-Fyn Fusion Protein—GST, GST-Fyn SH3 fusion protein, and GST-Fyn SH2 fusion protein were purified from bacteria as described (27). The fusion proteins were immobilized on Affi-Gel 15 beads (Bio-Rad) using methods recommended by the manufacturer.

SH2 Binding—Five hundred micrograms of solubilized bovine kidney membrane protein or 200 μl of solubilized HeLa cell protein were mixed with 20 μl of a 50% suspension of beads carrying the appropriate fusion protein in a total volume of 750 μl of solubilization buffer. The mixture was incubated at 4 °C for 1 h with gentle mixing. Beads were then collected by centrifugation. Unbound proteins were removed by 4 washes at 4 °C in 750 μl of solubilization buffer. Samples from HeLa cells were then suspended in SDS-PAGE loading buffer. Samples from bovine kidney were subjected to a further wash with 50 mM Tris, 1 mM EDTA, 0.1 mM vanadate, followed by a wash with 500 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.1 mM vanadate, before suspension in SDS-PAGE loading buffer. Samples were separated on a 10% SDS-PAGE gel, blotted to nitrocellulose, and probed with AP95 antibody specific for p64 (2, 4), and detected on x-ray film using the SuperSignal system (Pierce Biochemical).

For the alkaline phosphatase experiment, cells from a 3.5-cm dish were lysed by homogenization in 1 ml of 20 mM Tris, pH 8.0, 1 mM MgCl₂. The sample was divided into two 250-ml aliquots. Fifteen units of alkaline phosphatase, one fraction received EDTA (2 mM final) and NaVO₄ (0.1 mM final). Both were incubated at room temperature for 30 min prior to isolation of membranes by centrifugation as described in the preceding paragraph. The chloride efflux assay was performed as described previously (3). In brief, 200 μl of vesicles were passed through a Bio-Gel P-6 (Bio-Rad) spin column that had been equilibrated with 400 mM sucrose. The effluent from the column was added to 2 ml of 400 mM sucrose and the extravesicular chloride concentration was continuously monitored with a chloride selective electrode. Valinomycin was added to 1 μM final concentration to initiate voltage-dependent chloride efflux. Triton X-100 was then added to 0.1% final concentration to release any remaining intravesicular chloride. The initial rate of fractional chloride release after addition of valinomycin is taken as the chloride permeability of the reconstituted vesicles. Efflux rates pooled from several independent expression-reconstitution experiments for each group are reported as mean ± S.E. Comparisons between groups were analyzed using analysis of variance (31).

RESULTS

We initially looked for p64-Fyn interactions in a cell culture expression system. p64 and p59⁵⁷⁷ were expressed separately or together in HeLa cells using a vaccinia T7-driven system as we have previously reported for the functional expression of p64 (3). Using this expression system, we determined the tyrosine phosphorylation status of p64 when expressed alone or with p59⁵⁷⁷. Cells expressing p64 or p64 plus p59⁵⁷⁷ were solubilized in the presence of phosphatase inhibitors. Equal amounts of protein were separated by SDS-PAGE, blotted, and probed with antibodies specific for phosphotyrosine (Fig. 1A). There were no major tyrosine-phosphorylated proteins in cells expressing p64 alone. In cells expressing both p64 and p59⁵⁷⁷, two major tyrosine-phosphorylated proteins are apparent: one

The abbreviations used were: GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SH2, Src homology domain 2.
Regulation of p64 by Fyn

The size of p59\(^{\text{SH2}}\) and one the size of p64 (Fig. 1A, lanes 1 and 2). To determine whether the larger tyrosine-phosphorylated protein is indeed p64, the cell lysates were immunoprecipitated with p64 antisera and probed with phosphotyrosine antibodies or with antibodies directed at p64. The upper tyrosine-phosphorylated band is precipitated with anti-p64 antisera and this protein is indeed p64, the cell lysates were immunoprecipitated with p64 antisera and probed with phosphotyrosine antibodies specific for the SH2 domain of Fyn when co-expressed with Fyn. HeLa cells expressing p64 alone (lanes 1 and 3) or p64 plus p59\(^{\text{SH2}}\) (lanes 2, 4, 6, 7, and 8) were solubilized. Total protein (lanes 1 and 2) or immunoprecipitates with pre-immune serum (lanes 3, 4, and 7) or with antisera raised against p64 (lanes 5, 6, and 8) were separated, blotted, and probed with antibodies specific for phosphotyrosine (lanes 1–6) or p64 (lanes 7 and 8). The positions of p64 and p59\(^{\text{SH2}}\) are marked with arrows. The dense bands present in all immunoprecipitated lanes represent IgG heavy chain. B, p64 becomes a ligand for the SH2 domain of Fyn when co-expressed with Fyn. HeLa cells expressing p64 alone (lanes 1 and 3–5) or p64 plus p59\(^{\text{SH2}}\) (lanes 2 and 6–8) were solubilized. Total protein (lanes 1 and 2) or protein which bound to immobilized GST fusion proteins (lanes 3–8) were separated, blotted, and probed with affinity-purified anti-p64 antisera. The immunoprecipitated fusion proteins were GST itself (labeled C; lanes 3 and 6), GST-fynSH3 (labeled SH3; lanes 4 and 7), or GST-Fyn SH2 (labeled SH2; lanes 5 and 8). C, phosphatase treatment eliminates p64 binding to the Fyn-SH2 domain. p64 and p59\(^{\text{SH2}}\) were co-expressed in HeLa cells and cell lysates prepared. Total protein (lane 1) or protein which bound to immobilized GST-Fyn SH2 fusion protein (lanes 2 and 3) was separated and probed with affinity purified anti-p64 antisera. Prior to binding to fusion protein, the lysate was treated with alkaline phosphatase (lane 3) or subjected to identical incubation in the absence of added phosphatase (lane 2). In panels B and C, the arrows mark the migration position of p64.

The phosphotyrosine band co-migrates with p64 itself (Fig. 1A, lanes 1, 3, and 5) or p64 plus p59\(^{\text{SH2}}\) (lanes 2, 4, 6, 7, and 8) were solubilized. Total protein (lanes 1 and 2) or immunoprecipitates with pre-immune serum (lanes 3, 4, and 7) or with antisera raised against p64 (lanes 5, 6, and 8) were separated, blotted, and probed with antibodies specific for phosphotyrosine (lanes 1–6) or p64 (lanes 7 and 8). The positions of p64 and p59\(^{\text{SH2}}\) are marked with arrows. The dense bands present in all immunoprecipitated lanes represent IgG heavy chain. B, p64 becomes a ligand for the SH2 domain of Fyn when co-expressed with Fyn. HeLa cells expressing p64 alone (lanes 1 and 3–5) or p64 plus p59\(^{\text{SH2}}\) (lanes 2 and 6–8) were solubilized. Total protein (lanes 1 and 2) or protein which bound to immobilized GST fusion proteins (lanes 3–8) were separated, blotted, and probed with affinity-purified anti-p64 antisera. The immunoprecipitated fusion proteins were GST itself (labeled C; lanes 3 and 6), GST-fynSH3 (labeled SH3; lanes 4 and 7), or GST-Fyn SH2 (labeled SH2; lanes 5 and 8). C, phosphatase treatment eliminates p64 binding to the Fyn-SH2 domain. p64 and p59\(^{\text{SH2}}\) were co-expressed in HeLa cells and cell lysates prepared. Total protein (lane 1) or protein which bound to immobilized GST-Fyn SH2 fusion protein (lanes 2 and 3) was separated and probed with affinity purified anti-p64 antisera. Prior to binding to fusion protein, the lysate was treated with alkaline phosphatase (lane 3) or subjected to identical incubation in the absence of added phosphatase (lane 2). In panels B and C, the arrows mark the migration position of p64.

We determined whether p64 is a ligand for the SH2 or SH3 domains of Fyn, either when expressed in the absence or presence of p59\(^{\text{SH2}}\). p64 was expressed in HeLa cells either alone or with p59\(^{\text{SH2}}\). Cells were solubilized and proteins allowed to bind to immobilized GST (control), GST-Fyn SH3 fusion protein, or GST-Fyn SH2 fusion protein. Bound proteins were separated, blotted, and probed with p64 antibodies (Fig. 1B). When p64 is expressed in the absence of p59\(^{\text{SH2}}\), it does not bind to either SH2 or SH3 domains of Fyn. However, when co-expressed with p59\(^{\text{SH2}}\), p64 binds to GST-Fyn SH2 but not GST-Fyn SH3.

Typical SH2 binding requires tyrosine phosphorylation of the SH2 ligand (17, 18). To determine whether phosphorylation state affects the ability of p64 to bind to Fyn-SH2 domain, p59\(^{\text{SH2}}\) and p64 were coexpressed in HeLa cells and lysates prepared. Lysates were either treated with alkaline phosphatase or kept as control prior to exposure to immobilized Fyn-SH2. Bound proteins were separated and probed as before (Fig. 1C). Phosphatase pretreatment of the solubilized membranes from cells co-expressing p64 and p59\(^{\text{SH2}}\) eliminates the SH2 binding by p64. Taken together, these experiments indicate that 1) co-expression of p64 with p59\(^{\text{SH2}}\) results in tyrosine phosphorylation of p64, and 2) tyrosine phosphorylation of p64 renders it a ligand for the SH2 domain of Fyn.

There are a number of tyrosines within the p64 sequence which could serve as sites for phosphorylation and SH2 binding. A consensus for SH2 binding to Src family tyrosine kinases has been described. This consensus sequence is comprised of a phosphorylated tyrosine plus the 3 amino acids immediately C-terminal to it, the consensus sequence being Tyr(Glu, Asp, or Thr)(Glu, Asn, or Asp)Ile, Val, Met, or Leu) (17). Within the N-terminal half of p64, tyrosines at positions 20 (Tyr-Glu-Asp-Ser), 33 (Tyr-Asp-Glu-Val), and 56 (Tyr-Asp-Ser-Val) all show some similarity to this consensus, tyrosine 33 fitting most closely. Using site-directed mutagenesis, the codons for each of these tyrosines was changed to encode alanine in separate constructs, named p64(Y20A), p64(Y33A), and p64(Y56A), respectively. The resulting mutants were co-expressed with p59\(^{\text{SH2}}\) in HeLa cells and Fyn-SH2 binding was assessed (Fig. 2). We found that mutation of tyrosine 33 eliminates binding of p64 to Fyn-SH2 while mutation of tyrosines 20 and 56 have no effect on SH2 binding.

Proteins from cells expressing either p64 or the p64(Y33A) mutant with or without co-expression of p59\(^{\text{SH2}}\) were assessed for expression of p64 and p59\(^{\text{SH2}}\), for SH2 binding by p64, and for tyrosine phosphorylation (Fig. 3). In each panel of Fig. 3, lanes 1 and 2 are from cells not expressing p64, lanes 3–5 are from cells expressing wild type p64, and lanes 6 and 7 are from cells expressing p64(Y33A). Lanes 1, 3, and 6 are from cells without exogenous p59\(^{\text{SH2}}\), lanes 2, 4, and 7 are from cells expressing normal p59\(^{\text{SH2}}\), and lane 5 is from cells expressing a kinase-defective mutant of p59\(^{\text{SH2}}\) (28). Total protein probed for p64 (panel A) and p59\(^{\text{SH2}}\) (panel B) demonstrate the expected patterns of expression. In panel C, solubilized protein which bound to immobilized GST-Fyn SH2 fusion protein were separated, blotted, and probed for p64. As expected, only wild type.
Regulation of p64 by Fyn

The experiments using p64 and p59<sup>59k</sup> overexpressed in HeLa cells show that the interactions between p59<sup>59k</sup> and p64 are possible. To determine whether these interactions are relevant to the normal function of p64, we looked for evidence for p64-p59<sup>59k</sup> interactions using native material from bovine kidney. Bovine kidney membranes were solubilized in Triton X-100 in the presence of phosphatase inhibitors and solubilized protein was allowed to bind to immobilized GST (control) or GST-Fyn SH2 fusion protein. After extensive washing, protein was eluted from the beads, separated by SDS-PAGE, blotted, and probed with antibodies to p64 (Fig. 4A). We found a fraction of the total p64 in solubilized bovine kidney membranes binds to immobilized SH2 domain of Fyn.

To determine whether p64 may be stably associated with p59<sup>59k</sup> in vivo, solubilized bovine kidney membranes were subjected to an immunoprecipitation-in vitro kinase assay as shown in Fig. 4B. Solubilized bovine kidney membranes were immunoprecipitated with anti-p64 antisera (lane 1), non-immune sera (lane 2), or anti-Fyn antisera (lane 3). The immune complexes were collected on protein A beads, washed, and allowed to self-phosphorylate with [γ-<sup>32</sup>P]ATP. The proteins were then solubilized, separated by SDS-PAGE, and detected by autoradiography. Within such immunoprecipitates, p59<sup>59k</sup> and other Src family tyrosine kinases can phosphorylate themselves (32, 33). As expected, the Fyn antibody brought down an activity which phosphorylates a protein of about 59 kDa, consistent with the expected band of p59<sup>59k</sup> itself. The p64 antibody brought down an activity that labels a protein indistinguishable from the p59<sup>59k</sup> band, indicating that p59<sup>59k</sup> is stably associated with p64 in solubilized bovine kidney membranes. Of note, there is no apparent phosphorylation of p64 or of other proteins in these immunoprecipitates.

Many proteins are known to be ligands for various SH2 and SH3 containing proteins. In many cases, the functional significance of these interactions is unknown. To determine whether interaction with p59<sup>59k</sup> could have an effect on p64 channel activity, we expressed p64 in HeLa cells either alone or in combination with p59<sup>59k</sup>. Cells were lysed, membranes prepared, solubilized, reconstituted, and chloride channel activity assayed as described previously. In brief, the initial rate of valinomycin-dependent efflux of chloride from reconstituted vesicles is taken as the chloride permeability. We had previously demonstrated a small but significant increase in chloride permeability from vesicles reconstituted from p64-expressing cells as compared with control (3).

The results are shown in Fig. 5A. Reconstituted vesicles from control HeLa cells expressing neither exogenous p59<sup>59k</sup> nor p64 had a low rate of chloride efflux (0.628 ± 0.068%/s, n = 9). Expression of p59<sup>59k</sup> alone had no detectable effect on chloride efflux rate of reconstituted vesicles (0.601 ± 0.065%/s, n = 11). As noted previously (3), vesicles reconstituted with membrane protein from cells expressing p64 had a slightly elevated rate of efflux (0.755 ± 0.058%/s, n = 8) which, however, did not achieve statistical significance in these experiments. In contrast, reconstitution of membrane proteins from cells expressing both p59<sup>59k</sup> and p64 had a dramatically increased rate of chloride efflux (1.099 ± 0.141%/s, n = 17) which was significantly greater than that of vesicles reconstituted from cells either expressing p64 alone or no p64 (p < 0.01 in comparison with all other groups). Expression of p64(Y33A) resulted in no apparent increase in chloride permeability (efflux rate 0.6545 ± 0.054%/s, n = 11) and there was no effect on chloride permeability by co-expression of p64(Y33A) with p59<sup>59k</sup> (efflux rate 0.6545 ± 0.054%/s, n = 11).

FIG. 4. Native bovine p64 and Fyn. A, 20 µg of solubilized kidney membrane protein (lane 1) or those proteins which bound to immobilized GST (lane 2) or GST-Fyn SH2 fusion protein (lane 3) were separated, blotted, and probed with affinity purified anti-p64 antisera. The arrow marks the migration position of p64. B, solubilized kidney membrane protein was immunoprecipitated with antiserum raised against p64 (lane 1), with non-immune serum (lane 2), or with antiserum raised against p59<sup>59k</sup> (lane 3). Immunoprecipitates were allowed to self-phosphorylate using [γ-<sup>32</sup>P]ATP. Products were separated by SDS-PAGE and results detected by autoradiography. The arrow marks the expected migration position of p59<sup>59k</sup>. In both panels A and B, the migration position of molecular weight standards (in kilodaltons) is marked.

FIG. 3. SH2 binding and tyrosine phosphorylation of p64 when expressed with p59<sup>59k</sup>. Various combinations of p64 and p59<sup>59k</sup> were expressed in HeLa cells and lysates prepared. Each panel contains material from cells expressing neither exogenous p64 nor p59<sup>59k</sup> (lane 1), p59<sup>59k</sup> without p64 (lane 2), p64 without p59<sup>59k</sup> (lane 3), p64 with p59<sup>59k</sup> (lane 4), p64 with the kinase inactive form of p59<sup>59k</sup> (lane 5), p64(Y33A) without p59<sup>59k</sup> (lane 6), and p64(Y33A) with p59<sup>59k</sup> (lane 7). A, 25 µg of total lysate protein were separated, blotted, and probed with affinity purified antibody to p64. B, 25 µg of lysate protein were probed with antisera to p59<sup>59k</sup>. C, protein which bound to immobilized GST-Fyn SH2 fusion protein was separated, blotted, and probed for p64. D, 2 µg of total protein were separated, blotted, and probed with antibodies for phosphorysine. The arrowheads mark the migration position of p64 and p59<sup>59k</sup>.
To confirm that phosphorylation was critical to the enhanced chloride permeability of vesicles reconstituted from cells expressing p64 and p59\(^{\text{fyn}}\), HeLa cell extracts were treated with phosphatase inhibitors or with alkaline phosphatase prior to isolation of membrane fractions, solubilization, reconstitution, and chloride permeability assay. As presented in Fig. 5C, alkaline phosphatase treatment had no effect on chloride permeability of vesicles reconstituted from cells expressing no p64, or from cells expressing p64 in the absence of p59\(^{\text{fyn}}\). In contrast, alkaline phosphatase treatment of the whole cell lysate did decrease chloride permeability of vesicles reconstituted from cells expressing both p64 and p59\(^{\text{fyn}}\). Absolute rates of chloride efflux were: control 0.352 ± 0.032%/s, n = 4; control plus alkaline phosphatase, 0.363 ± 0.064%/s, n = 4; p64, 0.366 ± 0.032%/s, n = 5; p64 plus alkaline phosphatase, 0.374 ± 0.078%/s, n = 5; p64 + Fyn, 0.493 ± 0.029%/s, n = 5; and p64 + Fyn plus alkaline phosphatase, 0.323 ± 0.018%/s, n = 5. These results are from a single experiment.

A significant fraction of both native p64 in bovine kidney and exogenous p64 expressed in HeLa cells is present in a soluble, non-membrane-associated form. It has been recently proposed that regulation of one p64 homolog could be partially due to redistribution of the protein from a soluble to a membrane inserted form (13). We carried out initial experiments to determine whether the increased chloride channel activity conferred by Fyn could be due to redistribution of p64. In one set of experiments, HeLa cells expressing either p64 alone or p64 plus p59\(^{\text{fyn}}\) were fractionated and membrane and soluble fractions separated and probed for p64. We found no shift in distribution of p64 from the soluble to membrane-associated compartment when co-expressed with p59\(^{\text{fyn}}\) (not shown). In a second set of experiments, a stable transfected cell line expressing p64 (4) was transfected with a Fyn expression construct and the distribution of p64 determined using confocal immunofluorescence microscopy. Again, we detected no convincing redistribution of p64 in response to p59\(^{\text{fyn}}\) (not shown).

**DISCUSSION**

In this paper, we have explored the relationship between the Src family tyrosine kinase, p59\(^{\text{fyn}}\), and the bovine intracellular chloride channel, p64. We demonstrated that both native p64 from bovine kidney and exogenous p64 expressed in HeLa cells can interact with p59\(^{\text{fyn}}\) via SH2 binding, and that co-expression of p64 and p59\(^{\text{fyn}}\) results in enhanced p64-dependent chloride channel activity. These results identify a new type of protein-protein interaction for CLIC family proteins and provide new insights into how the channel activity of p64 might be regulated in vivo.

**p64 and p59\(^{\text{fyn}}\) in the HeLa Cell Overexpression System**—We have shown that p64 becomes tyrosine phosphorylated when co-expressed with p59\(^{\text{fyn}}\) in HeLa cells and that when co-expressed with p59\(^{\text{fyn}}\), p64 is a ligand for the SH2 domain of Fyn. p64 binding to Fyn-SH2 is phosphatase sensitive, consistent with the idea that tyrosine phosphorylation of p64 is necessary for p64 binding to Fyn-SH2.

We mapped the p64 domain necessary for Fyn-SH2 binding using directed mutagenesis and found that a single tyrosine residue at position 33 in the p64 sequence is necessary for p64 to be competent to bind Fyn-SH2. This tyrosine is the closest fit to the previously described consensus sequence for Src family SH2 binding partners. Taken together, this data strongly supports the notion that p64 can be phosphorylated at tyrosine 33 and that this phosphorylation renders p64 a ligand for the SH2 domain of Fyn.

Although tyrosine 33 is necessary for SH2 binding, it is apparent that other sites on the p64 molecule are tyrosine phosphorylated as well in the presence of p59\(^{\text{fyn}}\). There are
multiple other tyrosine residues in p64, some of which would fit the consensus for binding to other known SH2 domains. Whether these sites take part in binding interactions with SH2-containing proteins other than Fyn remains to be seen.

Our data show that p64 becomes tyrosine phosphorylated when co-expressed with p59\(^{\text{yn}}\) but we do not know whether p59\(^{\text{yn}}\) itself is directly responsible for phosphorylation of p64. p59\(^{\text{yn}}\) is known to activate cascades of kinases within cells and the phosphorylation of p64 could be carried out by an endogenous kinase which is activated directly or indirectly by the presence of p59\(^{\text{yn}}\).

In addition to potential SH2 binding sequences, we had noted repeated PXXP motifs in p64 which could be SH3-binding sites. In all the PXXP copies in p64, the XX residues are acidic, inconsistent with the known consensus for binding by Src family SH3 domains (14, 15). Nonetheless, we looked to see whether p64 could bind Fyn-SH3. As predicted, we found no binding. Whether the PXXP motifs of p64 are binding partners for other SH3 domains with distinct binding specificity is unknown.

The expression system used for these studies produces very high levels of both p64 and p59\(^{\text{yn}}\) in a short time in cells that are undergoing cytopathologic changes related to the vaccinia infection. Furthermore, the SH2 and SH3 binding experiments use high concentrations of both immobilized protein and soluble ligand. These conditions could drive low affinity interactions which might not be relevant to normal intracellular physiology. Therefore, the results from the HeLa expression system must be interpreted to mean that these interactions can happen, not that these interactions are necessarily significant under more normal conditions. Because of these considerations, we also looked for evidence of p64-p59\(^{\text{yn}}\) interactions using material from bovine kidney.

Native p64 and p59\(^{\text{yn}}\) in Bovine Kidney—We found that a fraction of the p64 in freshly solubilized bovine kidney membranes shows affinity for Fyn-SH2. Thus, the ability of p64 to act as an SH2 ligand is not limited to the overexpression system, but takes place with native material in a normal kidney. In contrast to the experiments with the overexpression system, the experiments with native material required larger amounts of starting material and more extensive washing of the beads to detect the results. In addition, Western blots of p64 immunoprecipitates from bovine kidney probed with anti-phosphotyrosine antibody did not detect phosphotyrosine in p64. This sort of experiment is particularly difficult since p64 runs only slightly larger than IgG heavy chain which would interfere with any Western blot signal. We interpret these results to mean that a smaller fraction of p64 is tyrosine phosphorylated and capable of SH2 binding in the native state than when overexpressed with p59\(^{\text{yn}}\) in HeLa cells.

Coupled immunoprecipitation-in vitro kinase labeling is an exquisitely sensitive method for detecting interactions between proteins and tyrosine kinases (32, 33). We found that immunoprecipitation of p64 from solubilized bovine kidney membranes brings down a kinase activity which labels a protein indistinguishable by SDS-PAGE mobility from authentic p59\(^{\text{yn}}\) brought down with anti-Fyn antisera. The simplest interpretation of this result is that a small fraction of p64 in bovine kidney membranes is associated with p59\(^{\text{yn}}\). The failure of the co-immunoprecipitated kinase to label p64 in these immunoprecipitates could indicate that only p64 which is already phosphorylated is stably associated with p59\(^{\text{yn}}\).

Although the labeled band in the p64 immunoprecipitate co-migrates with p59\(^{\text{yn}}\) on SDS-PAGE, we cannot be certain this band in fact represents p59\(^{\text{yn}}\). It could be another kinase of approximately the same molecular weight, or it could be a non-kinase protein which is the substrate for some other kinase, both of which were co-immunoprecipitated with p64. Because of its low abundance and the fact that it is almost the same size as IgG heavy chain, we have not been able to characterize this labeled protein in the p64 immunoprecipitation further. In light of all the data, our simplest explanation remains that this protein represents p59\(^{\text{yn}}\) which is stably associated with p64 in normal kidney membranes.

**Fyn and p64-related Channel Activity**—Expression of p64 in HeLa cells has been shown to result in the appearance of a novel chloride channel activity and a small increase in chloride permeability of whole cell membranes. In this study, we have shown that co-expression of p64 with p59\(^{\text{yn}}\) in HeLa cells leads to a dramatic increase in the p64-dependent chloride permeability. This result is dependent on the presence of both active Fyn tyrosine kinase and on the presence of tyrosine 33 in the p64 sequence.

Non-receptor tyrosine kinases and various SH2/SH3 interactions have now been implicated in regulation of a number of ion channels through a variety of mechanisms (19, 34–39). In general, non-receptor tyrosine kinases of the Src family are capable of interacting with other proteins in at least three ways, each of which could conceivably result in changes of activity of the target protein (20–23). The target protein may be directly phosphorylated by the tyrosine kinase and phosphorylation could directly alter activity or targeting of the protein. Second, phosphorylation of specific tyrosine residues within the sequence could render the target protein a ligand for other proteins containing an appropriate SH2 domain and this protein-protein interaction could alter target protein activity or targeting. Third, the kinase could bind to the protein directly via the kinase’s SH3 domain, or, in a tyrosine phosphorylation-dependent way, via the kinase’s SH2 domain, potentially altering activity or targeting. In both the SH2 and SH3 binding paradigm, activity or targeting could be directly effected by interaction with the kinase itself, or by clustering via the kinase with other regulatory molecules.

Our initial data provides some clues as to how Fyn might lead to increased p64 activity. Coexpression of p64 with normal p59\(^{\text{yn}}\) resulted in enhanced channel activity while coexpression with a p59\(^{\text{yn}}\) mutant which has lost its tyrosine kinase activity but retains capacity for SH2 and SH3 binding failed to enhance channel activity. Thus, the presence of intact Fyn-SH2 or -SH3 binding sites in the absence of the kinase is not sufficient to cause activation of p64. Second, treatment of cell lysates with alkaline phosphatase both eliminates the SH2 binding by p64 and eliminates the enhanced chloride permeability conferred by co-expression of p59\(^{\text{yn}}\) with p64, further supporting the importance of phosphorylation. However, alkaline phosphatase will act on many proteins in the cell lysate, and we cannot be certain that it is the dephosphorylation of p64 itself which is critical for the phosphatase effect on activity. Third, mutation at tyrosine 33 both eliminates the ability of p64 to bind to Fyn-SH2 domain when co-expressed with p59\(^{\text{yn}}\), and eliminates the Fyn-dependent activation of p64. These data indicate that phosphorylation of p64 at tyrosine 33 is necessary for Fyn-dependent activation of p64, but cannot distinguish whether phosphorylation alone is sufficient or whether subsequent binding of phosphorylated p64 to the Fyn-SH2 domain is also necessary for activation. Another possibility is that mutation of tyrosine 33 eliminates the activity of p64 altogether, independent of any effect of p59\(^{\text{yn}}\). Because of the low level of activity due to expression of p64 without Fyn in these experiments, we cannot eliminate this possibility. Finally, we did not find evidence for Fyn-dependent redistribution of p64, either assessing the fraction of p64 which is associated with mem-
Regulation of p64 by Fyn

branes, or by confocal immunofluorescent microscopy. These data strongly suggest that Fyn-dependent increased p64 channel activity is most likely due to a change in intrinsic activity of the protein rather than a change in distribution or targeting of p64. Our data cannot rule out a subtle shift in distribution, but we can be confident there is no large scale redistribution of p64 in the presence of p59Fyn.

Thus, activation of channel activity by p59Fyn requires the presence of active Fyn tyrosine kinase and phosphorylation of p64 at tyrosine 33. Furthermore, this activation is not due to some large scale redistribution of p64, but is likely due to direct activation of the channel itself.

In conclusion, we have found a novel mechanism by which p64 could be regulated in vivo. This information is of particular interest in that it links regulation of p64 and perhaps exocytosis or acidification of the regulated secretory vesicles in which it resides with one of the key regulatory cascades of the cell.

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