The Sites of Phosphorylation by Protein Kinase C and an Intact SH2 Domain Are Required for the Enhanced Response to β -Adrenergic Agonists in Cells Overexpressing *c-src*

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Previously we demonstrated that C3H10T $\frac{1}{2}$ murine fibroblasts overexpressing avian *c-src* exhibit elevated levels of cyclic AMP (cAMP) in response to β -adrenergic agonists compared with that in control cells and that this enhanced response requires *c-src* kinase activity (W. A. Bushman, L. K. Wilson, D. K. Luttrell, J. S. Moyers, and S. J. Parsons, *Proc. Natl. Acad. Sci. USA* 87:7462–7466, 1990). However, it is not yet known which components of the β -adrenergic receptor pathway, if any, interact with pp60^{*c-src*}. It has recently been shown that immune complexes of pp60^{*c-src*} phosphorylate recombinant G α proteins in vitro to stoichiometric levels, resulting in alterations of GTP binding and GTPase activity (W. P. Hausdorff, J. A. Pitcher, D. K. Luttrell, M. E. Linder, H. Kurose, S. J. Parsons, M. G. Caron, and R. J. Lefkowitz, *Proc. Natl. Acad. Sci. USA* 89:5720–5724, 1992), raising the possibility that the G α protein may be an in vivo target for the interaction with pp60^{*c-src*}. To further characterize the involvement of pp60^{*c-src*} in the β -adrenergic signalling pathway, we have overexpressed, in 10T $\frac{1}{2}$ cells, pp60^{*c-src*} containing mutations in several domains which are believed to be important for signalling processes. In this study we show that the sites of phosphorylation by protein kinase C (PKC) (Ser-12 and Ser-48) as well as the SH2 region of pp60^{*c-src*} are required for the enhanced response of *c-src* overexpressors to β -agonist stimulation. Mutation at the site of myristylation (Gly-2) results in a decrease in the enhanced response, while mutation at the site of phosphorylation by cAMP-dependent protein kinase (Ser-17) has no effect. Two-dimensional phosphotryptic analyses indicate that phosphorylation on Ser-12 and Ser-48 in unstimulated cells is associated with the ability of overexpressed pp60^{*c-src*} to potentiate β -adrenergic signalling. Cells overexpressing wild-type *c-src* also exhibit enhanced cAMP accumulation upon treatment with cholera toxin, an effect that is abated in cells overexpressing pp60^{*c-src*} defective in the kinase or SH2 domains or altered at the sites of phosphorylation by PKC. These studies provide the first evidence for the physiological significance of the pp60^{*c-src*} sites of PKC phosphorylation. In addition, they show that the SH2, Ser-12/48, and myristylation regions may be important for efficient interaction of pp60^{*c-src*} with components of the β -adrenergic pathway. Our data also support the possibility that the G α protein may be an in vivo target for alteration by pp60^{*c-src*}.

The *c-src* proto-oncogene encodes a tyrosine kinase of 60 kDa (10, 11). Cotranslational myristylation of pp60^{*c-src*} is required, though not sufficient, for attachment of the protein to the inner surface of the plasma membrane (6, 46), a location which places pp60^{*c-src*} proximal to transmembrane signalling events. Roles have been implicated for *c-src* in signalling pathways associated with several growth factor receptors. For example, pp60^{*c-src*} has been found to associate with activated platelet-derived growth factor receptors and to undergo alterations in phosphorylation which accompany increases in its tyrosine kinase activity (18, 28, 45). In response to stimulation by epidermal growth factor (EGF), C3H10T $\frac{1}{2}$ cells overexpressing *c-src* exhibit an enhanced mitogenic response associated with an increase in tyrosine phosphorylation of several cellular proteins (8, 33, 34, 55, 56).

Recent evidence obtained from a variety of cellular systems suggests that tyrosine kinases interact directly or indirectly with signalling pathways involving GTP-binding proteins. The GDP-bound α subunits of G α , G β , and transducin have been shown to be phosphorylated on tyrosine residues by the insulin receptor kinase (27, 59). Insulin

treatment also stimulates GTPase activity in human platelets (15). While the EGF receptor has been found to phosphorylate tyrosine residues of the α subunits of G β and G γ to substoichiometric levels, a low-molecular-weight GTP-binding protein appears to serve as a more effective substrate (21). The EGF-stimulated tyrosine phosphorylation results in a slight enhancement in the ability of the small GTP-binding protein to bind GTP γ S. In addition, a small GTP-binding protein has also been found associated with the CD4-p56^{*lck*} and CD8-p56^{*lck*} T-cell receptor complexes (51). Reports also suggest that heterotrimeric G proteins are involved in signalling mediated by the *v-fps* tyrosine kinase and the *fms* oncogene product (1, 24). Additionally, the *v-src* tyrosine kinase appears to play a role in signalling by the endothelin-1 receptor, a G protein-coupled receptor which contains seven membrane-spanning domains (35).

In C3H10T $\frac{1}{2}$ murine fibroblasts, we have found that the magnitude of cyclic AMP (cAMP) accumulation in response to β -adrenergic agonists is influenced by *c-src* overexpression (5). Cells which express abundant levels of wild-type (wt) *c-src* exhibit elevated levels of intracellular cAMP upon β -agonist treatment, while cells overexpressing kinase-defective *c-src* or pp60^{*v-src*} fail to exhibit this enhanced response. The stimulatory effects of β -adrenergic agonists are mediated through the G α protein which couples the receptor

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to adenylyl cyclase (16). Stimulation of the receptor results in the activation of adenylyl cyclase and intracellular formation of cAMP. Degradation of cAMP is catalyzed by cAMP phosphodiesterase (2). In experiments to determine which components of this pathway might be targets for interaction with the pp60^{c-src} kinase, Hausdorff et al. have shown that immune-complexed pp60^{c-src} is capable of stoichiometrically phosphorylating G_{sα}, as well as several other G_α subunit subtypes, on tyrosine residues (22). This phosphorylation results in functional alteration of the G_{sα} protein activity, increasing the rate of receptor-mediated GTPγS binding and GTP hydrolysis.

To further characterize the nature of the involvement of pp60^{c-src} in the β-adrenergic signalling pathway, we have overexpressed, in 10T½ cells, pp60^{c-src}-containing mutations in several domains which are believed to be of functional importance (37). The first mutation results in an alteration of the myristylation domain at the site of fatty acid modification which is required for membrane association of the protein. Two additional mutations lie within the N-terminal serine phosphorylation domain which contains the sites of phosphorylation by protein kinase C (PKC) and cAMP-dependent protein kinase. The final mutation results in an alteration of the SH2 domain which is thought to modulate the binding of pp60^{c-src} to other tyrosine-phosphorylated proteins (26, 41). Our results demonstrate that the sites of phosphorylation by PKC and an intact SH2 domain are critical for c-src potentiation of adenylyl cyclase activity. We also present evidence consistent with the notion that pp60^{c-src} alters the ability of G_{sα} to be stimulated in vivo.

MATERIALS AND METHODS

Cells, DNA, and transfections. The derivation of C3H10T½ murine fibroblast lines overexpressing wt avian c-src cDNA or c-src mutated at the site of myristylation or in the SH2 domain has been described previously (55). Cells were cotransfected with plasmids encoding G418 resistance (pSV2neo) (50) and avian c-src (pM5H) (55) by either the lipofectin (14) or the transfectam (30) method. Specific mutations at serine residues were generated by oligonucleotide-directed mutagenesis in M13 and verified by sequence analysis. Expression vectors were generated by replacing the *SalI*-*Bgl*III c-src-containing fragment from pM5H with a *SalI*-*Bgl*III fragment containing either the Ser-17 or the Ser-12 and Ser-48 mutations. Transfected G418-resistant cells were cultured in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal bovine serum and 0.4 mg of G418 per ml (GIBCO). Clonal cell lines were isolated and screened for c-src expression by Western immunoblotting (data not shown) with the ¹²⁵I-labelled 327 monoclonal antibody (MAb) (29) as previously described (55).

pp60^{c-src} immunoprecipitations and kinase assays. Immunoprecipitations and kinase assays, both auto- and transphosphorylation (with enolase as an exogenous substrate), were performed as described previously, with a mixture of the rodent and avian pp60^{c-src}-reactive MAb GD11 and MAb EC10, which is more specific for avian pp60^{c-src} (55). Immunoprecipitations used for two-dimensional phosphotryptic analyses were performed with MAb EC10.

Cell stimulations and cAMP assays. Assays for intracellular cAMP levels were performed as described previously (5) by the acetylated radioimmunoassay developed by Harper and Brooker (20). Assays were carried out by the University of Virginia Diabetes Core Laboratory. Confluent monolayers were serum starved for 20 to 24 h in DMEM containing 0.1%

bovine serum albumin (BSA; low-insulin-containing fraction V; Sigma) prior to refeeding with serum-free medium containing the indicated agents at 37°C for the times noted. Reactions were terminated by aspirating the medium and extracting cAMP from the monolayer with 0.1 N HCl for 1 h at room temperature. Long-term phorbol ester treatment was carried out by incubating cells for 24 h at 37°C in DMEM and 0.1% BSA media containing 200 nM 4-β-phorbol 12-myristate 13-acetate (PMA) (Sigma) in 0.1% dimethyl sulfoxide. Control cultures were treated with 0.1% dimethyl sulfoxide in DMEM and 0.1% BSA. Treatment with cholera toxin was carried out by incubating cells for 3 h at 37°C in DMEM containing 0.1% BSA and the indicated concentrations of cholera toxin (ICN Biochemicals) prior to preparation for cAMP assays.

In vivo PDE activity. Phosphodiesterase (PDE) activity was assessed in a two-step reaction as described by Sadler and Maller (49) in which [³H]cAMP is converted by PDE and 5'-nucleotidase of snake venom to [³H]adenosine. Product nucleosides are separated from unreacted substrate by anion-exchange chromatography. Briefly, confluent monolayers, either mock stimulated or treated for 2 min with 10 μM epinephrine, were scraped into ice-cold buffer (10 mM Tris-HCl [pH 7.4], 250 mM sucrose, 1 mM EDTA) and lysed by Dounce homogenization. Reactions were initiated by addition of 25 μl of the enzyme preparation to 100 μl of reaction mixture containing 40 mM Tris-HCl (pH 7.4), 0.5 μM cAMP, 10 mM MgCl₂, 0.04% BSA, and 30,000 to 50,000 cpm of [³H]cAMP. Following a 10-min incubation at 30°C, reactions were stopped by immersion in a boiling water bath. After cooling, 50 μg of *Crotalus atrox* venom (Sigma) was added to each tube. Following a 10-min incubation at 30°C, samples were placed in an ice bath. Nonhydrolyzed nucleotides were removed by addition of 1 ml of a 30% (wt/vol) suspension of Dowex AG1-X8 (Bio-Rad) in 3 mM acetic acid. Tubes were vortexed and then centrifuged briefly at 13,000 × g. Aliquots (400 μl) of each supernatant were removed and counted by liquid scintillation spectrophotometry. Results were corrected for nonspecific binding, which was determined by control reactions containing no enzyme.

Cell labelling. Cultures were labelled with 2 to 2.5 mCi of ³²P_i per ml for 16 to 20 h in phosphate-free DMEM supplemented with 0.1% BSA following a 30-min preincubation in phosphate-free DMEM without label. Stimulations were performed by addition of DMEM supplemented with 0.1% BSA and the desired final concentration of okadaic acid (Sigma), isoproterenol (Sigma), or PMA for the times indicated. Following stimulation, monolayers were washed with ice-cold phosphate-buffered saline (PBS), supplemented with 10 mM NaF and 100 μM Na₃VO₄, and then lysed in ice-cold radioimmunoprecipitation assay buffer (150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 7.2]) supplemented with 1% aprotinin, 100 μM Na₃VO₄, 10 mM NaF, and 50 μg of leupeptin per ml. Immunoprecipitations of pp60^{c-src} were carried out as described above.

Two-dimensional phosphotryptic analysis. For tryptic phosphopeptide analysis, in vivo ³²P-labelled pp60^{c-src}, immunoprecipitated with the avian-specific MAb EC10, was transferred from SDS-polyacrylamide gels onto Immobilon membranes. Trypsin digestion was carried out by the method of Luo et al. (31), and two-dimensional phosphotryptic analysis was performed at pH 8.9 for electrophoresis (4).

FIG. 1. Domains and mutations in pp60^{c-src} analyzed in this study. Mutations: pm2A, Gly-2 to Ala; Ser-12/48, Ser-12 to Cys and Ser-48 to Ala; Ser-17, Ser-17 to Cys; dl155, deletion of residues 155 to 157; and Ala-430, Ala-430 to Val.

RESULTS

Generation and characterization of cell lines overexpressing mutated c-src. In order to gain insights into the mechanism by which overexpression of pp60^{c-src} potentiates β -adrenergic signalling, C3H10T $\frac{1}{2}$ murine fibroblast cell lines that overexpress c-src mutated at regions thought to be important for signal transduction processes were generated. Figure 1 shows a schematic diagram of the regions of c-src that have been altered. The first mutant (pm2A) contains a Gly-to-Ala mutation at the site of myristylation, resulting in localization of pp60^{c-src} to the cytoplasm rather than to the plasma membrane (25, 55). The second mutant (dl155) contains a deletion in the SH2 domain, which is believed to mediate the interaction of pp60^{c-src} with other tyrosine-phosphorylated proteins or substrates (26, 41, 53, 55). The third mutant (Ser-17) contains a Ser-to-Cys alteration at residue 17, which has been shown to be the *in vitro* and *in vivo* site of phosphorylation by cAMP-dependent protein kinase (11, 19,

40). The fourth mutant (Ser-12/48) contains a Ser-to-Cys substitution at residue 12 and a Ser-to-Ala substitution at residue 48, the *in vitro* and *in vivo* sites of phosphorylation by PKC (19, 43).

It has been confirmed (see Fig. 6) that mutations at serine residues *in fact* have removed the predicted phosphorylation sites. pp60^{c-src} from cells overexpressing c-src mutated at either residue 17 or residues 12 and 48 was immunoprecipitated with MAb EC10, following labelling with ³²P_i. pp60^{c-src} from nonstimulated cells or cells stimulated for 10 min with the PKC-activating phorbol ester, PMA, was subjected to two-dimensional phosphotryptic analysis as described in Materials and Methods. While the phosphorylated residues 12 and 48 were present in pp60^{c-src} from PMA-stimulated wt c-src overexpressors (see Fig. 6A), tryptic peptide maps show the absence of these phosphorylations in the phorbol ester-treated Ser-12/48 mutant (see Fig. 6B). Phosphorylation of the tryptic peptide containing Ser-17 occurs *in vivo* in nonstimulated cells (19, 40) (see Fig. 6J) and is maintained in cells stimulated *in vivo* with phorbol ester (19) (see Fig. 6A). The tryptic peptide map from PMA-stimulated cells overexpressing c-src with a mutation at Ser-17 shows the presence of phosphorylated peptides containing Ser-12 and Ser-48 and the absence of phosphorylation of the peptide containing Ser-17 (see Fig. 6C).

Representative cell lines overexpressing mutated forms of c-src were characterized with respect to their level of c-src overexpression, specific tyrosine kinase activity, and cAMP accumulation in response to β -agonists. Table 1 shows that

TABLE 1. Characteristics of representative cell lines overexpressing c-src

DNA transfected (c-src mutation) and cell line	Relative expression ^a	Specific kinase activity ^b		cAMP ^c		
		Auto	Trans	NS	S	S/NS
pSV2neo						
NM	1	1.0	1.0	9.0 $\pm$ 0.8	99 $\pm$ 7	11 $\pm$ 1
Neod21	1	1.0	1.0	6.5 $\pm$ 0.7	78 $\pm$ 5	12 $\pm$ 1
pM5H (none) + pSV2neo						
5Hd47	35	1.3	1.1	8.0 $\pm$ 0.5	320 $\pm$ 21	40 $\pm$ 3
C5	15	1.6	0.8	7.7 $\pm$ 0.5	277 $\pm$ 30	36 $\pm$ 4
pM12/48 (Ser-12 to Ala, Ser-48 to Cys) + pSV2neo						
12/48L	30	0.8	1.3	7.4 $\pm$ 0.6	67 $\pm$ 5	9.0 $\pm$ 2
12/48F	24	1.4	1.2	6.8 $\pm$ 0.3	75 $\pm$ 7	11 $\pm$ 2
pM155 (deletion 155-157) + pSV2neo						
155L	18	1.5	1.0	6.9 $\pm$ 0.8	76 $\pm$ 9	11 $\pm$ 1
155J	11	1.3	0.9	8.3 $\pm$ 0.4	83 $\pm$ 10	10 $\pm$ 2
pM17 (Ser-17 to Cys) + pSV2neo						
17V	44	0.6	0.8	9.0 $\pm$ 0.3	396 $\pm$ 35	44 $\pm$ 12
17T	22	0.5	0.7	8.1 $\pm$ 0.4	259 $\pm$ 10	32 $\pm$ 6
pM2A (Gly-2 to Ala) + pSV2neo						
2AK	40	0.8	1.0	9.7 $\pm$ 0.7	204 $\pm$ 7	21 $\pm$ 4
2AL	15	1.1	0.7	9.1 $\pm$ 0.7	200 $\pm$ 23	22 $\pm$ 2

^a pp60^{c-src} was quantitated in each cell line by laser densitometry of Western immunoblot autoradiographs prepared by immunoprecipitation of pp60^{c-src} as described in Materials and Methods. Values were normalized to the level of endogenous pp60^{c-src} found in NM and Neod21 cells (arbitrarily set at 1).

^b Specific activity values represent the extent of autophosphorylation or phosphorylation of the exogenous substrate enolase, normalized to the amount of pp60^{c-src} present in the immune complex. The amount of pp60^{c-src} was determined by Western immunoblotting, and phosphotransferase activities were measured by the *in vitro* immune complex assay, as described in Materials and Methods. Values obtained were corrected for the contribution of endogenous pp60^{c-src}. Specific activities are calculated as the ratio of activity of overexpressed pp60^{c-src} to the specific activity of endogenous pp60^{c-src} from NM and Neod21 cells (arbitrarily set at 1). For comparison, auto- and transphosphorylation activities of pp60^{c-src} were 3.5 and 2.4, respectively.

^c Intracellular cAMP values (in picomoles per milligram of cell protein) were determined as described in Materials and Methods for nonstimulated cells (NS) or cells stimulated with 10 μ M epinephrine for 2 min at 37°C (S). Results are an average of at least two independent experiments, each performed in duplicate. Average fold increases in cAMP were determined by dividing stimulated values by nonstimulated values (S/NS).

FIG. 2. cAMP accumulation in cells overexpressing *c-src* mutants. Cell lines expressing various levels of mutated *c-src* were assayed for levels of intracellular cAMP following a 2-min treatment with 10 μ M epinephrine. cAMP levels were determined by averaging the results of at least two assays per clonal line; each assay was performed in duplicate. Results show the average fold increase in cAMP accumulation (mean $\pm$ standard error of the mean). The average responses of Neo control cell lines and cell lines expressing various levels of wt *c-src* are included for comparison. An analysis of these cell lines and cells expressing kinase-defective *c-src* has been shown previously (5).

both the basal auto- and transphosphorylation in vitro-specific activities of the pp60^{c-src} mutants, 2A, dl155, Ser-12/48, and Ser-17, are comparable to those of endogenous pp60^{c-src} and overexpressed wt pp60^{c-src}, while those of the transforming protein, pp60^{v-src}, were higher (see footnote c). No changes in specific activity were detected in wt or mutant *c-src* proteins upon stimulation with β -agonists (data not shown). Intracellular cAMP accumulation was measured following treatment of cell lines for 2 min with 10 μ M epinephrine, as described in Materials and Methods. As shown in Table 1, cells overexpressing abundant levels (≥ 10 -fold above endogenous) of unmyristylated pp60^{c-src} or the Ser-17 mutant sustained the enhanced response that was previously observed in wt *c-src* overexpressors (5). In contrast, cells that overexpressed high levels of pp60^{c-src} mutated in the SH2 region or at the sites of phosphorylation by PKC failed to exhibit the enhanced response. Each cell line exhibited nearly identical basal levels of intracellular cAMP. Similar results were obtained following stimulation with 10 μ M β -specific agonist isoproterenol (data not shown). From these data we conclude that the variation in responses to β -agonists exhibited by the different cell lines was not due to significant alterations in specific kinase activity of overexpressed pp60^{c-src} mutant proteins or to differences in basal levels of cAMP accumulation.

Effects of the level of *c-src* mutant expression on β -adrenergic stimulated cAMP accumulation. Previous results have shown that the enhanced response of wt *c-src* overexpressors to epinephrine stimulation is dependent on a threshold level of *c-src* expression (5). To determine whether the level of expression of mutated *c-src* affected epinephrine-stimulated cAMP accumulation, multiple cell lines expressing various levels of mutated *c-src* were derived and assayed for response to β -agonist treatment. As shown before with cells overexpressing wt *c-src* (5), the enhanced response of cells overexpressing unmyristylated and Ser-17-mutated pp60^{c-src} was dependent on a threshold level of *c-src* expression (Fig. 2). Cells expressing ≥ 10 -fold above endogenous levels of wt *c-src* or Ser-17 mutant *c-src* exhibited hormone-stimulated cAMP accumulations that averaged 4- and 4.5-fold, respectively, above control cells. Cells expressing abundant levels of the unmyristylated *c-src* exhibited a slightly lower enhanced response, averaging 2.6-fold above control cells. Cells expressing lesser amounts of mutated *c-src* failed to exhibit an enhanced response. In contrast, cells overexpressing the SH2 or Ser-12/48 mutant, even at levels ≥ 10 -fold above endogenous levels, did not show the enhanced response. No differences in the basal cAMP levels were found between the *c-src* transfectant lines and the neo controls. From these results we conclude that serine resi-

A.

B.

FIG. 3. Time course and agonist concentration dependence. The cell lines described in Table 1 were assayed for intracellular cAMP accumulation following treatment for various times with 10 μ M epinephrine (A) or for 2 min with the indicated concentrations of epinephrine (B). Results are expressed as fold increase in cAMP (A) or as percent maximal response (B) (mean $\pm$ standard error of the mean). Assays were performed in duplicate for each cell line.

dues 12 and 48, as well as an intact SH2 domain, are necessary for overexpressed pp60^{c-src} to potentiate β -agonist-induced signalling.

Time course and agonist concentration dependence of response of cells overexpressing mutated c-src. Since cell lines overexpressing the Ser-12/48- or dl155-mutated c-src failed to elicit an enhanced β -agonist-induced cAMP accumulation, we sought to determine whether these cell lines exhibited an altered time course of response to agonist stimulation. Representative clonal cell lines expressing ≥ 10 -fold above endogenous levels of wt or mutated c-src were assayed for cAMP accumulation at various times following epinephrine treatment. Figure 3A shows the average time course of stimulation of two clones of each mutant overexpressor cell line, control cell lines, and wt c-src overexpressors. Peak cAMP concentrations were achieved at similar times (1 to 3 min) after agonist treatment, followed by a

FIG. 4. Phosphodiesterase activity in c-src overexpressors. Control cells (Neo21) and a cell line overexpressing ≥ 10 -fold above endogenous levels of wt c-src (5Hd47) were mock stimulated or treated for 2 min with 10 μ M epinephrine before assaying for phosphodiesterase activity. Results are expressed as total counts per minute. Assays were performed in duplicate.

gradual decline approaching nonstimulated levels by 30 min. The lack of an elevated response in the dl155 and Ser-12/48 mutants, therefore, is not due to an alteration in the kinetics of response following stimulation of these cells.

In order to determine whether the agonist concentration dependence of the Ser-12/48 and dl155 mutant overexpressors differed from that of control cells and wt c-src overexpressors, the same clones used in the preceding experiment were stimulated for 2 min with various concentrations of epinephrine. While cAMP levels averaged 4-fold greater for the wt and Ser-17 overexpressors and 2.5-fold for cells overexpressing unmyristylated c-src (as in Table 1), Fig. 3B shows that the concentration-dependent responses of all cell lines were similar. We conclude that the inability of overexpressed Ser-12/48 and dl155 to potentiate agonist-induced signalling is not due to an altered agonist concentration dependence in cells overexpressing these mutants.

Phosphodiesterase activity of cells overexpressing c-src. Elevated levels of intracellular cAMP in c-src overexpressors may result from a potentiation of adenylyl cyclase activity in response to β -adrenergic agonists or a decrease in cAMP phosphodiesterase activity in comparison to that of control cells. In order to distinguish between these possibilities, we determined the *in vivo* PDE activity of control cells and c-src overexpressors. Basal and epinephrine-induced PDE activities were equivalent in both cell lines, with epinephrine treatment causing an approximate 37% increase in PDE activity (Fig. 4). Thus, the enhanced response of overexpressors to β -adrenergic agonists is not accounted for by differences in cAMP phosphodiesterase activity.

β -Adrenergic stimulation following down-regulation of PKC. Since stimulation of cells overexpressing c-src mutated at the sites of phosphorylation by PKC fails to elicit an enhanced cAMP accumulation, we were interested in determining whether PKC activity is needed for the C3H10T $\frac{1}{2}$ cell line to respond to β -agonist treatment. Control cells or cells overexpressing wt or Ser-12/48 mutant c-src were stimulated with 10 μ M epinephrine following down-regulation of PKC by long-term (24-h) treatment with the phorbol ester PMA. Prolonged treatment with phorbol esters has been shown to greatly decrease events requiring PKC activity in a number of cell types (12, 39, 47). As shown in Fig. 5, stimulation of cells with 10 μ M epinephrine, following chronic phorbol ester treatment, resulted in an average

FIG. 5. Effects of down-regulation of PKC on epinephrine-stimulated cAMP accumulation. Control cells and a cell line expressing ≥ 10 -fold above endogenous levels of wt or Ser-12/48-mutated *c-src* were treated for 24 h with 200 nM PMA prior to treatment for 2 min with 10 μ M epinephrine. Results of a representative experiment (among 3) show the average fold increase in cAMP accumulation (mean $\pm$ standard error of the mean). Assays for each cell line were performed in duplicate: Neo control, Neod21; Wt *c-src* overexpressor, 5Hd47; and Ser-12/48 mutant, 12/48L.

threefold inhibition in the agonist-induced cAMP accumulation in control cells and Ser-12/48 overexpressors and an average sixfold reduction in the response of wt *c-src* overexpressor cells. Similar results were obtained when cells were stimulated with isoproterenol following phorbol ester treatment (not shown). Thus, the enhanced response of cells overexpressing wt *c-src* was partially blocked following down-regulation of PKC. From these data we conclude that PKC activity, at least in part, is required for the ability of these cell lines to respond to β -agonist treatment and for the *c-src* overexpressors to exhibit the elevated response to β -agonist treatment.

Two-dimensional phosphotryptic peptide analysis of *c-src* overexpressors. Since the mutation of serines 12 and 48 abolishes the ability of overexpressed *c-src* to potentiate β -adrenergic signalling, we sought to determine whether these residues in wt pp60^{c-src} become phosphorylated on stimulation of cells with β -adrenergic agonists. Phosphorylation of these residues was barely detectable in wt pp60^{c-src} from nonstimulated cells (Fig. 6J) or following treatment with epinephrine or isoproterenol or when extracts were prepared in the presence of 10 mM general phosphatase inhibitor NaF (not shown). Panel E shows two-dimensional peptide analysis of overexpressed wt pp60^{c-src} from cells treated for 20 min with okadaic acid, an inhibitor of type 1 and 2a serine phosphatases (9). Treatment of cells with

FIG. 6. Tryptic phosphopeptides of pp60^{c-src} from cells expressing ≥ 10 -fold above endogenous levels of wt or mutated *c-src*. pp60^{c-src} from C3H10T $\frac{1}{2}$ cells following *in vivo* ³²P labelling and stimulation with various agents was subjected to two-dimensional phosphotryptic analysis as described in Materials and Methods. (A to D) Phosphopeptides of pp60^{c-src} following treatment for 10 min with 200 nM PMA of cells overexpressing wt *c-src* (5Hd47) (A), Ser-12/48 mutant (12/48L) (B), Ser-17 mutant (17V) (C), and unmyristylated *c-src* (2Ak) (D). (E to H) Phosphopeptides of pp60^{c-src} from cells overexpressing wt *c-src* (E), Ser-12/48 mutant (F), Ser-17 mutant (G), or unmyristylated *c-src* (H) treated for 20 min with 1 μ M okadaic acid. The lower relative phosphorylation of Tyr-527 and Tyr-416 in panel F is not reproducible. (I) Phosphotryptic pattern of wt pp60^{c-src} from cells which were pretreated with okadaic acid prior to stimulation for 2 min with 10 μ M isoproterenol. (J) Phosphotryptic pattern of wt pp60^{c-src} from untreated cells. (K) Schematic diagram of pp60^{c-src} phosphopeptides showing migration of fragments containing Tyr-527, Tyr-416, Ser-48, Ser-17, Ser-12, and Ser-12 (incomplete). Electrophoresis was performed in the horizontal dimension with the anode on the left. Arrows indicate spotting origins. Cerenkov counts per minute loaded onto thin-layer plates and exposure times (at -70°C with intensifying screens) are as follows: panel A, 1,740, 2 days; panel B, 1,220, 3 days; panel C, 1,070, 3 days; panel D, 1,538, 2 days; panel E, 1,880, 2 days; panel F, 390, 5 days; panel G, 1,395, 3 days; panel H, 1,688, 2 days; panel I, 1,548, 2 days; panel J, 1,670, 2 days.

FIG. 7. cAMP accumulation in *c-src* overexpressors treated with cholera toxin. Clonal cell lines overexpressing ≥ 10 -fold above endogenous levels of wt or mutated *c-src* were treated for 3 h with the indicated concentrations of cholera toxin, and cAMP concentrations were assayed as described in Materials and Methods. Results show the average (mean $\pm$ standard error of the mean) intracellular cAMP accumulation (picomoles per milligram of cell protein) of two clonal lines of each cell type represented in Table 1 and in reference 5 (kinase-defective mutant). Assays were performed in duplicate for each clone.

phosphatase inhibitor allowed the detection of phosphorylated peptides containing residues 12 and 48 (compare with panel A), though these residues are phosphorylated to a lesser extent than in the phorbol ester-treated cells. When cells overexpressing the Ser-12/48 mutant were similarly treated, these phosphopeptides were not detected (panel F). Panel G shows the presence of phosphopeptides containing Ser-12 and Ser-48 in the cell line overexpressing the Ser-17 mutant following okadaic acid treatment, while phosphopeptides containing Ser-17 were not detected. Okadaic acid treatment of cells overexpressing the unmyristylated mutant also resulted in phosphorylation of peptides containing Ser-12 and Ser-48 (panel H), as did treatment of these cells with phorbol ester (panel D). Stimulation of the wt *c-src* overexpressor with 10 μ M isoproterenol (panel I) or epinephrine (not shown), following okadaic acid pretreatment, yielded a phosphotryptic profile similar to that of cells treated with okadaic acid alone. From these data we conclude that $pp60^{c-src}$ from wt overexpressor cells is phosphorylated basally on Ser-12 and Ser-48, either transiently or at a low stoichiometry, such that it is readily apparent only upon inhibition of phosphatase activity by okadaic acid treatment.

Treatment of wt and mutant overexpressors with cholera toxin. The observation that both the kinase-defective (5) and SH2-defective *c-src* overexpressors fail to elicit an enhanced hormone-induced stimulation of adenylyl cyclase suggests that $pp60^{c-src}$ may be interacting with, and phosphorylating, one or more components of the β -adrenergic pathway. In order to determine whether the G_{sa} protein function might be altered by $pp60^{c-src}$ in vivo, we stimulated cells overexpressing wt *c-src* with cholera toxin, an agent that ADP-ribosylates and activates the G_{sa} subunit, and compared the levels of cAMP accumulation of control cells with those of cells overexpressing mutated forms of *c-src*. As shown in Fig. 7, in comparison to control cells or cells expressing abundant levels of Ser-12/48 or SH2-mutated *c-src*, cells that overexpress abundant levels of wt or Ser-17-mutated *c-src*

exhibited an enhancement in the levels of cAMP accumulated following treatment with various doses of cholera toxin. Cells overexpressing unmyristylated $pp60^{c-src}$ exhibited cAMP accumulations approximately twofold lower than that of wt overexpressors. From these results we suggest that overexpressed *c-src* may affect β -adrenergic signalling at the level of the G protein or further downstream in the pathway. In addition, the kinase activity, SH2 domain, and sites of phosphorylation by PKC are required for an enhanced cholera toxin-induced cAMP accumulation in cells overexpressing $pp60^{c-src}$.

DISCUSSION

The results presented here demonstrate that in order for $pp60^{c-src}$ to potentiate signalling through the β -adrenergic receptor pathway, the overexpressed protein must, in addition to being active as a kinase (5), contain the serine residues which are the targets of phosphorylation by PKC, as well as an intact SH2 domain. Although the mechanism by which $pp60^{c-src}$ affects β -adrenergic signalling remains unclear, analysis of these mutants provides us with some insights. Our previous studies showing the requirement for the kinase activity of $pp60^{c-src}$ for potentiation of this system, together with a requirement for an intact SH2 domain, suggest that $pp60^{c-src}$ interacts with and phosphorylates a component of this signalling pathway.

G_{sa} as a $pp60^{c-src}$ substrate. In vitro evidence suggests that one possible substrate is the G_{sa} protein (22). These data are supported by the evidence presented here which shows that cells overexpressing wt *c-src* exhibit an elevated level of G_{sa} activation by treatment with cholera toxin, suggesting that G_{sa} may be an in vivo target of $pp60^{c-src}$. Numerous attempts to demonstrate an in vivo phosphorylation of G_{sa} on tyrosine residues have yielded suggestive, but not definitive, evidence. G_{sa} has been detected in antiphosphotyrosine immunoprecipitates (by Western blotting with G_{sa} antisera) (22), but this result does not rule out the possibility that the G_{sa} protein is precipitated in a complex with other phosphotyrosine-containing proteins. In addition, some G_{sa} antibodies which are capable of immunoprecipitating unphosphorylated recombinant G_{sa} are substantially less efficient in precipitating G_{sa} which has been phosphorylated in vitro by $pp60^{c-src}$ (21a). These results suggest that phosphorylation of G_{sa} by $pp60^{c-src}$ reduces the ability of anti- G_{sa} to bind its cognate protein. Thus, other strategies may be required to pursue the question of G_{sa} tyrosine phosphorylation in vivo.

Other possible $pp60^{c-src}$ targets. That cholera toxin stimulation elicits elevated levels of cAMP in *c-src* overexpressors does not rule out the possibility that adenylyl cyclase itself, or other components downstream of G_{sa} , may be altered in these cells. In vivo stimulation with forskolin, a direct activator of adenylyl cyclase, does not clarify this issue, since numerous experiments measuring cAMP accumulation in response to forskolin treatment have yielded variable results in our hands (unpublished observations). Recent in vitro evidence suggests that the potentiating effects of *c-src* on this signalling system may, in fact, be mediated through more than one component of this pathway. In membranes from 10T $\frac{1}{2}$ cells overexpressing *c-src*, direct stimulation of G_{sa} with NaF results in a 30% greater increase in adenylyl cyclase activity in comparison to membranes from control cells, while no difference was detected upon activation of G_{sa} with GTP γ S (32). Stimulation of membranes with Mn $^{2+}$ and forskolin, direct activators of adenylyl cyclase, resulted in a slight reduction in the stimulation of cyclase in the

overexpressor relative to the control cell membranes. These data suggest that pp60^{c-src} interacts not only with the G_{sa} protein but also with adenylyl cyclase and possibly other components of the β -adrenergic pathway. A direct interaction with the receptor or β subunits appears to be unlikely, however, since pp60^{c-src} is unable to phosphorylate any of these proteins efficiently in vitro (21a). In addition, we have shown here that cAMP phosphodiesterase activity, in either unstimulated or stimulated cells, is not altered by overexpression of *c-src* (Fig. 4). Whether *c-src* is capable of affecting signalling through the inhibitory protein, G_i, remains to be clarified. Preliminary evidence from our laboratory indicates that the ability of pertussis toxin to reverse adenosine inhibition of isoproterenol-induced cAMP accumulation is impaired in *c-src* overexpressors compared with that in control cells (31a). These data suggest that pp60^{c-src} interacts with and functionally alters G_{ia} in a manner similar to G_{sa}.

Ser-12/48 and SH2 domains as potential sites of protein-protein interaction. Agents that activate phosphatidylinositol turnover (platelet-derived growth factor, serum, fibroblast growth factor, vasopressin, and prostaglandin F_{2a}) also induce the phosphorylation of the Ser-12 residue in pp60^{c-src} (37). Nevertheless, a functional role for these phosphorylations has not been found. It has previously been observed that alteration of Ser-12 of *v-src* has little effect on its transforming capabilities in chicken cells, while expression of elevated levels of *c-src* mutated at this residue results in subtle alterations in the morphology of Rat-1 cells and a concomitant increase in anchorage-independent growth (37). The data presented here suggest that Ser-12 and Ser-48 are important for the ability of overexpressed pp60^{c-src} to affect β -adrenergic receptor signalling and regulation of the second messenger, cAMP. Phosphorylation of pp60^{c-src} on Ser-12 and Ser-48 appears to occur in unstimulated cells transiently or at a low stoichiometry, since phosphorylated peptides were detectable only in the presence of the phosphatase inhibitor okadaic acid. Okadaic acid pretreatment, however, did not appear to significantly alter the cAMP response of control cells or cells expressing ≥ 10 -fold above endogenous levels of wt *c-src* following β -agonist treatment, while acute PMA pretreatment yielded variable results which were difficult to interpret (data not shown). The possibility remains, however, that pretreatment with okadaic acid or phorbol ester could lower the threshold level of *c-src* expression that is necessary in order to observe an enhancement in cAMP accumulation.

In addition to pp60^{c-src}, PKC interacts with several components of the β -adrenergic receptor pathway, being capable of phosphorylating the β -receptor (3), G_{sa} (44), and adenylyl cyclase (58) proteins. Thus, identifying the nature of the influence of PKC on the interaction of pp60^{c-src} with this signalling pathway is a complicated task. It appears that interaction between PKC and the *src* protein occurs independently of receptor stimulation, since we could detect no alteration in the levels of Ser-12 and Ser-48 phosphorylation upon β -agonist treatment.

The phosphorylation of pp60^{c-src} by PKC may represent a mechanism of regulating the responsiveness of cells to β -agonist stimulation, perhaps by affecting the ability of *src* to interact with one or more components of the pathway. This notion is supported by the finding that pretreatment of 10T $\frac{1}{2}$ cells with phorbol ester, prior to immunoprecipitation of pp60^{c-src}, results in an increase in the stoichiometry of G_{sa} phosphorylation in in vitro assays (22). Precedence for phosphoserine/phosphothreonine-dependent protein-protein

interactions is also provided by the BCR-ABL paradigm, in which phosphorylation of the BCR protein on serine and threonine residues appears to mediate binding to the SH2 domain of the *c-abl* protein (42). The possibility exists, however, that the Ser-12/48 mutations disrupt a conformation, rather than a phosphorylation event, necessary for the association of pp60^{c-src} with proteins involved in β -adrenergic signalling.

Binding of pp60^{c-src} with the same or other components of the pathway may also be mediated via the SH2 region, which must be intact in order for *c-src* to potentiate the β -agonist-induced signal. Studies have shown that peptides containing this region of pp60^{c-src} bind tyrosine-phosphorylated proteins which are believed to be components of signalling complexes (26, 41). In fact, recent crystal structure analyses show that Arg-155, which is deleted in the mutant used in these studies, is a crucial residue in the phosphate-binding pocket of the SH2 domain of *v-src* (52) and *c-abl* (36). The identities of the in vivo substrate(s) of pp60^{c-src} and the protein(s) interacting with the SH2 domain and the Ser-12/48 region are the focus of our current investigations.

Ser-17 mutants of pp60^{c-src}. The site of phosphorylation by cAMP-dependent protein kinase (Ser-17) is not critical for the transforming capabilities of *v-src* (13). Hirota et al. reported that overexpressed *c-src* mutated at this residue exhibits a slight elevation in kinase activity in chicken embryo fibroblasts with no morphological alteration (23). Similarly, mutation of pp60^{c-src} and pp60^{c-src}(F527) at Ser-17 does not affect the specific activity or transforming activity of these proteins in NIH 3T3 cells (57). We found that overexpression of *c-src* mutated at Ser-17 in 10T $\frac{1}{2}$ fibroblasts was not associated with a striking alteration in either specific kinase activity of the protein or agonist-induced cAMP accumulation, indicating that phosphorylation on this residue may not contribute to potentiation of β -agonist signalling mediated by *c-src* kinase.

Myristylation mutants of pp60^{c-src}. Membrane attachment is required for transformation by pp60^{c-src} (25) and for potentiation of EGF-induced mitogenic signalling by pp60^{c-src} (55). Overexpression of unmyristylated pp60^{c-src} is incapable of potentiating adenylyl cyclase activity to the same extent as the wild-type protein (Fig. 2, 3, and 7). Since unmyristylated pp60^{c-src} retains the ability to be phosphorylated on Ser-12 and Ser-48, either basally or following phorbol ester treatment (Fig. 6), the cytosolic localization of this mutant appears to be responsible for its inability to fully potentiate β -adrenergic signalling. Since we would expect that wt pp60^{c-src} exerts its effects at the membrane, it is surprising that the mutated protein can participate in the adrenergic pathway signalling events at all. We suggest two possibilities for this finding: first, the substrates for pp60^{c-src} are accessible for interaction with the cytosolic protein, and, alternatively, other regions of pp60^{c-src}, such as the Ser-12/48-containing region or SH2 domain, may themselves mediate interaction between pp60^{c-src} and membrane-bound proteins (see above), juxtaposing *src* with signalling events at the cell surface. Similar explanations were offered by Calothy et al. (7) for their observations that unmyristylated pp60^{v-src} was capable of stimulating proliferation but not transformation of chicken embryo cells.

Requirement for high levels of *c-src* overexpression. Finally, it is intriguing to note that a threshold level of pp60^{c-src} expression (≥ 10 -fold above endogenous levels) is necessary for wt *c-src* and the Ser-17 and unmyristylated mutants to potentiate β -adrenergic signalling. Such high levels of pp60^{c-src} in 10T $\frac{1}{2}$ cells could be required if the overex-

pressed protein interacted with other G_α subtypes in addition to G_{sa}. If pp60^{c-src} were acting catalytically, high levels of expression may not be required. If, however, pp60^{c-src} were acting in a noncatalytic manner, such as regulating protein-protein interactions through sites of PKC phosphorylation or the SH2 region, greater numbers of *src* molecules may be required to cause an observable effect on signalling pathways. It is of further interest to note that levels of pp60^{c-src} comparable to those found in *c-src* overexpressors are also found in platelets (17) and neuronally derived cells (38, 48, 54) in which G protein-mediated signalling events play a prominent role in cellular function. Additional studies are necessary to clarify the mechanism of pp60^{c-src} involvement in such signalling pathways.

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