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Overexpression of c-*src* enhances β-adrenergic-induced cAMP accumulation

(tyrosine kinase/guanine nucleotide-binding regulatory protein/adenylyl cyclase/signal transduction)

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ABSTRACT **During our investigations into the physiological role of c-*src* tyrosine kinase in normal cells, we found that clonal transfectants of C3H10T1/2 murine fibroblasts overexpressing chicken c-*src* exhibited strikingly elevated levels of cAMP accumulation in response to adrenergic stimulation as compared to control cells. Enhanced cAMP accumulations were detected when cells were treated with the β-agonists, epinephrine, isoproterenol, or terbutaline and were blocked by treatment with the β-specific antagonist propranolol, indicating action through β-adrenergic receptors. The hyperresponsiveness was not observed in cells overexpressing kinase-defective c-*src*. No differences in basal levels of cAMP, agonist concentration dependence, or kinetics of cAMP accumulation were detected between cells containing elevated levels of wild-type or kinase-defective c-src protein and control cells. To determine if the degree of c-*src* overexpression could influence the response, multiple clones, transfected with DNA encoding genes for wild-type or kinase-defective c-*src* plus neomycin resistance or neomycin resistance alone, were derived in parallel and assayed for the amounts of c-src protein produced and the levels of cAMP accumulated in response to epinephrine. Only clones with abundant wild-type c-src protein (>10-fold above endogenous) exhibited enhanced cAMP accumulation, averaging 3.3-fold above control cells. We conclude, therefore, that the enhanced degree of cAMP accumulation in cells overexpressing c-*src* is dependent upon activation of β-adrenergic receptors and upon a threshold level of $pp60^{c\text{-}src}$ that retains full tyrosine kinase activity.**

$pp60^{c\text{-}src}$ is a prototype of the non-receptor protein-tyrosine kinase family, whose members have been implicated in regulating transmembrane signaling events (1, 2). Evidence is accumulating to indicate a role for the src-like kinases not only in pathways involving receptors that exhibit intrinsic protein-tyrosine kinase activities, such as the epidermal growth factor (EGF) (3–5) and platelet-derived growth factor (6–8) receptors, but also in pathways involving receptors that lack such activities. This latter category is exemplified by the CD4/CD8 surface antigens of murine and human lymphocytes that form functional complexes with $p56^{lck}$ (9–13), the acetylcholine receptor and its associated modulatory tyrosine kinase of *Torpedo californica* synaptic membranes (14), and the nerve growth factor receptor, which upon activation induces an increased phosphotyrosine content of specific cellular proteins in PC-12 cells (15). The identity of the tyrosine kinases mediating the latter two phosphorylations remains to be elucidated; however, $pp60^{c\text{-}src}$ and/or members of the src-like family are excellent candidates to catalyze these reactions.

Upon receptor binding, virtually all ligands that activate tyrosine kinases also activate one or more serine/threonine kinases. The latter group of kinases includes the cAMP-dependent kinases, Ca^{2+}/phospholipid-dependent protein kinases [protein kinase C (PKC) family], microtubule-associated protein kinase, and Ca^{2+}/calmodulin-dependent protein kinases (16–19). However, not all ligands that induce the activation of serine/threonine kinases have been shown to activate tyrosine kinases. Examples of such ligands include the β-adrenergic agonists that bind receptors coupled through guanine nucleotide-binding regulatory proteins to adenylyl cyclase, resulting in increased cAMP production and stimulation of cAMP-dependent serine/threonine kinases (17, 20–22). Nevertheless, recent evidence supports the hypothesis that functionally relevant interactions do occur after receptor activation between components of pathways traditionally thought to contain either serine/threonine kinases or tyrosine kinases (cross-talk). In some instances both types of kinases may be contained within the same pathway. Evidence for these interactions includes: (*i*) phosphorylation of the EGF receptor by PKC, a modification that results in a reduction of ligand-binding affinity (23, 24); (*ii*) tyrosine phosphorylation and concomitant activation of phospholipase C after stimulation of cells with EGF and platelet-derived growth factor (25, 26); (*iii*) dissociation of the CD4 and $p56^{lck}$ complex upon activation of PKC (27); and (*iv*) activation of the raf and microtubule-associated protein serine/threonine kinases after tyrosine phosphorylation induced by treatment of multiple cell types with a variety of ligands (19, 28–30). $pp60^{c\text{-}src}$ itself can serve as a substrate for cAMP-dependent protein kinase (31), PKC (31, 32), maturation-promoting factor (33), and a 47-kDa tyrosine kinase (34), suggesting that it may also participate in the cross-talk between signaling pathways.

Our initial investigations into the role of $pp60^{c\text{-}src}$ in transmembrane signaling systems of C3H10T1/2 murine fibroblasts demonstrated an involvement of the c-src protein in the mitogenic response to EGF (3, 4), a ligand whose activity is mediated through a tyrosine kinase receptor. To determine if $pp60^{c\text{-}src}$ could modulate the action of other cell surface receptors, particularly those not exhibiting tyrosine kinase activity but known to stimulate serine/threonine kinases, we assessed the effect of overexpression of c-*src* on the response of 10T1/2 cells to β-adrenergic activation. Our results show that high levels of expression of wild-type (wt) but not kinase-defective c-*src* correlated with an enhanced accumulation of intracellular cAMP (an average of 3.3-fold above the accumulation observed in control cells) after treatment of cells with epinephrine, isoproterenol, or terbutaline. The response in all cells to these agents was abolished by propranolol, a specific β-adrenergic antagonist, confirming the identity of the receptors involved. These results suggest a

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Abbreviations: EGF, epidermal growth factor; PKC, protein kinase C; wt, wild type; neo^R, neomycin resistant.

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unique and unexpected functional link between a nonreceptor tyrosine kinase, pp60$^{c\text{-}src}$, and the well-characterized transmembrane signaling pathway involving β-adrenergic receptors and adenylyl cyclase.

MATERIALS AND METHODS

Cells, DNA, and Transfections. Neomycin-resistant (neo^R) cell lines were derived from C3H10T1/2 murine fibroblasts (CCL226, American Type Culture Collection) at passage 10 by DNA transfection using the calcium phosphate precipitation technique (35). Clones expressing either wt or kinase-defective c-*src* were obtained by cotransfecting a plasmid encoding neomycin resistance [pSV2neo (36)] with a plasmid encoding either wt avian c-*src* cDNA [pM5H(4)] or avian c-*src* cDNA with an inactivating point mutation in the kinase domain [pM430(4)], each gene being under the transcriptional control of the Moloney murine leukemia virus long terminal repeat. As a control, clones were also obtained by transfecting pSV2neo alone (neo^R). neo^R transfectants were selected, cloned, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol), fetal calf serum and G418 (400 μg/ml). Cells were maintained at subconfluence and assayed for c-*src* expression levels on second and third passages and for basal and hormone-stimulated cAMP accumulations on third and fourth passages.

pp60^{src} Western Immunoblots and Kinase Assays. pp60$^{c\text{-}src}$ immunoprecipitations, Western immunoblots, and *in vitro* kinase assays were performed as described by using the rodent/avian pp60$^{c\text{-}src}$-specific monoclonal antibodies GD11 and 327 (4, 37, 38). Kinase-defective pp60$^{c\text{-}src}$ exhibited an enzyme activity that was 10% or less of the unaltered protein (refs. 4 and 39 and data not shown).

cAMP Assays. Cells were grown to confluency in 60-mm tissue culture dishes and serum-starved for 20–24 hr in DMEM containing 0.1% bovine serum albumin (low-insulin-containing fraction V; Sigma). Cultures were refed with serum-free medium containing the indicated agent [in most instances, 10 μM epinephrine (Sigma)] and incubated at 37°C for 2 min, except where otherwise noted. Reactions were terminated by aspirating the medium and extracting cAMP from the monolayer with 1 ml of 0.1 M HCl for 1 hr at room temperature. The cAMP content of acetylated extracts was determined by radioimmune assay (40), as carried out by the University of Virginia Diabetes Core Laboratory. After removal of the acid extract, protein was solubilized in 1 ml of RIPA buffer (150 mM NaCl/1% deoxycholate/1% Triton X-100/0.1% SDS/50mM Tris·HCl, pH 7.2), clarified by centrifugation at 10,000 × *g* for 5 min at 4°C, and a sample was taken for determination of protein concentration by the BCA method (Pierce). Propranolol blockade of epinephrine stimulation was tested by incubating cells in serum-free medium containing 20 μM propranolol (Sigma) for 15 min prior to stimulating for 2 min with epinephrine in the continued presence of propranolol. The degree of enhanced cAMP accumulation seen in cells overexpressing wt c-*src* relative to the neo^R control cells was not altered by refeeding the cells with serum-containing medium either 1 hr or 24 hr prior to assay. The experimental conditions (i.e., serum starvation) were chosen to mimic those of EGF stimulation for comparison purposes.

RESULTS

Enhanced, β-Adrenergic-Dependent cAMP Accumulation in Cells Overexpressing wt c-*src*. To determine if c-*src* overexpression could influence transmembrane signaling through adrenergic receptors, we measured intracellular cAMP levels in response to epinephrine in the following C3H10T1/2 clonal cell lines: 5H14, which contains a 16-fold greater level of wt c-src protein than control cells; 430-4, which contains kinase-defective c-src protein at a level 45-fold above endogenous; and Neo-10, a control line resulting from transfection of the neomycin-resistance plasmid alone. The derivation and characterization of these lines has been described (4) and is briefly reviewed above. As shown in Fig. 1, cells containing elevated levels of wt c-src protein exhibited a striking enhanced accumulation of cAMP in response to epinephrine, ≈8-fold above the response in control cells. In contrast, the accumulation of cAMP in cells overexpressing kinase-defective c-*src* did not differ significantly from that of control cells. Each cell line exhibited the same basal cAMP level. Furthermore, C3H10T1/2 v-*src*-transformed cells (3) and control cells also contained the same basal and stimulated levels of cAMP (data not shown). Thus, the enhanced accumulation of cAMP in 10T1/2 cells appeared to be restricted to those clonal lines overexpressing wt c-*src*.

To determine if the enhanced hormone-stimulated cAMP accumulation was mediated through β-adrenergic receptors (since epinephrine is also an α-adrenergic agonist), we examined the effect of incubation of the same cells with propranolol, a competitive antagonist of β-adrenergic receptors, before treatment with epinephrine. Propranolol pretreatment completely abrogated the epinephrine-induced responses in all three cell lines (Fig. 1). Furthermore, isoproterenol (Fig. 1) and terbutaline (data not shown) (both β-specific agonists) mimicked the effect of epinephrine on all three cell lines, but prostaglandin E_1 [an agonist that activates an adenylyl cyclase-coupled receptor that is distinct from β-adrenergic receptors (22)] failed to stimulate cAMP accumulation in any of the cell lines (Fig. 1). We conclude, therefore, that the enhanced hormone-stimulated cAMP ac-

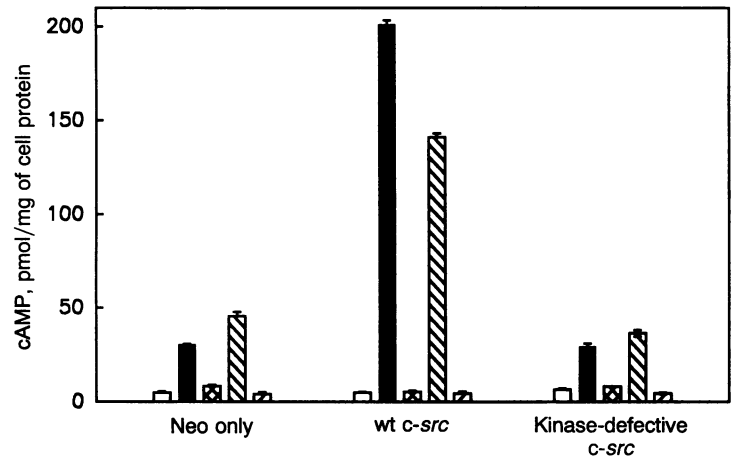

FIG. 1. Enhanced β-adrenergic-induced cAMP accumulation in cells overexpressing wt but not kinase-defective c-*src*. A representative neo^R C3H10T1/2 murine fibroblast clonal cell line (Neo-10), a wt c-*src* overexpressor (5H-14), and a kinase-defective c-*src* overexpressor (430-4) were tested for the amount of intracellular cAMP accumulated under conditions of mock-stimulation (□), within 2 min of treatment with 10 μM epinephrine (■), 10 μM epinephrine after a 15-min preincubation with 20 μM propranolol (▨), 10 μM isoproterenol (▧), or 10 μM prostaglandin E_1 alone (▨). 5H-14 exhibited a response to epinephrine that was ≈8-fold higher than that of either the Neo-10 or 430-4 cells. The enhanced response was competitively antagonized by propranolol and reproduced with isoproterenol, indicating that it was mediated through β-adrenergic receptors.

Table 1. Threshold level of wt c-src protein required for the enhanced β-adrenergic response

DNA transfected	c-*src* expression level, fold	Clones, no.	cAMP, pmol/mg of cell protein		cAMP stimulated/ unstimulated ratio
			Unstimulated	Stimulated	
neo^R	1	22	6.3 ± 0.2	62 ± 6	11 ± 1.0
wt c-*src*	1–2	6	6.6 ± 0.3	68 ± 8	11 ± 1.1
	3–4	11	7.2 ± 0.3	97 ± 16	15 ± 2.2
	5–10	6	6.9 ± 0.4	96 ± 20	14 ± 2.5
	11–25	13	6.8 ± 0.3	185 ± 24	31 ± 4.5*
	26–50	6	7.0 ± 0.7	250 ± 45	39 ± 6.0*
	>50	9	6.9 ± 0.3	270 ± 41	41 ± 5.5*
Kinase-defective c-*src*	1–2	7	6.1 ± 0.3	66 ± 6	12 ± 1.0
	3–4	6	6.3 ± 0.4	80 ± 16	13 ± 2.3
	5–10	7	7.2 ± 0.4	65 ± 10	10 ± 2.0
	11–25	8	6.9 ± 0.4	100 ± 15	15 ± 2.8
	26–50	6	7.0 ± 0.4	100 ± 23	16 ± 4.4

c-*src* expression levels were determined by averaging the results of two assays performed with passage 2 and 3 cells. cAMP levels were determined by averaging the results of assays performed with passage 3 and 4 cells. Assays were performed at 2 min after stimulation in duplicate for each clone.
*Significantly different from neo^R controls, $P < 0.0005$, using Duncan's multiple range test.

cumulation seen in cells overexpressing wt c-*src* reflects an exaggerated response mediated through β-adrenergic receptors.

Requirement for a Threshold Level of Enzymatically Active c-src for the Enhanced Response to β-Adrenergic Stimulation. To determine if the enhanced response to epinephrine was dependent upon the level of c-src protein, 51 clonal cell lines overexpressing wt c-*src*, 31 kinase-defective c-*src* overexpressors, and 22 neo^R control lines (all derived from parallel transfections) were characterized with respect to morphology, growth rate, levels of c-src protein, and levels of cAMP accumulation in response to epinephrine. No significant variations in morphology, growth rate, mean length of time to reach confluence or protein content per 1×10^6 cells were observed among the various cell lines at early passage, consistent with our observations (3). To assess levels of c-src protein, Western immunoblots of all clonal lines were analyzed. Cell lines containing either wt or kinase-defective c-*src* cDNA displayed a wide range of c-src protein levels as compared to endogenous pp60$^{c\text{-}src}$ and were grouped as indicated in Table 1. The basal and epinephrine-induced cAMP levels for each clonal line were also determined and are likewise summarized in Table 1. No differences in the basal (nonstimulated) cAMP levels between the neo^R control cells and any of the groups of c-*src* transfectants were found. Treatment of the neo^R control cells with 10 μM epinephrine for 2 min induced a mean rise in intracellular cAMP concentration of ≈11-fold. The wt c-*src* transfectants containing ≤10-fold endogenous levels of pp60$^{c\text{-}src}$ exhibited hormone-stimulated increases in cAMP of comparable magnitude. Cell lines with >10-fold endogenous c-src levels, on the other hand, showed augmented mean responses of 31- to 41-fold above nonstimulated levels. These values represent a mean enhancement of ≈3.3-fold (with a range of responses from 1- to 20-fold above the response in control cells). In contrast, cells containing kinase-defective c-src protein, even at levels

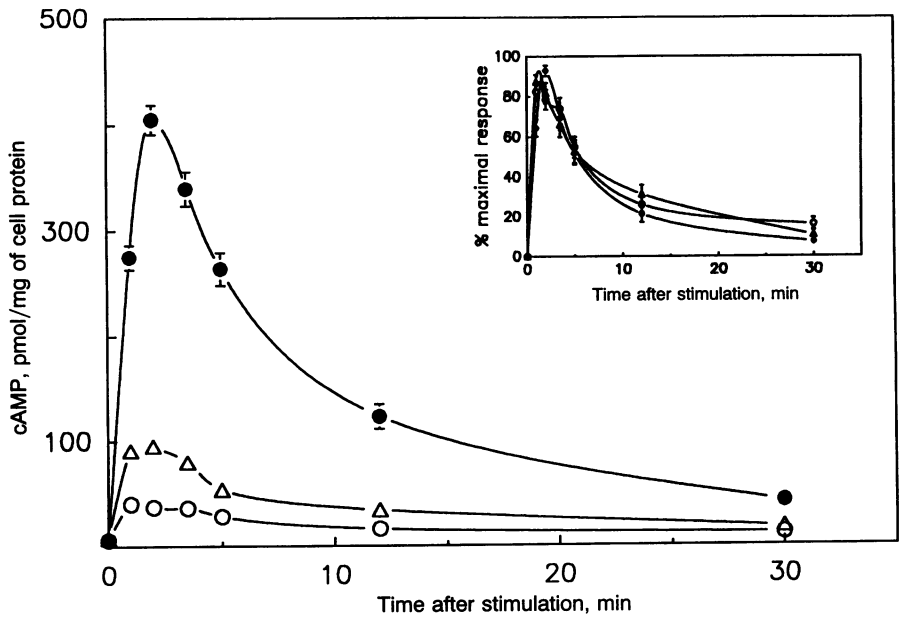

FIG. 2. Enhanced accumulation of cAMP in cells overexpressing wt c-*src* occurs with similar kinetics as in control cells. Four representative neo^R clones [Neo-5, -6, -10, and -14 (○)], four wt c-*src* overexpressors [5H-9, -14, -34, and -62 (●)], and four kinase-defective c-*src* overexpressors [430-4, -11, -17, and -60 (△)] were assayed for levels of intracellular cAMP at various times after addition of 10 μM epinephrine. Results are expressed as pmol of cAMP per mg of cell protein (mean ± SEM) of four similar transfectants. Error bars are contained within the symbols for the neo^R and kinase-defective c-*src* overexpressor clonal lines. (*Inset*) Time courses represent the results normalized to the percent maximal response of each group of cell lines.

≥10-fold above endogenous, showed cAMP accumulations that did not differ significantly from those of control cells. We conclude, therefore, that the enhanced responsiveness to epinephrine was dependent upon a threshold level of enzymatically active c-src protein that was ≈10-fold the level found in parental cells.

Time Course of Hormone-Stimulated cAMP Accumulation. To determine if overexpression of c-*src* altered either the rate of cAMP accumulation or the time of peak accumulation after adrenergic stimulation, four randomly selected neo^R clones, four wt c-*src* transfectants (containing >10-fold endogenous c-src protein), and four kinase-defective c-*src* transfectants (containing >10-fold endogenous c-src protein) were assayed for cAMP accumulation at several times after hormone stimulation. The composite time courses for each group of clones (Fig. 2) shows that at all early time points after stimulation, greater levels of cAMP were detected in the wt c-*src* overexpressors than in either the kinase-defective overexpressors or controls cells. Peak cAMP concentrations were achieved rapidly and at similar times (1–3 min after stimulation) for all three groups. After the achievement of peak cAMP levels, there was a gradual decline in cAMP concentrations that approached nonstimulated levels by 30 min. The slight enhancement over control cells seen in the kinase-defective c-*src* overexpressors was most likely due to the residual kinase activity of the altered enzyme observed in some cell lines expressing high levels of pm430. Noting that the peak cAMP levels in the wt c-*src* transfectants were 4-fold or greater than those in the clones expressing kinase-defective c-*src* or in the neo^R control clones, the similarity in the normalized curves (Fig. 2 *Inset*) could represent either a more rapid rate of cAMP accumulation and diminution in cells overexpressing wt c-*src* or a recruitment of more cells in the population responding to hormone stimulation with rates of accumulation nearly identical to those of control cells. Overall, however, the kinetics of adrenergic activation and inactivation appeared to be unaltered by c-*src* overexpression.

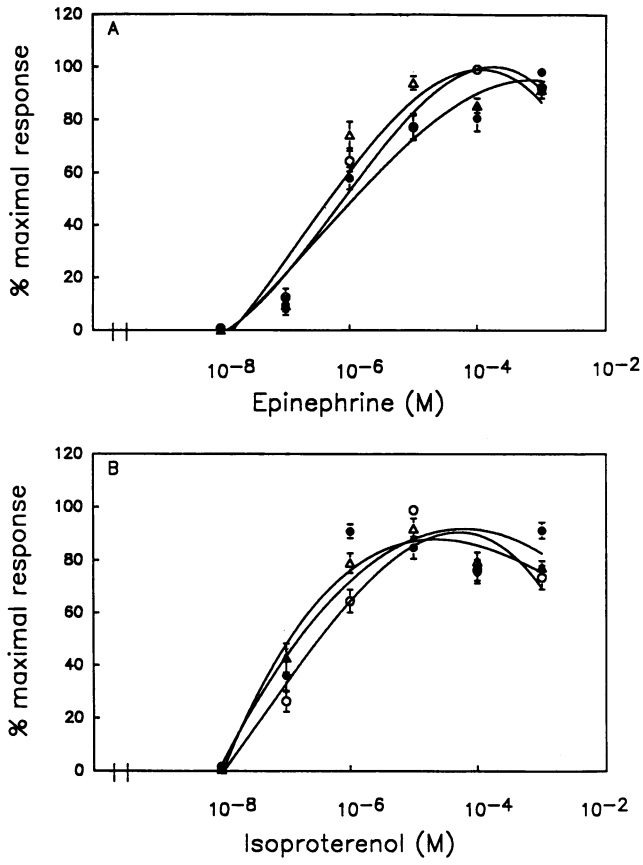

FIG. 3. Similar agonist concentration dependence of cAMP accumulation in cells overexpressing wt c-*src* and control cells. The 12 clones described in Fig. 2 were assayed for intracellular cAMP levels after a 2-min stimulation with various concentrations of epinephrine (*A*) or the β-specific adrenergic agonist isoproterenol (*B*). ○, neo^R clones; ●, wt c-src overexpressors; △, kinase-defective c-*src* overexpressors. Results are expressed as pmol of cAMP per mg of cell protein (mean ± SEM) of four similar transfectants, normalized to the percent maximal response of each group of cell lines.

Agonist Concentration Dependence of Hormone-Stimulated cAMP Accumulation. The dependence of hormone-stimulated cAMP accumulation upon agonist concentration was examined to determine if cells overexpressing c-*src* exhibited a greater sensitivity to agonist than control cells. The same clones used in the preceding experiment were assayed for cAMP levels after stimulation with various concentrations of epinephrine (10^{-8} to 10^{-3} M). Even though cAMP levels in the wt c-*src* transfectants were 4-fold or greater than the levels in the kinase-defective c-*src* or the neo^R transfectants, at all concentrations tested (as in Fig. 2), all cell lines exhibited similar concentration-dependent responses (Fig. 3*A*) with a half-maximal response of ≈0.3 μM. The composite dose–response curves for the β-specific agonist isoproterenol (Fig. 3*B*) were also similar, with a half-maximal response of ≈0.1/μM. From these data we conclude that cells overexpressing either wt or kinase-defective c-*src* do not display markedly different agonist concentration dependencies for hormone-stimulated cAMP accumulation than those of control cells and that the enhanced response of cells overexpressing wt c-*src* can be observed both at maximally and submaximally stimulating concentrations of the agonist.

DISCUSSION

From the studies described in this report, we conclude that overexpression of c-*src* potentiates the action of β-adrenergic agonists, ligands that initiate a cascade of events that have not been associated with the activity of a tyrosine kinase. Enhanced accumulation of intracellular cAMP in response to β-agonists was dependent upon a threshold level of wt c-src protein (≈10-fold or greater above endogenous levels) and was greatly reduced in cells overexpressing kinase-defective c-*src*, suggesting that the tyrosine kinase activity of the enzyme was important in the mechanism of enhancement. Furthermore, basal cAMP levels, time course of hormone-induced cAMP accumulation, and the dose–response to β-agonists were not altered by c-*src* overexpression, indicating that the response in overexpressors reflected an amplification of normal events.

What are the possible mechanisms by which pp60$^{c\text{-}src}$ could enhance the accumulation of cAMP in response to β-agonists? First, pp60$^{c\text{-}src}$ could intervene at several different points in the stimulatory arm of the β-adrenergic pathway, e.g., by enhancing β-adrenergic receptor–G_s interactions (where G_s is the stimulatory guanine nucleotide-binding regulatory protein), reducing the rate of receptor desensitization, augmenting G_s–adenylyl cyclase coupling, or activating adenylyl cyclase directly. Alternatively, pp60$^{c\text{-}src}$ could inhibit the breakdown of cAMP by phosphodiesterase or uncouple adenylyl cyclase from an inhibitory receptor (such as the adenosine receptor). Preliminary pharmacological studies designed to test these many alternatives suggest that pp60$^{c\text{-}src}$ mediates its effect through more than one component and utilizes both direct and indirect mechanisms to accomplish the full enhancement (data not shown).

Acting directly, pp60$^{c\text{-}src}$ could catalyze the tyrosine phosphorylation of one or more components of the β-adrenergic pathway. Although evidence exists for the phosphorylation

of β-adrenergic receptors, GTP-binding proteins, adenylyl cyclase, and phosphodiesterases (20, 21, 41–45), serine/threonine phosphorylations are cited more frequently than tyrosine phosphorylations in these studies. Nevertheless, the requirement for c-src kinase activity suggests that tyrosyl phosphorylation of some critical substrate(s) is necessary for the enhanced response. Whether these substrates are components of the β-adrenergic pathway or other pathways and whether c-*src* kinase effects the basal or β-adrenergic-dependent phosphorylation of these components remain to be investigated.

$pp60^{c\text{-}src}$ could also enhance the β-adrenergic response in an indirect fashion—e.g., by regulating factors that either modulate components of the β-adenergic pathway [cAMP-dependent protein kinase, PKC, β-adrenergic receptor kinase, and calcium/calmodulin and/or its dependent kinases (17, 20–22, 41, 42, 46)] or alter the expression level or ratio of various spliced forms of pathway components. Further pharmacological and biochemical analyses are required to clarify these events. Whatever mechanism may be elucidated, the findings from our laboratory (refs. 3 and 4 and L.K.W. and S.J.P., unpublished observations) as well as those from others (6, 7), that provide evidence for the involvement of $pp60^{c\text{-}src}$ in the action of the β-adrenergic, EGF, platelet-derived growth factor, and fibroblast growth factor receptors, implicate $pp60^{c\text{-}src}$ as a versatile potentiator of a variety of cell surface receptors, perhaps functioning as a "fine-tuner" of transmembrane signaling.

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