A Protein Kinase Cδ-binding Protein SRBC Whose Expression Is Induced by Serum Starvation*

Yasushi Izumi‡, Syu-ichi Hirai‡, Yoko Tamai‡, Ariko Fujise-Matsuoka‡, Yoshifumi Nishimura†, and Shigeo Ohno‡‡

West-Western screening of a cDNA expression library using 32P-labeled, autophosphorylated protein kinase Cδ (PKCδ) as a probe, led us to identify cDNA clones encoding a PKCδ-binding protein that contains a leucine zipper-like motif in its N-terminal region and two PEST sequences in its C-terminal region. This protein shows overall sequence similarity (43.3%) to the serum deprivation response (sdr) gene product, and we named it SRBC (sdr-related gene product that binds to c-kinase). PKCδ binds to the C-terminal half of SRBC through the regulatory domain and phosphorylates it in vitro. In COS1 cells, the phosphorylation of over-expressed SRBC is stimulated by 12-O-tetradecanoylphorbol-13-acetate and further enhanced by the over-expression of PKCδ. The mRNA for SRBC is detected in a wide variety of cultured cell lines and tissues and is strongly induced by serum starvation. Furthermore, SRBC mRNA is induced during retinoic acid-induced differentiation of P19 cells. These results suggest that SRBC serves as a substrate and/or receptor for PKCδ and might be involved in the control of cell growth mediated by PKC.

Protein kinase C (PKC) is a serine/threonine kinase thought to act in diverse cellular processes such as the secretion of hormones and neurotransmitters and the regulation of cell proliferation and differentiation. So far, more than 10 PKC isoforms have been reported, and these can be divided into three distinct classes based on differences in their structures and biochemical properties, conventional PKC (cPKC) members (α, βI, βII, and γ), novel PKC (nPKC) members (δ, ε, η, θ, and μ), and atypical PKC (aPKC) members (ζ and ι/λ). All PKC members consist of an N-terminal regulatory domain and a C-terminal catalytic domain; the co-factor binding site has been identified in the regulatory domain (1–4).

PKCδ is an nPKC member that is expressed in a variety of tissues and cultured cell lines (5–8). We have previously shown that a constitutively active mutant of PKCδ acts as a potent inducer of transcription factor activator protein 1/Jun and that a kinase-deficient mutant of PKCδ can inhibit the activity of the mutant (9). This dominant-negative effect of kinase-deficient PKCδ can be explained by the titration of an effector, substrate, or receptor molecule(s) that binds stably to PKCδ.

The stable interaction of PKC and its substrate has been demonstrated by using MARCKS, a well characterized PKC substrate (10). Furthermore, our kinetic analysis of the phosphorylation of MARCKS by PKC isoforms revealed a very low Km value (10–20 nM), supporting the high affinity interaction between MARCKS and PKC isoforms (α, δ, ε, and e) (11). Some other PKC-binding proteins have been identified by the screening of a cDNA expression library using a purified brain PKC mixture as a probe. One such protein is RACK1, which is thought to serve as a receptor for activated PKC (12). Others include F52/Mar-MARCKS and SH3/γ-aducin, which is a known substrate for PKC in vivo and in vitro (13, 14). A yeast two-hybrid system has also been used to identify PKCα-binding protein PICK1 (15). In addition to these proteins, several proteins are known to bind to a brain PKC mixture in vitro. These include annexin I, II, VI, vinculin, talin, and 72kDaRIG (16, 17). In addition, Bruton tyrosine kinase binds to cPKC (α, βI, and βII), nPKC (ε), and aPKC (ζ) in vitro (18). Actin binds to PKC in vitro (19) and PKCβII in vitro and in vivo (20).

Recently, it has been shown that ARAP79, an A kinase anchoring protein, can also bind to PKCα and PKCβII in vitro and in vivo (21), and human immunodeficiency virus Nef protein can bind to PKCδ in vitro and in vivo (22).

In the present study, we used West-Western screening to isolate cDNA clones encoding PKCδ-binding proteins. Here we describe a clone encoding as sdr-related gene product named SRBC, which serves as a substrate for PKCδ and whose expression is induced by serum starvation of NIH3T3 cells or differentiation of mouse embryonal carcinoma P19 cells.

EXPERIMENTAL PROCEDURES

Purification and 32P Labeling of PKCδ—PKCδ was purified from recombinant baculovirus-infected Sf21 cells as described elsewhere (11). Briefly, 3 days after infection, Sf21 cells were lysed, and PKCδ was purified by a series of chromatographic steps on DEAE-cellulose (Toyo, hydroxyapatite (Koken), and MonoQ (Pharmacia Biotech Inc.) columns. No additional proteins were obvious in the final PKCδ fraction as judged by SDS-PAGE, and the specific activity was 380 units/mg. One unit of PKCδ activity was defined as 1 nmol of 32P incorporated into

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A myelin basic protein (MBP<sub>4–14</sub>) peptide per min in a reaction buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM Mg(OAc)<sub>2</sub>, 10 μg/ml leupeptin, 25 μg/ml phosphatidylserine, 50 ng/ml TPA, 20 μM ATP, and 0.5 μCi of [γ<sup>32</sup>P]ATP (Amersham Corp.). Purified PKC<sub>d</sub> (400 ng) was labeled with <sup>32</sup>P by autophosphorylation in 155 μl of kinase buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 10% glycerol, 0.6 mM ATP, 40 μg/ml phosphatidylserine, and 0.5 μCi of [γ<sup>32</sup>P]ATP (Amersham Corp.). After the reaction, <sup>32</sup>P-labeled PKC<sub>d</sub> was separated from unreacted ATP by gel filtration on Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl.
NaCl, 0.1 mM dithiothreitol, and 0.5% Triton X-100 and used as a probe for screening an aEXlox cDNA expression library.

**Isolation of a cDNA Clone Encoding a PKCδ-binding Protein**—Poly(A)⁺ RNA was isolated from NIH3T3 cells, and randomly primed cDNA was synthesized using HindIII primer adapter (Novagen). cDNA was ligated to aEXlox vector arm (Novagen) and packaged in phage particles using a Phage Marker System (Novagen). This library contains approximately 0.95 x 10⁶ independent clones. Approximately 2 x 10⁵ clones were plated at 10,000 phages per plate using *Escherichia coli* strain BL21 (DE3) pLysE as host cells. After incubation for 6 h at 37°C, the plaques were covered with Hybond-C extra (Amersham Corp.) impregnated with 10 mM isopropyl-1-thio-D-galactopyranoside and allowed to grow for an additional 3.5 h at 37°C. The filters were then washed with TBS buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl), blocked with 5% skim milk at 4°C overnight, and incubated with 1 mM ATP solution (TBS buffer containing 1 mM ATP) at room temperature for 2 h to saturate any auto-phosphorylation sites or ATP-binding sites of the proteins. The filters were then incubated with 3²P-labeled PKCδ (10⁶ cpm/ml) in 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 50 μg/ml phosphatidylserine, and 1% bovine serum albumin for 5 h at room temperature. After washing in TBS containing 1% SDS buffer, the filters were exposed for autoradiography. Positive phage clones were converted to plasmids (pEXlox + cDNA insert) using *E. coli* strain BM25.5 according to the manufacturer's instructions. A cDNA insert was used as a probe to screen a 3Y1-lZAP II cDNA library to obtain a full-size SRBC coding sequence. Plaque hybridization with a lZAP II cDNA library and the isolation of positive phage clones were performed according to standard procedures.

**Phosphorylation of Proteins Fixed on PVDF Membranes**—PKCδ-binding proteins were subjected to SDS-PAGE, blotted onto PVDF membranes, and treated with a 5% skim milk solution. The phosphorylation reaction was carried out by incubating the PVDF membranes with phosphorylation buffer (83 ng/ml PKCδ, 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10% glycerol, 50 μg/ml phosphatidylserine, 400 ng/ml TPA, and 2.4 μCi/ml [γ²³P]ATP) at room temperature for 2 h. After washing with PBS containing 1% SDS buffer, the membranes were exposed for autoradiography.

**Overlay Assay Using ²³P-Labeled PKCδ as a Probe—Overlay assay was performed based on the procedure of Wolf and Sahyoun (23).** PKCδ-binding proteins were subjected to SDS-PAGE and blotted onto a PVDF membrane. After treatment with a 5% skim milk solution, the PVDF membrane was incubated with 3²P-labeled PKCδ (10⁶ cpm/ml) diluted in 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 50 μg/ml phosphatidylserine, and 1% bovine serum albumin at room temperature for 5 h. Excess ligand was removed by washing the PVDF membrane with TBS buffer, and the membrane was exposed for autoradiography.

**Phosphatidylserine Overlay Assay**—Protein-blotted PVDF membrane was overlaid with 20 μg/ml [¹⁴C]phosphatidylserine (specific activity 5 x 10⁵ μCi/mg, Amersham Corp.) diluted in 50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and 10 mg/ml bovine serum albumin at room temperature for 1 h. Membrane was washed briefly in phosphate-buffered saline and exposed for autoradiography.

**Overlay Assay Using MBP Fusion Proteins as Probes—one probe encoded mouse PKCδ and its deletion mutant (lacking amino acid residues 299–654, 209–654, and 111–654) were subcloned into *E. coli* expression vector pMAL-c2 (New England BioLabs) for expression of these proteins as fusion proteins with maltose-binding protein (MBP-PKCδ, MCD299, MCD209, and MCD111). The proteins were purified on amylose resin (New England BioLabs) and used as probes for the overlay assay.**

The protein-transferred PVDF membrane was incubated with 0.7 μM MBP-PKCδ or 1 μM MCD299, MCD209, or MCD111 in 50 mM Tris-HCl,
Phosphorylation of SRBC in COS1 Cells—An in vitro phosphorylation of GST-SRBC Protein by PKCδ—An E. coli lysate contains the T7 gene 10 product and the fusion protein of clone 53 product were separated by SDS-PAGE and transferred onto a PVDF membrane. Proteins were detected by Western blot analysis using anti-T7 tag antibody (lanes 1 and 2), and the binding of [32P]labeled PKCδ was tested by overlay assay in the presence (lanes 3 and 4) or absence (lanes 5 and 6) of phosphatidylycerine. The phosphorylation of proteins on the PVDF membrane is also shown (lanes 7 and 8). The upper arrowheads indicate the position of the T7 gene 10-fused clone 53 product, and the lower arrowheads indicate the position of the T7 gene 10 product.

**RESULTS**

Cloning of SRBC—A mouse NIH3T3-3EXloxd expression library was screened for proteins that bind to PKCδ in the presence of phosphatidylyserine. Using [32P]labeled PKCδ as a probe, two clones, clone 53 and 91, that appeared to originate from the same mRNA were isolated. To isolate full-length cDNAs, a rat 3Y1-ZAPII cDNA library was screened using clone 53 as a probe. The nucleotide sequence of a rat homologue of clone 53/91, and the structural features of the protein are shown in Fig. 1A. The ATG at position 1 is most likely an initiation codon because 1) the surrounding nucleotide sequence fulfills Kozak's criteria, and 2) there is an in-frame TAG codon in the 5' non-coding sequence. The open reading frame starting with this initiation codon encodes a protein with 263 amino acid residues. The calculated molecular mass of the protein, named SRBC, is 27,879. Mouse clone 53 encodes 105 amino acid residues corresponding to the C-terminal part of rat SRBC (Fig. 1A, lower panel), and the amino acid sequence identity in this region is 86%. SRBC contains a leucine zipper-like motif in the N-terminal part and two PEST regions, which are commonly found in short-lived proteins. A comparison of the SRBC nucleotide sequence with the GenBank data base revealed high homology to mouse sdr (29) and a chicken mRNA for the expressed sequence tag (CHKESTFLLE) (Fig. 1B). The overall amino acid sequence identities for SRBC to Sdr and CHKESTFLLE are 43.3 and 25.9%, respectively. Amino acid residues conserved among the proteins are clustered in two regions, SRBC conserved region 1 (SCR1) and SRBC conserved region 2 (SCR2). SCR1 contains the leucine zipper-like motif in its N-terminal part, and SCR2 contains one or two PKC phosphorylation sites (Fig. 1B). Amino acid identity in SCR1 is 43.8% between SRBC and Sdr and 29.0% between SRBC and CHKESTFLLE. In SCR2, it is 60.9% between SRBC and Sdr and 52.2% between SRBC and CHKESTFLLE. In addition, all of these proteins contain PEST regions even though their primary structures are not conserved. It is noteworthy that the sequence from 1 to 185 amino acids of SRBC shows 90% identity with the sequence from 1 to 185 amino acids of the human 7N-1 clone, identified as a gene fused to c-Raf-1 that results in the constitutive activation of the c-Raf-1 gene (30). In this clone, the N-terminal regulatory domain of c-Raf-1 is replaced by the human SRBC homologue.

**PKCδ binds to SRBC**—The original pEXloxd clone, clone 53, encodes only a C-terminal part of SRBC (156 to 263 amino acids) as fusion proteins with the T7 gene 10 product, indicating that the C-terminal part is sufficient for the interaction with PKCδ. The overlay assay showed that PKCδ binds to the product of the T7 gene 10 fused clone 53 product in the presence of G-Serphosphorylated (Pharmacia), for 1 h at 4 °C. The immunocomplexes on Sepharose were washed 5 times with lysis buffer and 2 times with final wash buffer containing 20 mM Hepes, pH 7.5, 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1.8 μg/ml aprotinin, and 0.1% Triton X-100. The proteins were then separated by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-T7 antibody to estimate the amount of tag-SRBC precipitated. The amount of [32P] incorporated into tag-SRBC was measured by a Bio-Image analyzer (BAS 2000 FUJI).

**Cell Culture Conditions for the Analysis of SRBC mRNA—NIH3T3 cells** were routinely cultured in DMEM supplemented with 10% fetal calf serum (FCS). For serum starvation, the medium was changed to 0.5% FCS when the cells were still confluent. For serum stimulation, fresh medium containing 20% FCS was added to the starved cells. Cells were harvested at the desired times for RNA isolation. Differentiation of P19 cells was performed following a standard procedure (24, 25).

Poly(A)+ RNA was isolated using a QuickPrep Micro mRNA Purification Kit (Pharmacia), and Northern blot analysis was performed according to the standard protocol.
ence of phosphatidylserine, a common activator of PKCα (Fig. 2, lanes 3 and 5). On the other hand, no such interaction was observed with the T7 gene 10 product itself (Fig. 2, lanes 3 and 4). Furthermore, the fusion protein but not the T7 gene 10 product itself can be phosphorylated by PKCδ on the membrane (Fig. 2, lanes 7 and 8), in agreement with the presence of putative PKC-phosphorylation sites in the clone 53 product (Fig. 1A).

To test whether full-length SRBC binds to PKCδ, SRBC was expressed as a GST-fusion protein in E. coli and tested for PKCδ binding by overlay assay. MARCKS, a known binding protein/substrate for PKC, and GST alone were also tested for PKCδ binding. GST-SRBC appeared as a 65.4-kDa band on SDS-PAGE, and MARCKS and GST appeared as 80- and 26-kDa bands, respectively (Fig. 3A, lanes 1–3). The positions of GST and GST-SRBC were confirmed by Western blot analysis using anti-GST antibody (Fig. 3A, lanes 4 and 5). Overlay assay was performed in the presence (lanes 6–8) or absence (lanes 9–11) of phosphatidylserine using 32P-labeled PKCδ as a probe. Arrowheads indicate the position of each protein. B, protein-transferred membrane was overlaid with [14C]phosphatidylserine. Phosphatidylserine-binding proteins were detected by autoradiography. Samples used are purified GST-SRBC (same as A) and crude extract of E. coli containing clone 53 products (same as Fig. 2).

**Phosphatidylserine Binds to SRBC and Clone 53 Product—**
Previous studies showed that most of the PKC-binding proteins are phosphatidylserine-binding proteins (10, 13, 16, 23, 31). Thus, phosphatidylserine may form a bridge between PKC and PKC-binding proteins and stabilize the binding. Since binding of SRBC to PKCδ also depends on phosphatidylserine, we next tested whether SRBC binds to phosphatidylserine by using [14C]phosphatidylserine overlay assay. As shown in Fig. 3B, GST-SRBC binds to phosphatidylserine, and clone 53 encoding only C-terminal part of SRBC also binds to phosphatidylserine. Note that the C-terminal part of SRBC was sufficient for the binding to PKCδ. These results suggest that the binding of SRBC and PKCδ depends on phosphatidylserine-bridging as suggested for other PKC-binding proteins.

**The Regulatory Domain of PKCδ Binds to SRBC—**
Since the binding of PKCδ to SRBC depends on the presence of phosphatidylserine, the co-factor binding site of PKCδ located in its N-terminal regulatory domain might be involved in the interaction with SRBC. It has been reported that the pseudosubstrate region, in the regulatory domain, directly mediated phosphatidylserine-dependent PKC binding to some PKC-binding proteins (16, 31). To test whether the N-terminal regulatory domain is sufficient for this interaction, whole PKCδ and its regulatory domain were expressed as MBP (maltose-binding protein kinase domain).
protein) fusion proteins in *E. coli* (Fig. 4A) and used as probes for the overlay assay. In these experiments, bound PKCδ was detected by anti-MBP antibodies. As shown in Fig. 4B, MBP-PKCδ bound to GST-SRBC or MARCKS similarly to intact PKCδ (Fig. 4B, lanes 1 and 2). Furthermore, MBP fused to the regulatory domain of PKCδ (MCD299) also bound to GST-SRBC and MARCKS to the same extent (Fig. 4B, lanes 4 and 5). The regulatory domain of PKCδ includes a cysteine-rich domain conserved among all cPKC and nPKC members. When the cysteine-rich domain was totally deleted (MCD111), no binding to SRBC or MARCKS was observed (Fig. 4B, lanes 10 and 11), whereas the C-terminal half of the cysteine-rich domain was dispensable for binding (MCD209 in Fig. 4B, lanes 7 and 8). These results suggest that amino acid residues 111–209 on PKCδ, a region that includes a pseudosubstrate and half of the cysteine-rich domain, are essential for the binding to GST-SRBC and MARCKS. When the cysteine-rich domain was totally deleted (MCD111), no binding to SRBC or MARCKS was observed (Fig. 4B, lanes 10 and 11), whereas the C-terminal half of the cysteine-rich domain was dispensable for binding (MCD209 in Fig. 4B, lanes 7 and 8). These results suggest that amino acid residues 111–209 on PKCδ, a region that includes a pseudosubstrate and half of the cysteine-rich domain, are essential for the binding to GST-SRBC and MARCKS. The binding of SRBC to the regulatory domain of PKCδ raises the possibility that GST-SRBC modulates the activity of PKCδ. However, we could not detect any effect of GST-SRBC on the myelin basic protein kinase activity of PKCδ in vitro (data not shown).

**SRBC Is Phosphorylated Upon PKC Activation in Vivo**—To monitor the phosphorylation of SRBC in *vivo*, we designed an epitope-tagged SRBCΔ15 expression vector. In this construction, the N-terminal 15 amino acids of SRBC are replaced by a T7 gene 10 epitope tag. The apparent molecular mass of this protein is 43 kDa on SDS-PAGE. The SRBCΔ15 was spontaneously phosphorylated in serum-starved COS1 cells, and TPA stimulation caused a 2-fold increase in the level of phosphorylation (Fig. 6). We next examined the effect of the co-expression of PKCδ in the presence or absence of TPA. No significant effect of PKCδ over-expression was observed in the absence of...
SRBC mRNA in starved cells (Fig. 7) was retained at least for 48 h (Fig. 7). The induced mRNA expression levels were low in growing NIH3T3 cells (Fig. 7) and autophosphorylated PKC is also induced upon serum starvation. The level of SRBC binding protein, SRBC, is ubiquitously expressed in almost all tissues tested except liver, and the SRBC mRNA level was even eight times higher in serum-starved cells compared with serum-stimulated or exponentially growing cells. The induction of SRBC mRNA was also observed upon retinoic acid-induced differentiation of mouse embryonal carcinoma P19 cells to neuron-like cells (Fig. 7). Taken together, these results show that the level of SRBC mRNA expression correlates with cell growth suppression.

DISCUSSION

We cloned a cDNA encoding a PKC-binding protein by Western screening using 32P-labeled, autophosphorylated PKCδ as a probe. This protein, called SRBC, binds to and is phosphorylated by PKCδ in vitro. In COS1 cells, the phosphorylation of over-expressed SRBC is stimulated by TPA and is further enhanced by the over-expression of PKCδ.

The Km value for the phosphorylation of SRBC by PKCδ is quite low (60 nM), which may reflect a strong interaction between the two molecules. On the other hand, the Vmax value is quite low indicating that SRBC is not a good substrate for PKCδ.

The binding of SRBC to PKCδ depends on the presence of phosphatidylserine; therefore, the active conformation of PKCδ might be necessary for the interaction. In addition, the fact that SRBC binds to phosphatidylserine suggests that phosphatidylserine mediates or stabilizes the interaction between SRBC and PKCδ through its bridging. Interaction with SRBC was also observed for MCD209, a deletion mutant of PKCδ lacking the kinase domain and the C-terminal half of the cysteine-rich domain that is responsible for the binding to phorbol ester or diacylglycerol (32, 33).

This indicates that these functional domains are dispensable for the interaction with SRBC. Since MCD111 cannot bind to SRBC, the N-terminal half of the cysteine-rich domain and the pseudosubstrate region may be essential for binding. Previous studies demonstrated that the
pseudosubstrate region is one of several motifs that mediate PKC binding (16, 31). However, from our results, we cannot conclude whether the pseudosubstrate region is important to the binding to PKCδ and SRBC or not. We need further experiments to determine the SRBC binding region on the PKCδ.

SRBC binds to PKCa, a cPKC family member, to the same extent as PKCδ. And it barely binds to PKCζ, an aPKC family member (data not shown). Thus, the binding of PKC to SRBC does not clearly show isotype specificity. We identified several PKCδ-binding proteins in addition to SRBC by subsequent screening of the cDNA expression library using PKCδ as a probe. These include MARCKS and several other proteins that do not include any of previously reported PKC-binding proteins. Among these novel PKC-binding proteins, we could find a PKCδ-specific binding protein.3

The primary structure of SRBC shows considerable similarity to Sdr and one of the chicken expression sequence tags (CHKESTFLLLE). sdr has been identified as a gene induced in NIH3T3 cells by serum starvation but whose function remains unknown. Most of the conserved amino acids in these proteins are clustered in the N-terminal region named SCR1, which includes a “leucine zipper”-like motif. Although this region is not required for the binding of SRBC to PKCδ, it might be involved in the formation of complexes with themselves or other proteins. The rest of the conserved amino acids are clustered in a small region called SCR2. This region in SRBC includes two putative PKC phosphorylation sites, one of which is conserved among the three proteins. Since only the C-terminal half of SRBC was encoded by the cDNA clones first identified in the expression library, SCR1 must be dispensable for the binding to PKCδ. No striking sequence homology is found among known PKC-binding proteins, AKAP79 (21), MARCKS (10), and the C-terminal part of SRBC including SCR2. However, it is noteworthy that SCR2 as well as the PKC-binding region in AKAP79 and MARCKS show a high content of basic amino acids and both SRBC and MARCKS show similar binding patterns to PKCδ (Figs. 3 and 4). Therefore, SCR2 could be responsible for the binding to PKC in the same way as the basic amino acid region of MARCKS.

The expression pattern of the src gene shares some common features with that of sdr; both src and sdr are induced by serum starvation of NIH3T3 cells and down-regulated by the addition of serum. This suggests that sdr and src form a family of genes that play a role in cell-growth control. In addition, other genes induced by growth arrest of cells have been identified in different systems (34, 35) but show no structural homology with SRBC. Noteworthy, the N-terminal 185 amino acids of SRBC, including SCR1, have been found in the N-terminal part of the oncogenic c-RAF-1 gene, replacing the N-terminal regulatory domain of c-RAF-1 (30). The significance of the SRBC sequence fused to c-RAF-1 is unclear since a variety of genes can activate the c-RAF-1 gene by similar gene fusion (36). However, since the SRBC-RAF fusion gene seems to be under the control of the src promoter, the fusion gene is most likely expressed at high levels in quiescent cells, and this could explain some of the oncogenic properties of the fusion gene. The ubiquitous expression of src in different tissues and its induction upon retinoic acid-induced differentiation of mouse embryonal carcinoma P19 cells suggest the involvement of SRBC in a variety of the cellular events that accompany growth arrest.

A mutant PKCδ lacking kinase activity shows a dominant-negative effect on the TPA-induced activation of the TRE-tk-CAT reporter gene in NIH3T3 cells (9). Furthermore, the same dominant negative mutant suppresses TRE-tk-CAT expression caused by the ectopic expression of a PKCδ-active mutant (9). Thus, we examined the effect of SRBC on TRE-tk-CAT expres-

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sion in NIH3T3 cells. However, we failed to detect any significant effect (data not shown). Another series of preliminary experiments to examine the effect of SRBC overexpression on the growth of NIH3T3 cells demonstrated the inhibition of DNA synthesis and colony formation, supporting the idea that SRBC is involved in the regulation of cell growth. We cannot conclude that PKC is involved in these events; however, the further characterization of SRBC might provide a clue to understanding the signaling pathway by which PKC controls cell growth and differentiation.

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REFERENCES

1. Nishizuka, Y. (1992) Science 258, 607–614
2. Nishizuka, Y. (1995) FASEB J. 9, 484–496
3. Ohno, S., Akita, Y., Hata, A., Osada, S., Kubo, K., Konno, Y., Akimoto, K., Mizuno, K., Saado, T., Kuroki, T., and Suzuki, K. (1991) Adv. Enzyme Regul. 31, 287–303
4. Hug, H., and Sarre, T. F. (1993) Biochem. J. 291, 329–334
5. Ohno, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932
6. Mizuno, K., Kubo, K., Saado, T. C., Akita, Y., Osada, S., Kuroki, T., Ohno, S., and Suzuki, K. (1991) Eur. J. Biochem. 202, 931–940
7. Borner, C., Guadagno, S. N., Fabbro, D., and Weinstein, I. B. (1992) J. Biol. Chem. 267, 12892–12899
8. Ohno, S., Mizuno, K., Adachi, Y., Hata, A., Akita, Y., Akimoto, K., Osada, S., Hirai, S., and Suzuki, K. (1994) J. Biol. Chem. 269, 17495–17501
9. Hirai, S., Izuimi, Y., Higa, K., Kaibuchi, K., Mizuno, K., Osada, S., Suzuki, K., and Ohno, S. (1994) EMBO J. 13, 2231–2240
10. Hyatt, S. L., Liao, L., Chapline, C., and Jaken, S. (1995) J. Biol. Chem. 270, 931–940
11. Fujise, A., Mizuno, K., Ueda, Y., Osada, S., Hirai, S., Takayanagi, A., Shimizu, N., Osada, M. K., Nakajima, H., and Ohno, S. (1994) J. Biol. Chem. 269, 31642–31648
12. Ron, D., Chen, C., Caldwell, J., Jamieson, L., Orr, E., and Monchly-Rosen, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 839–843
13. Chapline, C., Ramsay, K., Klauck, T., and Jaken, S. (1993) J. Biol. Chem. 268, 6855–6861
14. Dong, L., Chapline, C., Mouesseau, B., Fowler, L., Ramsay, K., Stevens, J. L., and Jaken, S. (1995) J. Biol. Chem. 270, 25534–25540
15. Staudinger, J., Zhou, J., Burgess, R., Eledge, S. J., and Olson, E. N. (1995) J. Cell Biol. 129, 263–271
16. Hyatt, S. L., Liao, L., Chapline, C., and Jaken, S. (1994) Biochemistry 33, 1223–1228
17. Chapline, C., Mouesseau, B., Ramsay, K., Duddy, S., Li, Y., Kiley, S. C., and Jaken, S. (1996) J. Biol. Chem. 271, 6417–6422
18. Yao, L., Kawakami, Y., and Kawakami, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9175–9179
19. Prekeris, R., Mayhew, M. W., Cooper, J. B., and Terrian, D. M. (1996) J. Cell Biol. 132, 77–90
20. Blobe, G. C., Stibring, D. S., Fabbro, D., Stabel, S., and Hainun, Y. A. (1996) J. Biol. Chem. 271, 15823–15830
21. Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996) Science 271, 1589–1592
22. Smith, B. L., Krushelnycky, B. W., Mochly-Rosen, D., and Berg, P. (1996) J. Biol. Chem. 271, 16753–16757
23. Wolf, M., and Sahyoun, N. (1986) J. Biol. Chem. 261, 13327–13332
24. Scheibe, R. J., Moeller-Range, L., and Mueller, W. H. (1991) J. Biol. Chem. 266, 21308–21305
25. Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., Suzuki, K., and Ohno, S. (1994) J. Biol. Chem. 269, 12677–12683
26. Kozak, M. (1987) J. Mol. Biol. 196, 947–950
27. Rogers, S., Wells, R., and Rechsteiner, M. (1986) Science 234, 364–368
28. Rechsteiner, M., Rogers, S., and Rote, K. (1987) Trends Biochem. Sci. 12, 390–394
29. Gustinich, S., and Schneider, C. (1993) Cell Growth & Differ. 4, 753–760
30. Tahir, U., Izuimi, M., Hayashi, K., Nagao, M., and Sugimura, T. (1987) Nucleic Acids Res. 15, 4809–4820
31. Izuimi, S., Hyatt, S. L., Chapline, C., and Jaken, S. (1994) Biochemistry 33, 1229–1233
32. Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4868–4871
33. Zhang, G., Kazanietz, M. G., Blumberg, P. M., and Hurley, J. H. (1995) Cell 81, 917–924
34. Schneider, C., King, R. M., and Philipson, L. (1988) Cell 54, 787–793
35. Sal, G. D., Ruaro, M. E., Philipson, L., and Schneider, C. (1992) Cell 70, 595–607
36. Ishikawa, F., Takaku, F., Nagao, M., and Sugimura, T. (1987) Mol. Cell. Biol. 7, 1226–1232