Signal transduction in T lymphocytes using a conditional allele of Sos

LESLIE J. HOLSINGER*, DAVID M. SPENCER*, DAVID J. AUSTIN†, STUART L. SCHREIBER†, AND GERALD R. CRABTREE*

*Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305; and †Howard Hughes Medical Institute, Department of Chemistry, Harvard University, Cambridge, MA 02138

Contributed by Stuart L. Schreiber, June 14, 1995

ABSTRACT While Ras activation has been shown to play an important role in signal transduction by the T-lymphocyte antigen receptor, the mechanism of its activation in T cells is unclear. Membrane localization of the guanine nucleotide exchange factor Sos, but not Vav or Dbp, was sufficient for Ras-mediated signaling in T lymphocytes. Activation of Sos appears to involve membrane recruitment and not allosteric changes, because interaction of Sos with the linking molecule Grb-2 was not required for Ras activation. To extend this analysis, we constructed a modified Sos that could be localized to the membrane inducibly by using a rationally designed chemical inducer of dimerization, FK1012. The role of Grb-2 in signaling was mimicked with this technique, which induced the association of a modified Sos with the membrane, resulting in rapid activation of Ras-induced signaling. In contrast, inducible localization of Grb-2 to the membrane did not activate signaling and suggests that the interaction of Grb-2 with Sos in T cells is subject to regulation. This conditional allele of Sos demonstrates that membrane localization of Sos is sufficient for Ras activation in T cells and indicates that the role of Grb-2 is to realize the biologic advantages of linker-mediated dimerization: enhanced specificity and favorable kinetics for signaling. This method of generating conditional alleles may also be useful in dissecting other signal transduction pathways regulated by protein localization or protein-protein interactions.

Many signal transduction pathways induced by cell surface receptors, including the T-lymphocyte antigen receptor (TCR), involve activation of the small guanine nucleotide-binding protein Ras (1). These receptors, by association with the adaptor molecules Grb-2 and Shc, can activate Ras by membrane recruitment of the guanine nucleotide exchange factor (GEF) Sos. Here at the membrane, in close proximity to its substrate, Sos activates Ras by catalyzing the exchange of GDP for GTP (2–7). This is followed by membrane recruitment and activation of the serine/threonine kinase Raf and subsequent activation of the mitogen-activated protein kinase cascade (8–10). After stimulation of the antigen receptor in T lymphocytes, Ras may be activated by a protein kinase C-dependent inactivation of a Ras guanine nucleotide-activating protein, as well as by a protein kinase C-independent mechanism involving tyrosine kinases (11). Although the formation of a Sos–Grb2–Shc–TCR ζ chain complex has been detected after T-cell activation (12), the precise mechanism of Ras activation in T lymphocytes and the role of GEFs is not clear.

Studies of Ras, other Ras-related GTP-binding proteins, and their respective GEFs have been hampered by the absence of conditional alleles that permit the temporal dissection of events following guanine nucleotide-binding protein activation in mammalian cells. While both dominant-negative and con-

stitutively active alleles have been defined for Ras, putative feedback regulation and the prolonged period of their activation have confounded interpretation. To avoid these difficulties, we have developed an approach to conditionally activate mammalian genes by regulating their intracellular localization with a chemical inducer of dimerization (CID), FK1012, and its respective ligand-binding domain FKBP12, an FK506-binding protein. To illustrate the utility of this approach, we have created a conditional allele of Sos by mimicking the actions of Grb-2 with FK1012, which regulates the membrane localization of Sos. In addition, we demonstrate that membrane localization of Sos in T cells is sufficient for Ras-dependent signaling.

MATERIALS AND METHODS

Cell Lines and Transfection Assays. COS cells used in this study were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and penicillin/streptomycin. Jurkat-TAG cells (13) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin. For transfections, 10^7 Jurkat-TAG cells were electroporated and assayed for secreted alkaline phosphatase (SEAP) activity as described (14).

Protein Analysis. To monitor protein expression, cells were lysed 40 hr after transfection in RIPA buffer [150 mM NaCl/20 mM Tris, pH 7.5/0.5% sodium deoxycholate/0.1% SDS/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/1 mM benzamide/aprotinin (1 μ g/ml)/antipain (1 μ g/ml)/leupeptin (1 μ g/ml)]; lysates were immunoprecipitated with monoclonal antibody (mAb) 12CA5 (Babco, Richmond, CA) prebound to protein A-Sepharose, analyzed on SDS/10% PAGE, and electrotransferred to nitrocellulose. Blots were probed with 12CA5 and horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed), and proteins were visualized by ECL (Amersham). For coimmunoprecipitation assays, transfected Jurkat-TAG cells were lysed in HNTG lysis buffer [50 mM Hepes, pH 7.5/150 mM NaCl, 10% (vol/vol) glycerol/1% Triton X-100/1.5 mM $MgCl_2$ /1 mM EGTA/bovine serum albumin (1 mg/ml)/protease inhibitors as above]. Lysates were immunoprecipitated with 12CA5 as described above and washed extensively in HNTG buffer (50 mM Hepes/10% glycerol/0.1% Triton X-100/150 mM NaCl). Identical immunoprecipitates were analyzed on SDS/10% PAGE gels, and blots were probed with 12CA5, or immunoprecipitates were analyzed on SDS/12% PAGE gels and blots were probed with anti-Grb-2 mAb (Transduction Laboratories, Lexington, KY).

Immunofluorescence Microscopy. Fifty percent confluent monolayers of COS cells were transfected with the indicated

plasmid DNAs using LipofectAmine (GIBCO/BRL) on coverslips. Twenty-four hours after transfection, cells were fixed with 4% (vol/vol) paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, and stained with 12CA5 primary antibody followed by rabbit anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (PharMingen). Immunofluorescence was visualized using confocal microscopy.

RESULTS

Membrane Localization of Sos in T Lymphocytes. Suggestive evidence that membrane localization of Sos can activate signaling in T cells was obtained by fusing the *v-src* myristoylation sequence N-terminal to the coding region of Sos (MSosE; Fig. 1A). A control vector encoding soluble Sos (SSosE) was also constructed in which the myristoylation sequence was eliminated. Localization of the soluble and myristoylated Sos protein was confirmed in COS cells by confocal microscopy. Expression of the soluble Sos construct

was localized exclusively in the cytosol (Fig. 2A and B). As expected, myristoylation of Sos resulted in localization predominantly at the plasma membrane (Fig. 2C and D). To assess the role of Sos localization in signaling, these molecules were cotransfected into a human T-cell line (Jurkat-TAG) with a SEAP reporter gene under the control of an NF-AT-responsive promoter element (14). The NF-AT transcription complex responds to signaling through the TCR as well as by pharmacological agents such as phorbol ester and ionomycin, a calcium ionophore, which synergize to activate NF-AT-dependent transcription, as reviewed in ref. 18. Transfection of a constitutively active calcineurin can functionally replace calcium-dependent events (14, 17), whereas expression of constitutively active Ras (v-Ha-Ras) can bypass the need for phorbol ester (19) in T-cell signaling. Activities of the soluble and myristoylated Sos constructs were assayed for their ability to provide a signal complementary to the calcium signal induced by ionomycin. Both constitutively active Ras and myristoylated Sos, in contrast to soluble Sos, synergize with ionomycin to activate NF-AT-dependent transcription in T

FIG. 1. (A) Construction of chimeric Sos molecules. The *v-src* myristoylation sequence was cloned in-frame with Sos (amino acids 2–1333) in a derivative of the vector pBJ5 (15, 16) to give MSosE. SSosE is identical to MSosE but lacks the myristoylation sequence. All constructs contain a C-terminal influenza hemagglutinin (HA) epitope tag. SF3SosE and MF3E were generated by introducing three tandem copies of FKBP12 into the *Xho* I site of SSosE and the pBJ5 vector containing a myristoylation sequence, respectively. (B) Activation of T-cell signal transduction by membrane-targeted Sos. Jurkat-TAG cells (10^7) were transfected with 2 μ g of NF-AT-SX SEAP reporter plasmid together with 8 μ g of expression constructs containing MSosE, SSosE, v-Ha-Ras, or RSV-N17Ras. Twenty-four hours after transfection, cells were stimulated with ionomycin (1 μ M), and after an additional 20 hr SEAP activity was measured. The data are presented as the percentage of maximal activity obtained by stimulation with ionomycin plus phorbol 12-myristate 13-acetate (PMA) (25 ng/ml). The data represent the average of at least three independent transfections $\pm$ SEM. (Inset) Protein expression levels were verified by immunoblotting with the 12CA5 mAb specific for the HA epitope tag. (C) Myristoylated Sos synergizes with constitutively active calcineurin (CNMUT2B). All cells were cotransfected with CNMUT2B (17), NF-AT-SX, and either v-Ha-Ras, MSosE, SSosE, or pBJ5 vector control. Forty-four hours after transfection SEAP activity was measured. Data are presented as maximal stimulation obtained with CNMUT2B plus PMA stimulation. (D) MDblE contains amino acid residues 2–498 from oncogenic Dbl, while McVavE and ScVavE contain residues 2–845 of the Vav protooncogene. These genes were cloned into the myristoylated and soluble vectors, respectively, described in A. Cells were cotransfected with NF-AT-SX and the indicated expression constructs, stimulated with or without ionomycin, and analyzed for SEAP activity. Activation is presented as in B. Ion., Ionomycin. (Inset) Protein expression verified as in B. The asterisk denotes a nonspecific band recognized by the 12CA5 antibody.

FIG. 2. Soluble and myristoylated Sos exhibit distinct subcellular localization. COS-1 cells were transfected on coverslips with SSosE (A and B) or MSosE (C and D) plasmids, fixed, stained with 12CA5 mAb and fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody, and analyzed by confocal microscopy.

cells (Fig. 1B). Similar results were obtained with these constructs in activating transcription of an AP-1-dependent reporter gene independent of phorbol ester stimulation (data not shown). The signaling induced by expression of myristoylated Sos was inhibited by cotransfection of dominant-negative N17Ras (Fig. 1B). These data are consistent with the idea that signaling by membrane-bound Sos is mediated by activation of the Ras pathway. Constitutively active Ras and myristoylated Sos also synergized with constitutively active calcineurin to activate NF-AT in the absence of any additional stimulation (Fig. 1C), indicating that Sos functions exclusively on the Ras-dependent pathway of NF-AT activation. Myristoylated Sos was inactive without either ionomycin stimulation or cotransfection of the calcineurin gene (data not shown). These data are consistent with recently published observations that membrane localization of full-length Sos (20) and the catalytic domain of Sos (21) in fibroblasts also activates Ras-dependent signaling.

To investigate the specificity of Sos for Ras activation in T cells, we compared the activity of Sos to that of the Dbl

oncoprotein, a GEF specific for members of the Rho/Rac subfamily of small GTP-binding proteins (22–24). In addition, we investigated the activity of the product of the Vav protooncogene (25), a hematopoietic-specific protein implicated in a variety of signal transduction pathways (reviewed in ref. 26). Vav was of particular interest since it has been shown to be tyrosine phosphorylated after TCR activation (27), and some reports suggest that it exhibits a Ras-specific GEF activity (28). This is unexpected as the catalytic domain of Vav is homologous to the Rho/Rac family of exchange factors (29) and exhibits transformation with phenotypic changes similar to RhoA and Dbl and distinct from Ras (30, 31). As expected, myristoylated Dbl was unable to activate the Ras-dependent pathway in T-cell activation (Fig. 1D), indicating that specificity for Ras by a membrane-targeted GEF is essential. Interestingly, expression of the wild-type soluble Vav protooncogene (ScVavE) activated NF-AT-dependent transcription in the absence of ionomycin or PMA to levels $\approx 15\%$ of maximal stimulation (Fig. 1D), indicating that in contrast to Sos, its overexpression alone provides a weak but sufficient signal for NF-AT-directed transcription. Myristoylated Vav (McVavE) did not activate NF-AT-dependent transcription in the absence of ionomycin. As the McVavE protein appeared to be less stable than ScVavE (Fig. 1D *Inset*), the lowered activity of myristoylated Vav may simply be the result of lower protein expression. These results indicate that Vav plays a role in TCR signaling, manifested by NF-AT-dependent transcription, but that its role appears to be distinct from that of Sos.

The Role of Grb-2 in Ras Activation. To eliminate Grb-2 interaction with myristoylated Sos while maintaining an intact Sos C-terminal domain, the two proline-rich sequences found to be essential for Grb-2 interaction (32) were mutated (Fig. 3A). MSosE immunoprecipitates were analyzed for Grb-2 coimmunoprecipitation by Western blotting. Mutation of the consensus sequence at residues 1147–1156 reduced constitutive Grb-2 binding by $\approx 50\%$, whereas an additional mutation of the site at residues 1290–1295 eliminated detectable Grb-2 interaction (Fig. 3B). This is consistent with the observation that a high-affinity Grb-2–Sos interaction requires the coordinate binding of both Grb-2 Src homology domains (32). To identify the role of Grb-2 interactions with Sos, these myristoylated Sos mutants were assayed for their ability to activate Ras-dependent signaling

FIG. 3. (A) Construction of Sos molecules containing mutations in the C-terminal proline-rich regions. Using PCR-mediated site-specific mutagenesis, proline-rich sequences were changed to alanine residues as illustrated to abolish the consensus for Src homology 3 binding. The altered versions of Sos were cloned into the vector described in Fig. 1 to generate MSosP1E and MSosP1,2E. (B) Membrane-bound Sos molecules with mutant C-terminal proline-rich domains no longer bind to Grb-2. Cells were cotransfected with the illustrated Sos constructs or vector alone with excess soluble Grb-2, immunoprecipitated in duplicate with 12CA5 antibody, and analyzed by SDS/PAGE. Samples were immunoblotted with either 12CA5 mAb to detect Sos (α Sos) or anti-Grb-2 mAb (α Grb-2). (C) Activity of membrane-bound Sos is independent of interaction with Grb-2. Cells were cotransfected with NF-AT-SX and the illustrated mutants. Stimulation with ionomycin and analysis for SEAP activity were done as described in Fig. 1.

in T cells. The elimination of Grb-2 interaction had no effect on membrane-bound Sos signaling (Fig. 3C). This is consistent with a role for Grb-2 in T cells to localize the GEF Sos to the membrane but not to induce a conformational change. These results demonstrate that Sos can function independent of the Grb-2 interaction when constitutively localized to the membrane.

Development of a Conditional Allele of Sos. Constitutive activation of Sos results in prolonged activation of all signals downstream in the Ras activation pathway, potentially initiating feedback pathways and masking immediate effects downstream of Ras. Moreover, imposing membrane localization on Sos by the addition of a myristoylation sequence may activate its GEF activity by inducing a fortuitous conformational change. To avoid these objections, we devised a conditional allele of Sos in which membrane localization is induced with a rationally designed CID, FK1012, thereby mimicking the role of Grb-2 in membrane recruitment of Sos (Fig. 4A). This allele of Sos was designed by modifying a technique recently developed to induce intracellular oligomerization of cell surface receptors (15, 16). Soluble Sos was fused to three domains of FKBP12 (SF3SosE) and coexpressed in Jurkat-TAg cells with membrane-bound myristoylated FKBP12s (MF3E). Addition of the CID FK1012 resulted in the localization of soluble Sos with membrane-bound FKBP12s and the activation of Ras (Fig. 4B). Fusion of fewer FKBP12s on the myristoylated sequence at the membrane or on Sos resulted in less optimal membrane localization (data not shown). The activation in-

duced by Sos recruited to the membrane by FK1012 was inhibited by the coexpression of a dominant-negative Ras (Fig. 4C). These data provide compelling evidence that the signal induced by the addition of the CID FK1012 is indeed due to the activation of Ras-dependent signaling and that targeting Sos to the plasma membrane *per se* facilitates the activation of Ras.

Previous work in fibroblasts has indicated that Sos exists prior to stimulation in a previously existing complex with Grb-2, suggesting that localization of Grb-2 to the membrane might also recruit Sos and activate Ras-dependent signals. To assess this possibility in T cells, a modified human Grb-2 molecule containing three tandem copies of the FKBP12 module SF3GrbE was generated. Cells were cotransfected with MF3E and SF3GrbE and treated with FK1012 to recruit the modified Grb-2 to the membrane in the presence of ionomycin. In contrast to membrane recruitment of Sos, this localization of Grb-2 to the membrane did not activate Ras-dependent signal transduction in T cells (Fig. 4D). Placing the FKBP12 module C-terminal to Grb-2 (SGrbF3E) or constitutive localization of Grb-2 to the membrane by addition of an N-terminal myristoylation sequence (MGrbE) likewise did not activate Ras (data not shown). These results suggest that another level of regulation exists in T-cell activation regulating Sos-Grb-2 interaction. Although these data do not rule out that modification of Grb-2 might prevent its interaction with Sos, this seems unlikely, as three separate modifications of Grb-2 did not result in Ras activation.

FIG. 4. (A) Use of FK1012 to mimic the role of Grb-2-induced localization of Sos. (Upper) Proposed physiologic role of Grb-2 to localize Sos to the cell membrane. (Lower) FK1012 induces the localization of a chimeric Sos-FKBP12 to a myristoylated FKBP12 at the cell membrane. (B) Activation of Ras by inducible membrane localization of Sos. Cells were cotransfected with NF-AT-SX, SF3SosE or SF3E, and MF3E. Twenty-four hours after transfection, cells were stimulated with ionomycin plus the illustrated dilutions of FK1012 for 20 hr, and cell supernatants were analyzed for SEAP activity as described in Fig. 1. The data are presented as the percent activation after stimulation with ionomycin plus FK1012 relative to activation after stimulation with ionomycin plus PMA. Expression of all FKBP12-containing constructs was confirmed by immunoblotting (data not shown). (C) Activation of Ras by inducible membrane localization of Sos is inhibited by dominant-negative Ras. Ionomycin plus the illustrated dilutions of FK1012 were added to cells cotransfected as described in B with either dominant-negative Ras or control vector. Activation is presented as described in B. (D) Inducible localization of Grb-2 does not activate Ras. Ionomycin plus the illustrated dilutions of FK1012 were added to cells cotransfected with NF-AT-SX, SF3GrbE or SF3E, and MF3E. Activation is presented as described in B.

DISCUSSION

A role for Ras in T-cell activation has been clearly established (33); however, the mechanism of its activation is unclear. Our studies indicate that at least one mechanism in T cells may be mediated by membrane recruitment of Sos. A 36- to 38-kDa tyrosine phosphoprotein (34, 35) that is localized to the particulate fraction of cells has been found associated with Grb-2/Sos after TCR stimulation, and it may be responsible for the translocation of Grb-2/Sos to the membrane after receptor stimulation. Alternatively, recruitment of Sos to the membrane in T cells may be more complex than the localization of a preformed Grb-2-Sos complex to the membrane demonstrated in fibroblasts. Recent work indicates that in T cells the interaction between Grb-2 and Sos may be inducible after activation of the antigen receptor (36). This is consistent with our observation that membrane recruitment of Grb-2 alone, in both the constitutive and inducible systems, did not result in Ras activation. The possibility that Grb-2 induces a conformational change in Sos and thereby activates its catalytic activity is unlikely in light of the ability of MSosP1,2E to fully activate the Ras pathway. This is consistent with data indicating that Grb-2 binding to Sos *in vitro* has no measurable effect on guanine nucleotide exchange activity (37). Although elimination of the Sos C-terminal domain has been shown to accentuate Sos activity, suggesting that Grb-2 binding may eliminate a negative regulatory region (18), deletion of this domain may not activate Sos but rather eliminate sites of phosphorylation by a mitogen-activated protein kinase family member, which may negatively regulate Sos (38) or possibly induce an errant conformational change resulting in higher catalytic activity or accessibility to Ras.

The role of Vav in TCR activation also remains unclear. An analysis utilizing blastocyst complementation with embryonic stem cells containing a mutant *vav* allele has recently implicated a role for Vav in signaling by the TCR (39–41). Although the activation of Vav may require additional signals normally present after TCR receptor stimulation, our data suggest that Vav is not a membrane-recruited GEF for Ras in T cells. The precise definition of Vav GEF substrate specificity and the roles of its various domains are needed to define the precise role of Vav in T-cell activation.

The role of Grb-2 and the rationale for its insertion in the signaling pathway to mediate interaction between the growth factor receptor and Sos may initially be viewed as redundant. However, this form of inducible linker-mediated dimerization improves the biologic specificity of the interaction of receptor with Sos since specific contacts must be maintained on either side of Grb-2 to mediate a biologic response. To make a conditional allele of Sos, we have mimicked this natural form of linker-mediated dimerization by using a rationally designed CID to induce localization of Sos to the membrane, clearly demonstrating that recruitment of Sos alone to the membrane results in Ras activation. Inducible activation also minimizes compensatory or feedback signals that might result from prolonged activation of this pathway and will allow the temporal dissection of events following both the immediate and prolonged activation of Ras. In addition to the inducible activation of Ras demonstrated here, inducible regulation of members of the Src-like family of tyrosine kinases (42) by membrane localization has also been achieved, which illustrates the utility of CID-mediated protein localization in studying a variety of signal transduction events.

We thank D. Bar-Sagi, A. Eva, A. Weiss, M. Barbacid, T. Pawson, and D. Cantrell for their generous gifts of reagents. This work was supported by Grants CA39612 and GM-52067 from the National Institutes of Health (S.L.S. and G.R.C.) and from the Jane Coffin

Childs Memorial Fund for Medical Research (L.J.H.). S.L.S. and G.R.C. are Investigators at the Howard Hughes Medical Institute.

1. Boguski, M. S. & McCormick, F. (1993) *Nature (London)* **366**, 643–654.
2. Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. & Pawson, T. (1993) *Cell* **73**, 179–191.
3. Baltensperger, K., Kozma, L. M., Cherniack, A. D., Klarlund, J. K., Chawla, A., Banerjee, U. & Czech, M. P. (1993) *Science* **260**, 1950–1952.
4. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T. & Pelicci, P. G. (1992) *Cell* **70**, 93–104.
5. Chardin, P., Camonis, J. H., Gale, N. W., van Aelst, L., Schlessinger, J., Wigler, M. H. & Bar-Sagi, D. (1993) *Science* **260**, 1338–1343.
6. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J. & Pelicci, P. G. (1992) *Nature (London)* **360**, 689–692.
7. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. & Bowtell, D. (1993) *Nature (London)* **363**, 83–85.
8. Zhang, X. F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R. & Avruch, J. (1993) *Nature (London)* **364**, 308–313.
9. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. (1993) *Cell* **74**, 205–214.
10. Wood, K. W., Sarnecki, C., Roberts, T. M. & Blenis, J. (1992) *Cell* **68**, 1041–1050.
11. Izquierdo, M., Downward, J., Graves, J. D. & Cantrell, D. A. (1992) *Mol. Cell. Biol.* **12**, 3305–3312.
12. Ravichandran, K. S., Lee, K. K., Songyang, Z., Cantley, L. C., Burn, P. & Burakoff, S. J. (1993) *Science* **262**, 902–905.
13. Northrop, J. P., Ullman, K. S. & Crabtree, G. R. (1993) *J. Biol. Chem.* **268**, 2917–2923.
14. Clipstone, N. A. & Crabtree, G. R. (1992) *Nature (London)* **357**, 695–697.
15. Spencer, D. M., Wandless, T. J., Schreiber, S. L. & Crabtree, G. R. (1993) *Science* **262**, 1019–1024.
16. Pruschy, M. N., Spencer, D. M., Kapoor, T. M., Miyake, H., Crabtree, G. R. & Schreiber, S. L. (1994) *Chem. Biol.* **1**, 163–172.
17. Clipstone, N. A. & Crabtree, G. R. (1993) *Ann. N.Y. Acad. Sci.* **696**, 20–30.
18. Crabtree, G. R. & Clipstone, N. A. (1994) *Annu. Rev. Biochem.* **63**, 1045–1083.
19. Woodrow, M., Clipstone, N. A. & Cantrell, D. A. (1993) *J. Exp. Med.* **178**, 1517–1522.
20. Aronheim, A., Engelberg, D., Li, N., al-Alawi, N., Schlessinger, J. & Karin, M. (1994) *Cell* **78**, 949–961.
21. Quilliam, L. A., Huff, S. Y., Rabun, K. M., Wei, W., Park, W., Broek, D. & Der, C. J. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8512–8516.
22. Eva, A., Vecchio, G., Rao, C. D., Tronick, S. T. & Aaronson, S. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2061–2065.
23. Hart, M. J., Eva, A., Zangrilli, D., Aaronson, S. A., Evans, T., Cerione, R. A. & Zheng, Y. (1994) *J. Biol. Chem.* **269**, 62–65.
24. Hart, M. J., Eva, A., Evans, T., Aaronson, S. A. & Cerione, R. A. (1991) *Nature (London)* **354**, 311–314.
25. Katrav, S., Martin-Zanca, D. & Barbacid, M. (1989) *EMBO J.* **8**, 2283–2290.
26. Puil, L. & Pawson, T. (1992) *Curr. Biol.* **2**, 275–277.
27. Margolis, B., Hu, P., Katrav, S., Li, W., Oliver, J. M., Ullrich, A., Weiss, A. & Schlessinger, J. (1992) *Nature (London)* **356**, 71–74.
28. Gulbins, E., Coggeshall, K. M., Baier, G., Katrav, S., Burn, P. & Altman, A. (1993) *Science* **260**, 822–825.
29. Adams, J. M., Houston, H., Allen, J., Lints, T. & Harvey, R. (1992) *Oncogene* **7**, 611–618.
30. Khosravi-Far, R., Chrzanowska-Wodnicka, M., Solski, P. A., Eva, A., Burridge, K. & Der, C. J. (1994) *Mol. Cell. Biol.* **14**, 6848–6857.
31. Bustelo, X. R., Suen, K. L., Leftheris, K., Meyers, C. A. & Barbacid, M. (1994) *Oncogene* **9**, 2405–2413.
32. Li, N., Batzer, A., Daly, R., Yaynik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. & Schlessinger, J. (1993) *Nature (London)* **363**, 85–88.
33. Downward, J., Graves, J. D., Warne, P. H., Rayter, S. & Cantrell, D. A. (1990) *Nature (London)* **346**, 719–723.
34. Sieh, M., Batzer, A., Schlessinger, J. & Weiss, A. (1994) *Mol. Cell. Biol.* **14**, 4435–4442.
35. Buday, L., Egan, S. E., Rodriguez Vician, P., Cantrell, D. A. & Downward, J. (1994) *J. Biol. Chem.* **269**, 9019–9023.
36. Ravichandran, K. S., Lorenz, U., Shoelson, S. E. & Burakoff, S. J. (1995) *Mol. Cell. Biol.* **15**, 593–600.
37. Buday, L. & Downward, J. (1993) *Cell* **73**, 611–620.
38. Cherniack, A. D., Klarlund, J. K. & Czech, M. P. (1994) *J. Biol. Chem.* **269**, 4717–4720.
39. Tarakhovskiy, A., Turner, M., Schaal, S., Mee, P. J., Duddy, L. P., Rajewsky, K. & Tybulewicz, V. L. J. (1995) *Nature (London)* **374**, 467–470.
40. Zhang, R., Alt, F. W., Davidson, L., Orkin, S. H. & Swat, W. (1995) *Nature (London)* **374**, 470–473.
41. Fischer, K.-D., Zmuidzinas, A., Gardner, S., Barbacid, M., Bernstein, A. & Buidos, C. (1995) *Nature (London)* **374**, 474–477.
42. Spencer, D. M., Graef, I., Austin, D. J., Schreiber, S. L. & Crabtree, G. R. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9805–9809.