Sphingosine 1-Phosphate Stimulates Tyrosine Phosphorylation of Crk*

Vicky A. Blakesley†, Dana Beitner-Johnson‡, James R. Van Brocklyn¶, Sheela Rani¶, Zila Shen-Orr‡, Bethel S. Stannard†, Sarah Spiegel†, and Derek LeRoith‡

From the †Diabetes Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892 and ¶Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, D. C. 20007

The proto-oncogene molecule e-Crk plays a role in growth factor-induced activation of Ras. Sphingosine 1-phosphate (SPP), a metabolite of cellular sphingolipids, has previously been shown to play a role in growth factor receptor signaling (Olivera, A., and Spiegel, S. (1993) Nature 365, 557–560). SPP was found to strongly induce tyrosine phosphorylation of Crk, but not Shc, in NIH-3T3 parental, insulin-like growth factor-I receptor-overexpressing and Crk-overexpressing (3T3-Crk) fibroblasts. Sphingosine, a metabolic precursor of SPP, also produced a slight increase in tyrosine phosphorylation of Crk. In contrast, other sphingolipid metabolites including ceramide did not alter Crk tyrosine phosphorylation. Furthermore, Crk enhanced SPP-induced mitogenesis, as measured by SPP-stimulated [³H]thymidine incorporation in a manner proportional to the level of Crk expression in 3T3-Crk cells. This stimulation appears to be Ras-dependent, whereas SPP stimulation of MAP kinase activity is Ras-independent. These data indicate that SPP activates a tyrosine kinase that phosphorylates Crk and that Crk is a positive effector of SPP-induced mitogenesis.

Increasing evidence suggests that the sphingolipid ceramide and its metabolites sphingosine and sphingosine 1-phosphate (SPP) represent a new class of intracellular second messengers that mediate a variety of cellular functions (1–5). Sphingosine and SPP have been shown to induce mitogenesis in a wide range of cell types (5–7). Platelet-derived growth factor (PDGF), a potent mitogen, increases cellular levels of sphingosine and SPP (8, 9). Moreover, inhibition of the PDGF-induced increase in SPP levels markedly decreased PDGF-induced cellular proliferation (8).

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† Supported by a Pharmacology Research Associate Training fellowship from the NIGMS, National Institutes of Health. Present address: Dept. of Cellular and Molecular Physiology, University of Cincinnati, Box 0576, Cincinnati, OH 45267-0576.

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§ To whom correspondence should be addressed: Bldg. 10, Rm. SS-235A, 10 Center Dr., MSC 1770, National Institutes of Health, Bethesda, MD 20892-1770. Tel.: 301-496-8090; Fax: 301-480-4386.

The abbreviations used are: SPP, sphingosine 1-phosphate; PDGF, platelet-derived growth factor; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; IGF-I, insulin-like growth factor-I; C₂-Cer, N-acetylsphingosine; C₆-Cer, N-hexanoylsphingosine; DHS, di-[threo]-dihydroxyphosphatidylcholine; PAGIE, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; Erk, extracellular signal-regulated kinase; SH2 and SH3, Src homology regions 2 and 3, respectively; G-protein, guanine nucleotide-binding protein; Sph, sphingosine; DN-Ras, dominant negative Ras.

The downstream signaling pathways utilized by these sphingolipids have not been fully elucidated. Sphingosine and SPP are known to increase intracellular calcium (6, 10–12) and phosphatidic acid levels (13, 14) and decrease cAMP levels (6, 15). SPP has also been shown to stimulate the Raf/MEK/MAP kinase signaling pathway (16). In an attempt to understand the molecular mechanisms underlying sphingosine and SPP-induced mitogenesis, we investigated the effect of these sphingolipids on intracellular signaling molecules upstream of the MAP kinase cascade, and then we examined the role of the MAP kinase signaling pathway.

It has previously been shown that activation of either Shc- or Crk-related pathways leads to activation of the MAP kinase cascade. Shc is an SH2 domain-containing protein that becomes tyrosine-phosphorylated and associates with Grb2 in response to growth factor receptor stimulation (17–19). Signaling through Shc appears to be a common pathway by which both tyrosine kinase growth factor receptors and certain G-protein-coupled receptors lead to activation of Ras (20). Crk is a noncatalytic SH2 and SH3 domain-containing adaptor molecule that shares structural homology with Grb2 and Nck (21–23). Like Grb2, Crk associates with the guanine nucleotide exchange factor mSos and a related molecule called C3G (23, 24). Thus, Crk provides an alternate pathway by which growth factor receptors can signal Ras. Crk has been specifically implicated in IGF-I receptor signaling (25). Stimulation of the IGF-I receptor induces tyrosine phosphorylation of Crk (25), and the mitogenic effects of IGF-I are enhanced in Crk-overexpressing cells (26). To explore possible interactions of the sphingolipid and Crk signaling pathways, we have investigated the effects of sphingolipid metabolites on Crk and Sh signaling.

EXPERIMENTAL PROCEDURES

Materials—Sphingomyelinase (Streptomyces sp.) was obtained from Sigma. Sphingosine-1-phosphate, sphingosine, N-hexanoylsphingosine (C₆-Cer), N-acetylsphingosine (C₂-Cer), and di-[threo]-dihydroxyphosphatidylcholine (DHS) were obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Sphingolipids were added to cells as a complex with 0.4% bovine serum albumin (Siga) in serum-free DMEM (6). IGF-I was from Genentech (San Francisco, CA). The plasmid encoding the dominant negative Ras was a gift of Dr. Silvio Gutkind (NIDR, National Institutes of Health, Bethesda, MD).

Cell Culture—Both parental and transfected NIH-3T3 mouse fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (Upstate Biotechnology Inc., Lake Placid, NY). 3T3-Crk and 3T3-Neo cells were, respectively, stably transfected with either pCWX2-CRK II, a Crk expression vector driven by the cytomegalovirus promoter and carrying neo (kindly provided by Dr. M. Matsuda, Tokyo, Japan) or the pCWX2 vector from which the Crk cDNA had been excised, as described previously (26). Before stimulation, subconfluent cultures of cells in 100-mm dishes were switched to serum-free DMEM supplemented with 0.1% insulin-free bovine serum albumin (Intergen Co., Purchase, NY) and 20 mM HEPES (pH 7.5) for 18 h. Cells were treated with IGF-I (100 nM), SPP (10 μM), sphingosine (20 μM), DHS (20 μM), C₂-Cer (20 μM), C₆-Cer (20 μM), and C₆-Cer (20 μM).
entire immunoprecipitated samples were then boiled for 2 min in
sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml
lyophilization for measurement of MAP kinase activation.

Immunoprecipitation—After treatment with growth factors, cells
were washed twice with ice-cold phosphate-buffered saline and
harvested in a lysis buffer containing 50 mM HEPES (pH 7.4), 2 mM
sodium phosphate (pH 7.2), 0.5% Nonidet P-40, 10 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml
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Tyrosine phosphorylation of Crk.

SH3 domain-mediated interactions of the Crk protein with the Ras family guanine nucleotide exchange proteins mSos and C3G represent an alternative pathway to the Shc-Grb2-mSos mechanism of Ras activation (23–25, 29, 30). To determine whether Crk was tyrosine-phosphorylated in response to sphingosine or SPP, NIH-3T3 cells were treated for 5 min with various sphingolipids. Cleared whole cell lysates were immunoprecipitated for Crk and immunoblotted for phosphotyrosine. Stimulation of NIH-3T3 cells with SPP strongly increased the tyrosine phosphorylation of Crk, as shown in Fig. 2A. This suggests that SPP activates a tyrosine kinase that specifically phosphorylates Crk. It was of interest to compare the effects of SPP with those of other biologically active sphingolipid metabolites. Sphingosine, a metabolic precursor of SPP, slightly increased the tyrosine phosphorylation of Crk, whereas DHS, a mitogenically inactive analog of sphingosine, and sphingosylphosphorylcholine, a phosphocholine ester of C2-Cer, did not alter Crk phosphorylation, treatment with ceramide did not. These results were obtained in three separate experiments.

FIG. 2. A. effect of sphingolipids on tyrosine phosphorylation of Crk in NIH-3T3 cells. NIH-3T3 cells were either unstimulated (Control) or stimulated for 5 min with DHS (20 μM), Sph (20 μM), SPP (10 μM), C2-Cer (Cer2, 20 μM), C6-Cer (Cer6, 20 μM), sphingomyelinase (SMase, 0.1 unit/ml), sphingosylphosphorylcholine (Sph, 20 μM) for 30 min. Lysates were immunoprecipitated with an anti-Crk antibody, fractionated through 9% SDS-PAGE, and immunoblotted for phosphotyrosine. The results shown are representative of those obtained in three separate experiments. B. time course of the effect of SPP on tyrosine phosphorylation of Crk in NIH-3T3 cells. Cells were stimulated with 10 μM SPP for 0, 2, 5, 10, 20, or 30 min as indicated. Lysates were immunoprecipitated for Crk then immunoblotted for phosphotyrosine. Similar results were obtained in two separate experiments, each performed in duplicate.

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FIG. 3. Effect of SPP on tyrosine phosphorylation of Crk and its association with C3G in Crk-overexpressing cells. A, 3T3-Crk7 cells were either unstimulated (Control) or stimulated with SPP (10 μM) or C2-Cer (Cer2, 20 μM) for 5 min. Lysates were immunoprecipitated with an anti-Crk antibody, fractionated through 9% SDS-PAGE, and immunoblotted for phosphotyrosine. B, 3T3-Crk7 cells were either unstimulated (Control) or stimulated with SPP (10 μM), DHS (20 μM), or Sph (20 μM) for 5 min. Lysates were immunoprecipitated with anti-C3G antisera, fractionated through 9% SDS-PAGE, and immunoblotted for Crk. The results shown are representative of those obtained in three separate experiments.

FIG. 4. Effect of sphingolipids on phosphorylation of Erk1 and Erk2. Cells were incubated for 5 min with IGF-I (100 nM), Sph (20 μM), SPP (10 μM), DHS (20 μM), and DHS plus IGF-I. Lysates were fractionated by 7.5% SDS-PAGE and immunoblotted with an antibody that recognizes both Erk1 (E1) and Erk2 (E2). The results are representative of three separate experiments.

Immunoblots for phosphotyrosine. As shown in Fig. 3A, SPP, but not ceramide, induced tyrosine phosphorylation of Crk in these cells. This Crk-overexpressing cell line also enabled us to study the effect of SPP on interactions of Crk with the guanine nucleotide exchange protein C3G, which is difficult to visualize in NIH-3T3 parental cells.2 In these experiments, cleared whole cell lysates were immunoprecipitated with an antibody against C3G and immunoblotted for Crk. In Fig. 3B, it can be seen that treatment of cells with SPP, DHS, or sphingosine for 5 min had no effect on the amount of Crk protein associated with C3G. Identical results were obtained in a second Crk-overexpressing clone, 3T3-Crk9 (data not shown). IGF-I and other growth factors have also been found not to alter Crk association with C3G (data not shown).3 Thus, alterations in the amount of Crk associated with C3G do not appear to be involved in the mechanism by which SPP and other mitogens mediate signaling through Crk.

We have previously shown that SPP, sphingosine, and ceramide induced mitogenic responses in Swiss 3T3 fibroblasts (6, 31). We examined in detail the dose responses of different sphingolipid metabolites (6, 8, 14, 31) that are mitogenic for those cells and used optimal concentrations to ensure that the different responses are not caused by differences in uptake. We have shown in the present study that, whereas SPP induced Crk phosphorylation, treatment with ceramide did not. These results suggest that the mitogenic signaling pathways utilized by SPP and ceramide differ. We have previously shown that SPP stimulates the Raf/MEK/MAP kinase pathway in Swiss

2 V. A. Blakesley, D. Beitner-Johnson, S. Spiegel, and D. LeRoith, unpublished observations.

3 B. Knudsen, personal communication.
Tyrosine Phosphorylation of Crk by SPP

FIG. 5. Effect of Crk overexpression on the mitogenic response to SPP and various sphingolipid metabolites. A, subconfluent monolayers of cells were made quiescent in reduced serum medium for 6 h and then stimulated with the indicated concentrations of SPP for 18 h at 37 °C. [3H]Thymidine was added to a final concentration of 1 μCi/well, and the incubation was continued for 1 h. Incorporation of radioactivity was measured as described under “Experimental Procedures.” All assays were carried out in triplicate, and the standard error for each cell line in individual experiments was within 5%. The data shown are representative of three separate experiments. For each cell line, thymidine incorporation (cpm) was normalized to protein content (μg of protein). Cell lines: ○, 3T3-Neo; □, 3T3-Neo11; ○, 3T3-Crk7; and ■, 3T3-Crk9. B, subconfluent serum-starved 3T3-Crk7 cells were incubated for 18 h with 10% serum or various concentrations of SPP (10 μM), Sph (20 μM), C2-Cer (Cer2, 10 μM), or sphingomyelinase (SMase, 0.1 unit/ml), and [3H]thymidine incorporation was measured. Data are expressed as the average (cpm/μg of protein) of four determinations, with error bars (S.E.). Control, unstimulated.

3T3 cells (16). Moreover, in myeloid HL-60 cells, ceramide activated MAP kinase activity (34) and activated a protein kinase that phosphorylates Raf1 on Thr-269, enhancing its activity toward MEK and linking ceramide signaling to the MAP kinase pathway (27). Therefore, it was of interest to examine the activation state of MAP kinases by various sphingolipid metabolites. Whereas IGF-I, SPP, and sphingosine induced a mobility shift in Erk1 and Erk2 consistent with phosphorylation and activation of these MAP kinases (Fig. 4), DEHS and Cer-Cer did not induce a mobility shift (Fig. 4 and data not shown). Thus, in mouse fibroblasts, SPP and ceramide appear to act through distinct signaling pathways leading to Erk phosphorylation.

To further evaluate the functional effects of Crk overexpression on SPP signaling, the mitogenic effects of SPP were compared in two Crk-overexpressing (3T3-Crk) and two neomycin-resistant (3T3-Neo) control clones using a [3H]thymidine incorporation assay. In Fig. 5A it can be seen that SPP produced a dose-dependent increase in thymidine incorporation in both the 3T3-Neo and 3T3-Crk cell types. However, SPP-induced mitogenesis was significantly enhanced in Crk-overexpressing cells compared with control 3T3-Neo cells. Furthermore, SPP-induced mitogenesis was dependent on the level of Crk expression in these cell lines. That is, the highest level of SPP-induced mitogenesis was exhibited by 3T3-Crk7, which expresses 10-fold higher Crk levels than parental cells. Intermediate levels were found in 3T3-Crk9, which expresses 3-fold higher Crk levels than parental, and 3T3-Neo cells were least responsive to SPP. Interestingly, in contrast to SPP and sphingosine, which stimulate DNA synthesis by 8- and 4-fold, respectively, C2-ceramide and sphingomyelinase were not potent mitogens in Crk-overexpressing cells (Fig. 5B). To determine whether Shc also plays an important role in mitogenesis induced by SPP, we examined the effect of SPP on NIH-3T3 cells overexpressing Shc (Fig. 6). The SPP response in the Shc-overexpressing cell line, Shc/Myc, was similar to that seen in parental NIH-3T3 cells. Thus overexpression of Shc did not enhance the mitogenic response to SPP as did overexpression of Crk.

To determine if SPP induced Erk1 and Erk2 activation in cells overexpressing Crk was mediated via the Ras/MAP kinase pathway, we measured MAP kinase activity and thymidine incorporation in 3T3-Crk7 cells transiently transfected with either vector encoding dominant negative Ras (DN-Ras) or neo vector. Overexpression of Crk by 10-fold (3T3-Crk7 cells) increased the basal levels of MAP kinase activity by 2.3-fold. Furthermore, in NIH-3T3 cells with neo vector alone, MAP kinase activity was increased 4.3-fold in response to SPP, whereas 3T3-Crk7 cells exhibited only a 1.4-fold increase in MAP kinase activity after SPP stimulation. Expression of dominant negative Ras in 3T3-Crk7 cells did not affect basal MAP kinase activity nor did it block the effect of SPP on MAP kinase activity. This lack of effect is likely
due to the high basal activity. The presence of endogenous Ras in 3T3-Crk7 cells and dominant negative Ras in cells transfected with the construct encoding this protein is shown in Fig. 7. These results are consistent with the hypothesis that Crk, at least in part, induces increased MAP kinase activity by a pathway other than the Ras pathway.

We considered the possibility that the increased mitogenesis in response to SPP may not have been mediated entirely by Ras in the 3T3-Crk7 cells. As shown in Fig. 8, the basal level of thymidine incorporation in 3T3-Crk7 cells expressing dominant negative Ras was drastically reduced as compared with 3T3-Crk7 cells transfected with the neo' vector. In addition, there was an attenuation of the SPP stimulation of thymidine incorporation when dominant negative Ras was expressed. Thus, although Crk mediation of SPP-induced MAP kinase activation was shown to be Ras-independent, functional Ras is essential for SPP-stimulated mitogenesis in cells expressing Crk. Results from other investigative groups indicate that Crk may function in Ras-independent as well as Ras-dependent signal transduction pathways, leading to increased mitogenesis. Early reports of downstream activators of Crk showed that Crk complexed with the Ras guanine nucleotide exchange factors mSos and C3G, suggesting that Crk may activate the Ras pathway and, thus, MAP kinase, similar to Grb2 (24, 35, 36). More recently, it has become clear that stimulation of Crk phosphorylation and concomitant MAP kinase activation is not solely via the Ras/Raf/MEK pathway. Expression of dominant negative Ras did not abrogate enhanced MAP kinase activity, and treatment with the MEK1 inhibitor PD98059 did not reduce the number of colonies in soft agar in NIH-3T3 cells expressing v-Crk (37, 38). The finding that v-Crk, an oncogenic protein, could transform cells even in the presence of dominant negative Ras, however, may not predict the ability of c-Crk to be totally independent of the Ras pathway in mediating mitogenic or tumorigenic phenotypes. In fact, in the present report, the results indicate that c-Crk also mediates activation of MAP kinase by pathways other than the Ras pathway, but it further shows that mitogenesis is severely impaired if the Ras pathway is disrupted.

In summary, we show that the sphingolipid metabolite SPP, and to a lesser extent, sphingosine induces tyrosine phosphorylation of the signaling molecule Crk. This occurred in both parental and Crk-overexpressing NIH-3T3 cells as well as in Swiss 3T3 cells (data not shown). In contrast, ceramide, which has been implicated in cell growth, differentiation, and apoptosis, did not induce phosphorylation of Crk. Interestingly, SPP did not induce tyrosine phosphorylation of the SH2 domain-containing signaling molecule Shc. Thus, SPP appears to activate an as yet unidentified tyrosine kinase that specifically phosphorylates Crk. One potential candidate for such a kinase is c-Abl, which is known to phosphorylate Crk (39). In addition, SPP-induced mitogenesis was significantly enhanced in Crk-overexpressing cells compared with the control, and SPP-induced mitogenesis was proportional to the level of Crk expression. SPP-induced mitogenesis occurred in parallel with activation of MAP kinase, but these SPP-stimulated events were variably affected by the expression of a dominant negative Ras. Taken together, these data strongly suggest that Crk is a positive effector of SPP signaling, and furthermore, Crk sends multiple signals in response to upstream stimuli.

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REFERENCES

1. Hakomori, S. (1990) J. Biol. Chem. 265, 18713–18716
2. Merrill, A. H. (1991) J. Bioenerg. Biomembr. 23, 83–104
3. Hanuman, T. A. (1994) J. Biol. Chem. 269, 3125–3128
4. Kolesnick, R. N., and Golde, D. W. (1994) Cell 77, 325–328
5. Spiegel, S., and Milstein, S. (1995) J. Membr. Biol. 146, 225–237
6. Zhang, H., Desai, N. N., Murphy, J. M., and Spiegel, S. (1990) J. Biol. Chem. 265, 21309–21316
7. Gomez-Munoz, M. A., Martin, A., O'Brien, L., and Brandley, D. N. (1994) J. Biol. Chem. 269, 5847–5856
8. Olivera, A., and Spiegel, S. (1993) Nature 365, 557–560
9. Bornfeldt, K. E., Graves, L. M., Raines, E. W., Igashashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sibone, J. S., Krebs, E. G., Hakomori, S., and Ross, R. (1995) J. Cell Biol. 130, 193–206
10. Ghosh, T. K., Bhan, J., and Gill, D. L. (1994) J. Biol. Chem. 269, 22628–22635
11. Mattie, M., Brooker, G., and Spiegel, S. (1994) J. Biol. Chem. 269, 3181–3188
12. Seemayer, G., Laulederkiz, S. J., and Baliu, L. R. (1994) J. Biol. Chem. 269, 5847–5856
13. Lavie, Y., and Lisovitch, M. (1990) Biochem. Biophys. Res. Commun. 167, 607–613
14. Desai, N. N., Zhang, H., Olivera, A., Mattie, M. E., and Spiegel, S. (1992) J. Biol. Chem. 267, 23122–23128
15. Goodemote, K. A., Mattie, M. E., Berger, A., and Spiegel, S. (1995) J. Biol. Chem. 270, 10722–10727
16. Wu, J., Spiegel, S., and Sturgill, T. W. (1995) J. Biol. Chem. 270, 11484–11488
17. Li, W., Hu, P., Skolnick, E. Y., Ullrich, A., and Schlessinger, J. (1992) Mol. Cell. Biol. 12, 5824–5833
18. Buday, L., and Downward, J. (1995) Cell 73, 611–620
19. Pawson, A. J. (1995) Nature 373, 573–580
20. van Biesen, T., Hawes, B. E., Lutrillier, D. K., Krueger, K. M., Touhara, K., Portelli, E., Sakane, M., Lutrillier, L. M., and Lefkowitz, R. J. (1995) Nature 376, 781–784
21. Mayer, J. B., Hamauchi, M., and Hanafusa, H. (1988) Nature 332, 272–275
22. Reichman, C. T., Keshau, S., and Hanafusa, H. (1992) Cell Growth Diff. 3, 451–460
23. Matsuda, M., Hashimoto, Y., Muroya, K., Hasegawa, H., Kurata, T., Tanaka, S., Nakamura, S., and Hattori, S. (1994) Mol. Cell. Biol. 14, 5485–5500
24. Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Masabumi, S., Matouka, K., Takenawa, T., Kurata, T., Nagashima, K., and Matsuda, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3443–3447
25. Beirne-Johnson, D., and LeRoith, D. (1995) J. Biol. Chem. 270, 5387–5390
26. Huang, H. Y., Zhang, Y., Delikat, S., Mathias, S., Basu, S., and Kolesnick, R. (1995) J. Biol. Chem. 270, 6080–6089
27. Venable, M. E., Glue, C. G., and Obeid, L. M. (1994) J. Biol. Chem. 269, 26040–26044
28. Knudsen, B. S., Zheng, J., Feller, S. M., Mayer, J. P., Burrell, S. K., Cowburn, D., and Hanafusa, H. (1995) EMBO J. 14, 2191–2198
29. Olivera, A., Buckley, N. E., and Spiegel, S. (1992) J. Biol. Chem. 267, 36121–36127
30. Haueter, J. M., Buehrer, B. M., and Bell, R. M. (1994) J. Biol. Chem. 270, 26040–26044
31. Raines, M. A., Kolesnick, R. N., and Golde, D. W. (1993) J. Biol. Chem. 268, 14572–14575
32. Knudsen, B., Fell, S., and Hanafusa, H. (1994) J. Biol. Chem. 270, 7693–7699
33. Venable, M. E., Glue, C. G., and Obeid, L. M. (1994) J. Biol. Chem. 269, 13927–13930
34. Hauser, J. M., Buehrer, B. M., and Bell, R. M. (1994) J. Biol. Chem. 269, 6803–6809
35. Greulich, H., and Hanafusa, H. (1996) Cell Growth Diff. 7, 1443–1451
36. Tanaka, S., and Hanafusa, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2356–2361
37. Feller, S. M., Knudsen, B., and Hanafusa, H. (1994) EMBO J. 13, 2341–2351