Calcineurin is essential for DNA synthesis in Swiss 3T3 fibroblasts

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DNA synthesis was measured 16 h after stimulation of Swiss 3T3 fibroblasts in the resting phase with various growth factors (platelet-derived growth factor, fibroblast growth factor, lysophosphatidic acid and thrombin). When extracellular Ca^{2+} was chelated by EGTA, or when the influx of Ca^{2+} from outside to inside the cell was blocked by cobalt, DNA synthesis was completely inhibited. As there was no effect whatsoever on DNA synthesis when Ca^{2+} was chelated, or when the influx of Ca^{2+} was blocked up to the first 4 h after growth stimulation, it was concluded that, at an early stage, Ca^{2+} influx from outside to inside the cell is not related to the transition from

the G_1 to the S phase. A Ca^{2+} /calmodulin-dependent protein kinase II inhibitor (KN-62) had no effect on DNA synthesis. However, cyclosporin A and FK-506, which are inhibitors of Ca^{2+} /calmodulin-dependent protein phosphatase 2B (calcineurin), markedly inhibited DNA synthesis stimulated by all of the growth factors. These results indicate that calcineurin plays a role, not only in activation of T-cells of the immune system in the initial phase, but also in DNA synthesis in fibroblasts. It was concluded that Ca^{2+} influx from outside to inside the cell during the mid-to-late G_1 phase, followed by calcineurin activation, is essential as a mechanism of growth signal transduction.

INTRODUCTION

A considerable number of factors concerning the mechanism of signal transduction in cell growth are now understood, including the discovery of the mitogen-activated protein (MAP) kinase cascade [1–4], but numerous factors still remain to be clarified. In order for information from the growth factors to be transmitted into the nucleus, causing the cell to undergo transition from the G_1 to the S phase, it is essential that the intracellular Ca^{2+} concentration increases before the cell enters the S phase [5]. Conventionally, it has been thought that stimulation by growth factors causes activation of phospholipase C, causing $\text{PtdIns}(4,5)\text{P}_2$ to undergo hydrolysis, resulting in the production of $\text{Ins}(1,4,5)\text{P}_3$ and promoting Ca^{2+} mobilization from the endoplasmic reticulum to the cytoplasm, with a link between protein kinase C activation and DNA synthesis [6,7]. In recent years, however, there have been successive discoveries, including the fact that no inhibition of DNA synthesis occurs at all, even at platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors to which phospholipase C cannot bind [8–11], and doubts have been raised about the role of PtdIns metabolic turnover in the cell growth signal transduction process. In fact, the production of $\text{Ins}(1,4,5)\text{P}_3$ after stimulation by growth factors and the increase in cytoplasmic Ca^{2+} concentration are completed in a matter of a few minutes [6], and it is therefore highly unlikely that these processes are linked with DNA synthesis, which takes over 10 h following growth-factor stimulation. As DNA synthesis is completely inhibited when extracellular Ca^{2+} is chelated, or when the influx of Ca^{2+} from outside to inside the cell is blocked using substances such as cobalt [12,13], it is thought that the essential requirement for cellular G_1/S transition is not the transient mobilization of Ca^{2+} from the intracellular organelles to the cytoplasm, which occurs in the initial phase following stimulation with growth factors,

but the influx of Ca^{2+} from outside to inside the cell occurring in the late G_1 phase.

In recent years, it has become clear that Ca^{2+} /calmodulin-dependent protein phosphatase 2B (calcineurin) plays a vital role in the mechanism of T-cell activation occurring in the initial phase. Calcineurin activates the nuclear antigen of activated T-cells (NFAT) via dephosphorylation, stimulating the genetic transcription of cytokines, such as interleukin-2 (IL-2), and activating T-cells [14–16].

In this paper, we report on the results of a study in which, using Swiss 3T3 fibroblasts, we discovered that DNA synthesis due to various growth factors is markedly inhibited by a calcineurin inhibitor, raising the possibility that calcineurin is a protein phosphatase that is vital not only to the immune system, but also to more universal cell growth signal transduction pathways.

EXPERIMENTAL

Materials

PDGF, FGF, lysophosphatidic acid (LPA), thrombin, EGTA, Triton X-100 and cobalt chloride were purchased from Sigma. The Ca^{2+} /calmodulin-dependent protein kinase II inhibitor (KN-62) was obtained from Seikagaku Kogyo. Cyclosporin A was from Sandoz, FK-506 (an inhibitor of calcineurin) was from Fujisawa Pharmaceuticals, and quin-2/acetoxymethyl ester (quin-2/AM) was from Amersham.

Cell culture

Swiss 3T3 fibroblasts were continuously cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (50 units/ml), kanamycin (50 $\mu\text{g}/\text{ml}$) and glutamine (2 mM).

Abbreviations used: PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; LPA, lysophosphatidic acid; NFAT, nuclear antigen of activated T-cells; IL-2, interleukin-2; MAP kinase, mitogen-activated protein kinase; LDH, lactate dehydrogenase; quin-2/AM, quin-2/acetoxymethyl ester.

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[³H]Thymidine uptake

Swiss 3T3 fibroblasts were grown in 12-well tissue-culture dishes to about 70–80% confluency in the presence of 10% (v/v) serum. This was followed by incubation of the fibroblasts in serum-free medium for 24 h, and then by treatment with growth factors. Finally, incubations were continued in the presence of [*methyl*-³H]thymidine (5 μ Ci/ml) for 60 min. The fibroblasts were washed twice with PBS and then four times with 5% trichloroacetic acid. The acid-insoluble material was redissolved in 0.3% sodium hydroxide and an aliquot was taken to measure the DNA-associated ³H-activity in a liquid scintillation counter.

Measurement of quin-2 fluorescence

Measurement of quin-2 fluorescence evoked by various growth factors [17,18] was carried as follows. To 10 ml of Swiss 3T3 fibroblasts suspension (1 $\times$ 10⁶ cells/ml), quin-2/AM was added at a concentration of 40 μ M, and the mixture was incubated for 120 min at 37.0 °C. It was then centrifuged at 600 rev./min ($r_{av} \approx 15$ cm) for 5 min and the supernatant was discarded. The cell deposits were dispersed in fresh PBS and this washing process was repeated five times to eliminate extracellular quin-2. A Swiss 3T3 fibroblast suspension (3 ml) impregnated with quin-2 was placed in a fluorophotometric cuvette and the intensity was measured with a Shimadzu RF-500 fluorophotometer. To 3 ml of the same cell suspension, various growth factors were added, stirred and then subjected to fluorophotometry to detect changes in fluorescence intensity. After the measurement, 0.2% (v/v) Triton X-100 was added to the suspension to solubilize the cells. CaCl₂ was then added at a concentration of 1 nM and the maximum fluorescence was determined. Subsequently, the minimum fluorescence was determined after addition of 0.5 mM MnCl₂. The cytoplasmic Ca²⁺ concentration was calculated from these values.

Measurements of extracellular free Ca²⁺, Na⁺, K⁺ and lactate dehydrogenase (LDH)

The extracellular free Ca²⁺, Na⁺, K⁺ and LDH concentrations in the culture medium were measured by the minimum electrode method using an ISE automatic instrument (Nova[CRT8]).

RESULTS

Effects of extracellular Ca²⁺ on DNA synthesis

When Ca²⁺ in the extracellular fluid was chelated with 2 mM EGTA, DNA synthesis caused by stimulation with PDGF, FGF, LPA and thrombin was completely inhibited (Table 1). When the free Ca²⁺ concentration of the extracellular medium was de-

Table 2 Free Ca²⁺ concentration of the extracellular medium measured by the minimum electrode method using various concentrations of EGTA

The results are representative of an experiment which was performed five times.

EGTA	Free Ca ²⁺ concentration in the extracellular medium (mM)
0	1.98 $\pm$ 0.26
10 μ M	1.46 $\pm$ 0.17
100 μ M	1.02 $\pm$ 0.11
1 mM	0.37 $\pm$ 0.08
2 mM	0.04 $\pm$ 0.01

terminated using various concentrations of EGTA, it was confirmed that when 2 mM EGTA is used, chelation of almost all of the free Ca²⁺ occurs (Table 2). Moreover 2 mM cobalt, which blocks the influx of Ca²⁺, also completely inhibited DNA synthesis (Table 3). When chelation of extracellular Ca²⁺, or blocking of Ca²⁺ influx was investigated by adding growth factors to cells in the resting phase, we found that there was no inhibition of DNA synthesis during the first 4 h after the addition (Tables 1 and 3).

Transient intracellular Ca²⁺ mobilization by various growth factors

Intracellular Ca²⁺ concentration when FGF or LPA was added to quiescent Swiss 3T3 fibroblasts was measured using quin-2, with the extracellular free Ca²⁺ being chelated by 2 mM EGTA. As a result, a transient rise in intracellular Ca²⁺ was observed after addition of the growth factor, even though all of the extracellular Ca²⁺ had been chelated (Figure 1).

Effect of a Ca²⁺/calmodulin-dependent protein kinase II inhibitor (KN-62) on DNA synthesis

It has been clearly shown that the influx of Ca²⁺ from outside to inside the cell from the mid-to-late G₁ phase is essential to the G₁/S transition of Swiss 3T3 fibroblasts. We conducted the following experiment in order to determine why Ca²⁺ is required. KN-62 is known to be a specific inhibitor of Ca²⁺/calmodulin-dependent protein kinase II [19]. As a multifunctional protein kinase, Ca²⁺/calmodulin-dependent protein kinase II is thought to play various physiological roles in the body [20,21], but it is not clear whether it plays a role in the G₁/S transition of fibroblasts. We therefore added KN-62 to the culture medium at

Table 1 Measurement of [³H]thymidine uptake 16 h after adding various growth factors to Swiss 3T3 fibroblasts in the resting phase while varying Ca²⁺ chelating time using 2 mM EGTA

The control, growth-factor free, had a value of 543 $\pm$ 45 c.p.m./10³ cells. The results are representative of an experiment which was performed five times.

Ca ²⁺ chelating time (h) ...	[³ H]Thymidine uptake (c.p.m./10 ³ cells)				
	0	4	8	12	16
5 ng/ml PDGF	34758.7 $\pm$ 2852	32856.1 $\pm$ 2609	16980.4 $\pm$ 1342	811.4 $\pm$ 54	578.9 $\pm$ 31
5 ng/ml FGF	36274.4 $\pm$ 3120	32570.2 $\pm$ 2467	15368.9 $\pm$ 1278	764.5 $\pm$ 43	586.5 $\pm$ 37
100 μ M LPA	32515.6 $\pm$ 2564	31328.9 $\pm$ 1769	14650.8 $\pm$ 1143	754.1 $\pm$ 32	565.1 $\pm$ 61
100 μ M Thrombin	30084.1 $\pm$ 2001	30087.4 $\pm$ 1648	12976.4 $\pm$ 1076	732.1 $\pm$ 38	587.4 $\pm$ 49

Table 3 Measurement of [^3H]thymidine uptake 16 h after adding various growth factors to Swiss 3T3 fibroblasts in the resting phase while varying the time of treatment with 2 mM cobalt

The control, growth factor free, had a value of 558.5 ± 67 c.p.m./ 10^3 cells. The results are representative of an experiment which was performed five times.

Cobalt-treatment time (h) ...	$[\text{H}]$ Thymidine uptake (c.p.m./ 10^3 cells)				
	0	4	8	12	16
5 ng/ml PDGF	32865.8 ± 4975	29865.4 ± 3143	16879.4 ± 1556	1123.0 ± 113	578.0 ± 43
5 ng/ml FGF	35161.2 ± 5716	32312.5 ± 3431	15987.6 ± 2243	1245.9 ± 145	589.8 ± 21
100 μM LPA	30123.2 ± 2843	28796.0 ± 2978	14657.9 ± 1805	1008.9 ± 122	590.6 ± 65
100 μM Thrombin	29612.7 ± 3165	28031.4 ± 2658	16547.1 ± 1509	944.0 ± 101	558.9 ± 32

Figure 1 Intracellular Ca^{2+} concentration when 5 ng/ml FGF or 100 μM LPA was added to quiescent Swiss 3T3 fibroblasts

Intracellular Ca^{2+} concentration was measured using quin-2 with the extracellular free Ca^{2+} being chelated by 2 mM EGTA

various concentrations, but this kinase inhibitor showed virtually no inhibition of DNA synthesis stimulated by growth factors, even at high concentrations (Figures 2a and 2b).

Effect of Ca^{2+} /calmodulin-dependent protein phosphatase 2B inhibitors (cyclosporin A and FK-506) on DNA synthesis

In addition to Ca^{2+} /calmodulin-dependent protein kinase, another protein, which is activated by Ca^{2+} /calmodulin, is Ca^{2+} /calmodulin-dependent protein phosphatase 2B (calcineurin). Using cyclosporin A and FK-506, which are specific inhibitors of calcineurin, we investigated the effect on DNA synthesis in Swiss 3T3 fibroblasts. At concentrations of 1 μM or less, cyclosporin A and FK-506 both inhibited the stimulation of DNA synthesis by all of the growth factors by approx. 50 %, and at 10 μM or above, DNA synthesis was completely inhibited (Figures 3a, 3b, 4a and 4b).

Viability of cells

In order to demonstrate whether or not there is irreversible damage to cells following prolonged exposure of Swiss 3T3 fibroblasts to cobalt chloride, KN-62, cyclosporin A and FK-506, the concentrations of LDH, Na^+ and K^+ in the extracellular

Figure 2 Measurement of [^3H]thymidine uptake in the presence of KN-62

(a) [^3H]Thymidine uptake was measured 16 h after adding growth factors which use tyrosine kinase receptors ($\square$, 5 ng/ml PDGF; $\circ$, 5 ng/ml FGF) to Swiss 3T3 fibroblasts in the resting phase in the presence of various concentrations of KN-62. The results correspond to a representative experiment which was performed five times. (b) [^3H]Thymidine uptake was measured 16 h after adding growth factors which use receptors coupled to G_i ($\blacksquare$, 100 μM LPA; $\bullet$, 100 μM thrombin) to Swiss 3T3 fibroblasts in the resting phase in the presence of various concentrations of KN-62. The results correspond to a representative experiment which was performed five times.

medium were determined 16 h after the addition of these reagents. The extracellular LDH, Na^+ and K^+ concentrations were also determined when cells were irreversibly damaged by 0.5 % Triton X-100, as a control. As a result, when the above-mentioned reagents were added in a known concentration range (cobalt, 2 mM, KN-62, 10–100 μM , cyclosporin A, 10–100 μM FK-506, 10–100 μM), practically no difference was observed compared with the levels in the medium of normal cells when nothing was

Figure 3 Measurement of $[^3\text{H}]$ thymidine uptake in the presence of cyclosporin A

(a) $[^3\text{H}]$ Thymidine uptake was measured 16 h after adding growth factors which use tyrosine kinase receptors ($\square$, 5 ng/ml PDGF; $\circ$, 5 ng/ml FGF) to Swiss 3T3 fibroblasts in the resting phase in the presence of various concentrations of cyclosporin A. The results correspond to a representative experiment which was performed five times. (b) $[^3\text{H}]$ Thymidine uptake was measured 16 h after adding growth factors which use receptors coupled to G_i ($\blacksquare$, 100 μM LPA; $\bullet$, 100 μM thrombin) to Swiss 3T3 fibroblasts in the resting phase in the presence of various concentrations of cyclosporin A. The results correspond to a representative experiment which was performed five times.

added. There was an obvious marked rise in the LDH, Na^+ and K^+ concentrations in the medium when the cells were irreversibly damaged with 0.5% Triton X-100 (Table 4).

DISCUSSION

We found that a transient rise in intracellular Ca^{2+} was observed after adding various growth factors to quiescent Swiss 3T3 fibroblasts, even though all of the extracellular Ca^{2+} had been chelated by 2 mM EGTA. Based on the fact that even though this type of Ca^{2+} mobilization from organelle to cytoplasm is induced, DNA synthesis is blocked after 16 h when the extracellular Ca^{2+} is chelated, it is concluded that a transient Ca^{2+} rise in the initial phase following stimulation with growth factors is unrelated to DNA synthesis. Furthermore, we found that there was no inhibition of DNA synthesis when chelation or blocking of the Ca^{2+} influx was carried out during the first 4 h. This finding indicates that the influx of Ca^{2+} from outside to inside the cell during the mid-to-late G_1 phase is of vital importance for DNA synthesis.

It has been shown that DNA synthesis is strongly inhibited by a calmodulin inhibitor [5] so Ca^{2+} taken up from outside the cell

Figure 4 Measurement of $[^3\text{H}]$ thymidine uptake in the presence of FK-506

(a) $[^3\text{H}]$ Thymidine uptake was measured 16 h after adding growth factors which use tyrosine kinase receptors ($\square$, 5 ng/ml PDGF; $\circ$, 5 ng/ml FGF) to Swiss 3T3 fibroblasts in the resting phase in the presence of various concentrations of FK-506. The results correspond to a representative experiment which was performed five times. (b) $[^3\text{H}]$ Thymidine uptake was measured 16 h after adding growth factors which use receptors coupled to G_i ($\blacksquare$, 100 μM LPA; $\bullet$, 100 μM thrombin) to Swiss 3T3 fibroblasts in the resting phase in the presence of various concentrations of FK-506. The results correspond to a representative experiment which was performed five times.

is thought to exert its action via calmodulin. Ca^{2+} /calmodulin is now known to have at least 30 targets, two of which are Ca^{2+} /calmodulin-dependent protein kinase II and Ca^{2+} /calmodulin-dependent protein phosphatase 2B (calcineurin). As a multifunctional protein kinase, Ca^{2+} /calmodulin-dependent protein kinase II is especially well known. Therefore we added KN-62, which is known to be a specific inhibitor of Ca^{2+} /calmodulin-dependent protein kinase II, to the culture medium at various concentrations. However, this kinase inhibitor showed virtually no inhibition of DNA synthesis due to growth factors, even at high concentrations. This finding indicated that Ca^{2+} /calmodulin-dependent protein kinase II does not play an important role in the G_1/S transition of fibroblasts. Then we added cyclosporin A and FK-506, which are specific inhibitors of calcineurin, to the culture medium. In the initial phase of activation of T-cells, calcineurin is thought to activate NFAT via dephosphorylation, thus promoting the genetic transcription of cytokines such as IL-2. Even at low concentrations, cyclosporin A and FK-506 both strongly inhibited the stimulation of DNA synthesis by all of the growth factors. This finding indicated that calcineurin may play a vital role, not only in the specific phenomenon of T-cell activation, but also in more general cell

Table 4 Measurement of the concentrations of LDH, Na⁺ and K⁺ in the extracellular medium by the minimum electrode method 16 h after the addition of various reagents to Swiss 3T3 fibroblasts

The extracellular LDH, Na⁺ and K⁺ concentrations were also determined when cells were irreversibly damaged by 0.5% Triton X-100, as the control. The results are representative of an experiment which was performed five times.

Reagent	LDL (units/l)	Na ⁺ (m-equiv./l)	K ⁺ (m-equiv./l)
None	183.7 ± 2.6	129 ± 1.2	6.0 ± 0.08
Cobalt chloride (2 mM)	182.6 ± 3.7	128 ± 0.8	5.9 ± 0.07
KN-62 (10 µM)	179.5 ± 4.9	130 ± 1.1	5.9 ± 0.07
KN-62 (100 µM)	185.5 ± 4.8	131 ± 1.3	5.8 ± 1.03
Cyclosporin A (10 µM)	186.3 ± 2.6	129 ± 0.7	6.0 ± 0.09
Cyclosporin A (100 µM)	183.2 ± 3.5	130 ± 1.4	5.9 ± 1.04
FK-506 (10 µM)	179.6 ± 4.9	130 ± 1.7	5.9 ± 1.01
FK-506 (100 µM)	178.8 ± 4.0	128 ± 1.6	6.0 ± 0.08
Triton X-100 (0.5%)	303.1 ± 13.5	151 ± 2.7	10.5 ± 1.89

growth signal transduction mechanisms, including those in fibroblasts.

Richter et al. have also recently reported that cyclosporin A and FK-506 inhibit DNA synthesis [22]. However, these researchers state that other specific inhibitors of calcineurin, pyrethroid insecticides, do not inhibit DNA synthesis, suggesting that the inhibitory effects of cyclosporin A and FK-506 on DNA synthesis are not via the inhibition of calcineurin but via another pathway. The theory seems to be established at present that cyclosporin A and FK-506 bind with cyclophilin and FK-506 binding protein respectively, to form complexes that inhibit the activity of calcineurin when bound with calcineurin [23,24]. But the existence of other unknown pathways cannot be excluded, as Richter et al. [22] suggested. In the case of both cyclosporin A and FK-506 or pyrethroid insecticides, it seems impossible to determine in experiments with specific inhibitors whether calcineurin plays a direct role in DNA synthesis. Rasmussen et al. demonstrated in research using *Aspergillus nidulans* that the destruction of the calcineurin gene inhibited G₁/S transition [25]. We think it is also necessary to conduct such gene destruction experiments with mammalian cells, including Swiss 3T3 fibroblasts, for direct proof. We are currently planning experiments in this direction. Takuwa et al. have also reported that extracellular Ca²⁺ and calmodulin are essential for G₁/S transition and have suggested that both Ca²⁺/calmodulin-dependent protein kinase and Ca²⁺/calmodulin-dependent protein phosphatase 2B possibly play roles in the G₁/S transition [26]. In our present experiments, KN-62, which is a specific inhibitor of Ca²⁺/calmodulin-dependent protein kinase II, hardly suppressed DNA synthesis, even at high concentrations. Means and co-workers showed that Ca²⁺/calmodulin-dependent protein kinase activated p34^{cdc2} and NIMA protein kinase and played roles in the G₂/M transition in cells [27–29]. While various data, including the aforementioned, have elucidated that Ca²⁺/calmodulin-dependent protein kinase plays a direct role in the G₂/M transition, its role in the G₁/S transition remains unclear. As there are many kinds of Ca²⁺/calmodulin-dependent protein kinases other than II, we plan to study the relationship between each one of these and the G₁/S transition.

When cobalt chloride, KN-62, cyclosporin A and FK-506 were added to Swiss 3T3 fibroblasts for an extended period of time (16 h) in a known concentration range, practically no differences in the LDH, Na⁺ and K⁺ concentrations in the extracellular medium were observed compared with the levels in the medium of normal cells when nothing was added. Therefore, it appears that even when exposure is prolonged, cells are not

irreversibly damaged by the above-mentioned reagents if they are used within the concentration range employed here.

It is now known that the activation of ras and the subsequent MAP kinase cascade are necessary as mechanisms of signal transduction in cell growth. However, when extracellular Ca²⁺ is chelated, DNA synthesis in the cells does not take place, regardless of the activation of the MAP kinase cascade by growth factors. It is therefore thought that, in Swiss 3T3 fibroblasts, after the growth factors bind to receptors, there are some as yet unknown mechanisms by which Ca²⁺ flows from outside to inside the cell during mid-to-late G₁ phase. As a result, the Ca²⁺/calmodulin–calcineurin pathway is activated, and this activation, accompanying the activation of the MAP kinase cascade, causes a transition from the cellular G₁ phase to the S phase. However, more detailed research will be required in the future in order to determine whether this hypothesis is correct.

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REFERENCES

- Nishida, E. and Gotoh, Y. (1993) Trends Biochem. Sci. **18**, 128–131
- Pages, G., Lenormand, P., L'Allemand, G., Chambard, J. C., Moloché, S. and Pouyssegur, S. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8319–8323
- Cook, S. J., Rubinfeld, B., Albert, I. and McCormick, F. (1993) EMBO J. **12**, 3475–3485
- Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. (1994) Cell **77**, 841–852
- Anraku, Y., Ohya, Y. and Iida, H. (1991) Biochim. Biophys. Acta. **1093**, 169–177
- Berridge, M. J. and Irvine, R. F. (1989) Nature (London) **341**, 197–205
- Nishizuka, Y. (1984) Nature (London) **308**, 693–697
- Mohammadi, A. M., Dionne, C. A., Li, W., Li, N., Spivak, T., Honegger, A. M., Jaye, M. and Schlessinger, J. (1992) Nature (London) **358**, 681–684
- Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Rosario, M., Mirda, D. and Williams, L. T. (1992) Nature (London) **358**, 678–681
- Cuadrado, A. and Molloy, C. J. (1990) Mol. Cell. Biol. **10**, 6069–6072
- Margolis, B., Zilberstein, A., Franks, C., Felder, S., Kremer, S., Ullrich, A., Rhee, S. G., Skorecki, J. and Schlessinger, J. (1990) Science **248**, 607–610
- Nishimoto, I., Hata, Y., Ogata, E. and Kojima, I. (1987) J. Biol. Chem. **262**, 12120–12126
- Kojima, I., Matsunaga, H., Kurosawa, K., Ogata, E. and Nishimoto, I. (1988) J. Biol. Chem. **263**, 16561–16567
- McCaffrey, P. G., Perrino, B. A., Soderling, T. R. and Rao, A. (1993) J. Biol. Chem. **268**, 3747–3752
- McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain, J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A. and Hogen, P. G. (1993) Science **262**, 750–754
- Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A. and Crabtree, G. R. (1994) Nature (London) **369**, 497–502
- Tsien, R. Y. (1981) Nature (London) **290**, 527–528
- Tsien, R. Y., Pozzan, T. and Rink, T. J. (1982) J. Cell. Biol. **94**, 325–334

- 19 Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. and Hidaka, H. (1990) *J. Biol. Chem.* **265**, 4315–4320
- 20 Schulman, H. and Lou, L. L. (1989) *Trends Biochem. Sci.* **14**, 62–66
- 21 Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y. L., Rich, D. P., Smith, M. K. and Soderling, T. R. (1989) *Biochem. J.* **258**, 313–325
- 22 Richter, A., Davies, D. E. and Alexander, P. (1995) *Biochem. Pharmacol.* **49**, 367–373
- 23 Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I. and Schreiber, L. (1991) *Cell* **66**, 807–815
- 24 Liu, J., Albers, M. W., Luan, W. S., Alberg, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B. and Schreiber, S. L. (1992) *Biochemistry* **31**, 3896–3901
- 25 Rasmussen, C., Garen, C., Brining, S., Kincaid, R. L., Means, R. L. and Means, A. R. (1994) *EMBO J.* **13**, 2545–2552
- 26 Takuwa, N., Zhou, W. and Takuwa, Y. (1995) *Cell. Signalling* **7**, 93–104
- 27 Lu, K. P., Osmani, S. A. and Means, A. R. (1993) *J. Biol. Chem.* **268**, 8769–8776
- 28 Lu, K. P., Osmani, S. P., Osmani, A. H. and Means, A. R. (1993) *J. Cell. Biol.* **121**, 621–630
- 29 Means, A. R. (1994) *FEBS Lett.* **347**, 1–4

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