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Rapid Stimulation of Ser/Thr Protein Kinases following Treatment of Swiss 3T3 Cells with Bombesin

INVOLVEMENT OF CASEIN KINASE-2 IN THE SIGNALING PATHWAY OF BOMBESIN*

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Treatment of quiescent Swiss 3T3 mouse fibroblasts with bombesin resulted in a rapid 6–8-fold stimulation of cytosolic Ser/Thr kinase activities toward the S_6 peptide (RRLSSLR), myelin basic protein (MBP), and the G peptide (SPQPSRRGSESSEE). Anion exchange Mono Q chromatography resolved multiple S_6 peptide- and G peptide kinase activities and two MBP kinase peaks. Both MBP- and several S_6 peptide kinase peaks could be inactivated by PCS_L ($PP2A_2$) phosphatase action. This indicates that the bombesin-induced activation of these enzymes is mediated by a Ser/Thr phosphorylation event. The S_6 peptide kinases as well as the two MBP kinases stimulated in response to bombesin are similar to those activated by epidermal growth factor in Swiss 3T3 fibroblasts which suggests that the early events of the signal transduction pathway mediated by these growth factors in Swiss 3T3 cells may converge in the activation of common Ser/Thr kinases. Bombesin, which acts as a sole mitogen for Swiss 3T3 fibroblasts, also produced a several-fold increase in the kinase activity toward the RRREEESEEE peptide, a specific substrate for CK-2. This kinase activity was heparin-sensitive and also measurable with the G peptide (SPQPSRRGSESSEE) and GS-1 peptide (YRRAAVPPSPSPSLSRHSSPHQSEDEE), which contain consensus sequences for phosphorylation by CK-2. The bombesin-stimulated CK-2 activity could not be measured in whole cytosols but was revealed by the anion exchange chromatography step. The activation of CK-2 was not reversed by PCS_L phosphatase action. The implication of CK-2 in the signal transduction pathway of bombesin is discussed.

Cells respond to a variety of extracellular signals through changes in the level of protein phosphorylation. Stimulation of quiescent mammalian cells by mitogens results in a cascade of phosphorylation events involving protein tyrosine kinases as well as protein serine/threonine kinases, leading to DNA synthesis and cell division.

A number of hormones and growth factors such as insulin, insulin-like growth factor 1 (IGF-1),[1] epidermal growth factor (EGF), platelet-derived growth factor, and colony-stimulating factor 1 (CSF-1), act through receptors with an intracellular tyrosine kinase domain (reviewed in Refs. 1 and 2). Activation of this intrinsic tyrosine kinase upon ligand binding is supposed to be the initial step in the signaling pathway triggered by those growth factors (1, 2). Since most of the subsequent phosphorylations occur on serine and threonine residues, some link should exist between receptor tyrosine kinases and cytosolic serine/threonine kinases. This could point to a direct activation of a serine/threonine kinase by tyrosine phosphorylation although other mechanisms may occur (3–5).

The amphibian tetradecapeptide bombesin, as well as the bombesin-related mammalian peptides including gastrin-releasing peptide and the neuromedins (for a review see Ref. 6), are potent mitogens for Swiss 3T3 cells (6), human small cell lung cancer cells (7), and bronchial epithelial cells (8). The bombesin receptor has been cloned from Swiss 3T3 fibroblasts: it is a member of the family of seven hydrophobic-region, G-protein-linked neuropeptide receptors (9) and its sequence does not reveal a protein kinase domain. Upon binding to its receptor, bombesin evokes a complex cascade of early biochemical events including inositol 1,4,5-triphosphate-induced mobilization of intracellular Ca^{2+}, Na^+, and K^+ fluxes, protein kinase C activation, transmodulation of the EGF-receptor, accumulation, and expression of the proto-oncogenes c-Fos and c-Myc and cAMP production (6). Although this complex signaling pathway is still largely unknown, it promises to have some striking features.

First of all, bombesin by itself, just like platelet-derived growth factor or fibroblast growth factor, can act as a sole mitogen for Swiss 3T3 cells (10). The fact that many hormones and neuropeptides which have similar receptors with seven membrane spanning domains, are by themselves not mitogenic for Swiss 3T3 cells, suggests that bombesin is acting

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[1] The abbreviations used are: IGF-1, insulin-like growth factor type 1; EGF, epidermal growth factor; MBP, myelin basic protein; CK-2, casein kinase 2; MAP2, microtubule-associated protein 2; PK-A, cAMP-dependent protein kinase; GS, glycogen synthase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PCS, polycation-stimulated phosphatase; PP2A, protein phosphatase type 2A; GSK_3, glycogen synthase kinase 3; PK-I, protein kinase inhibitor; FPLC, fast protein liquid chromatography; RSK, ribosomal S_6 kinases; PMSF, phenylmethylsulfonyl fluoride; PDGF, platelet-derived growth factor.

through a rather special signaling pathway.

Second, although its receptor does not contain a cytoplasmic tyrosine kinase domain, bombesin stimulates the tyrosine phosphorylation of multiple substrates in quiescent Swiss 3T3 fibroblasts (11). However, as recently reported by Zachary *et al.* (11) none of these substrates corresponds by molecular weight or by immunological or biochemical criteria to known tyrosine kinase substrates suggesting that the tyrosine phosphorylation events initiated by this neuropeptide may be different from the classical receptor tyrosine kinase induced phosphorylations.

We were therefore interested to know whether the bombesin-induced mitogenesis in Swiss 3T3 cells encompasses any of the signaling mechanisms common of receptor tyrosine kinases. In particular, we examined the effect of bombesin on the activity of already known growth factor-activated kinases (recently reviewed in Refs. 12–14). The present study shows that, in quiescent Swiss 3T3 fibroblasts, bombesin rapidly stimulates the activity of several S_6 peptide kinases as well as MBP kinases. Furthermore, our results implicate casein kinase 2 (CK-2) in the early signal transduction pathway of this mitogen.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Time-dependent Effect of Bombesin on Soluble Kinase Activities

Cytosolic extracts of either control cells or cells treated for various lengths of time with bombesin (50 nM) were assayed for their ability to phosphorylate a number of peptide- and protein substrates such as the S_6 peptide, the G peptide, MBP, and the RRREEESEEE peptide (Fig. 1, Miniprint).

Fig. 1 shows that kinase activities toward the S_6 peptide, MBP, and the G peptide peaked after 5–10 min of bombesin treatment. The rate of phosphorylation of the S_6 peptide and the G peptide declined by about 40% at the 40-min time point (Fig. 1, *A* and *B*), whereas the 7-fold maximal stimulation of the MBP kinase, seen at 5-min incubation with the growth factor, was more drastically reduced at the later time points (Fig. 1*C*). Kinase activities toward the RRASVA peptide and the Kemptide followed the pattern of the S_6 peptide phosphorylation in the time-course experiment (not shown).

The rate of phosphorylation of the RRREEESEEE peptide (CK-2 activity) did not change very dramatically (Fig. 1*D*): a 30% increase in CK-2 activity was observed at the very most. Similar results were also obtained using casein as a substrate for CK-2 (not shown).

Dose Dependence of Protein Kinase Activation by Bombesin

In order to characterize the dose dependence of the protein kinase stimulations, quiescent Swiss 3T3 cells were treated for 5 min with various concentrations of bombesin. As shown in Fig. 2, a maximal response was observed with concentrations of bombesin around 10^{-8} to 10^{-9} M, which corresponds to the concentration range which elicits the mitogenic response in Swiss 3T3 fibroblasts (6). The small CK-2 activation measured with the RRREEESEEE peptide as illustrated in Fig. 1 was not always evident. The higher level of activations observed in these experiments as compared to the ones reported in the time courses of Fig. 1 are possibly due to the stabilizing effect of 100 nM microcystin which was included in the extraction buffer of the dose-dependence experiments.

Separation of Bombesin-activated Protein Kinase Activities by Mono Q FPLC Column Chromatography

The kinase activities in extracts from Swiss 3T3 control cells and from cells treated for either 5 or 40 min with bombesin, were resolved by Mono Q FPLC chromatography.

Early Responses (5 Min of Bombesin Treatment)—Fig. 3*A* shows that the S_6 peptide kinase activities in the cells treated for 5 min with bombesin were separated into several peaks: a major part of the activity came in the breakthrough of the column and four overlapping activity peaks eluted early in the gradient: at 0.12, 0.14, 0.17, and 0.2 M NaCl, respectively. All the S_6 peptide kinase peaks could phosphorylate the Kemptide and, with the exception of the 0.2 M NaCl peak, also the RRASVA peptide (not shown).

The breakthrough, the 0.12 M, and the 0.17 M eluate peaks revealed comparable kinase activities toward the G peptide as substrate (Fig. 3*B*), suggesting that the activated kinases may phosphorylate both peptides at very similar rates. The stimulated G peptide kinase peak eluting at 0.09 M NaCl was occasionally also seen using the S_6 peptide as substrate. However, the major stimulated G peptide kinase eluted later in the gradient at 0.35 M NaCl where no S_6 peptide kinase activity was measurable (Fig. 3*A*). Usually more G peptide kinase activity (200%) was recovered after Mono Q chromatography than originally applied to the column, suggesting that a large fraction of kinase activity toward this peptide is masked in whole cytosolic extracts.

The MBP kinase activity (Fig. 3*C*) was resolved by the gradient in two peaks eluting at 0.11 M (MBP kinase I) and at 0.17 M NaCl (MBP kinase II): the 8-fold stimulation by bombesin measured in the cytosols (Fig. 2*C*) was quantitively

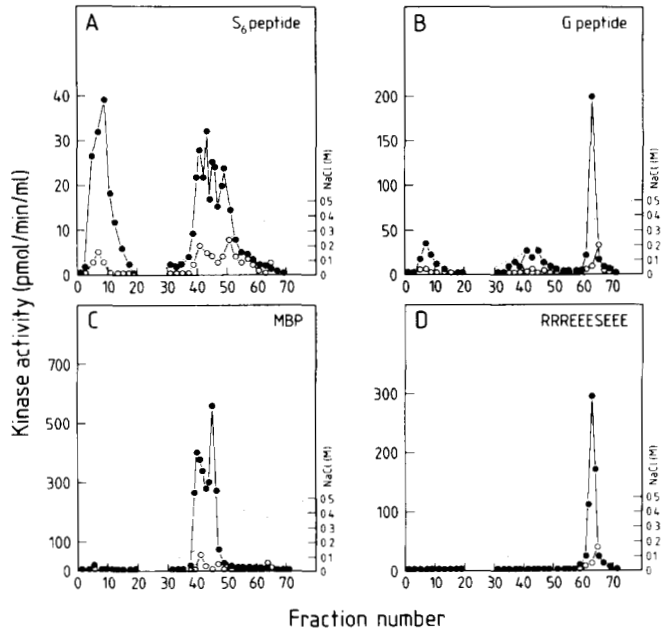

FIG. 3. **Elution profile of bombesin-stimulated kinase activities on Mono Q (FPLC) anion exchange chromatography.** Cytosols from control cells (○) or from cells incubated for 5 min with 50 nM bombesin (●) were applied to a Mono Q anion exchange column. The column was developed with a linear gradient of NaCl from 0 to 0.5 M (*dashed line*), and aliquots of the fractions were assayed for kinase activities as in Fig. 1. The elution pattern depicted is representative of results obtained in four independent experiments with different cell preparations.

[2] Portions of this paper (including "Experimental Procedures" and Figs. 1, 2, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

recovered in the gradient fractions.

Surprisingly, when the RRREEESEEE peptide was used to specifically detect the CK-2 activity in the Mono Q eluted fractions, a many-fold stimulation was observed, coeluting exactly with the G peptide kinase activity at 0.35 M NaCl (Fig. 3*D*). This observation together with the results depicted in Fig. 1*C* and Fig. 2*D* strongly suggests that the Mono Q chromatography step may separate substances that interfere in the assay: recovery of the bombesin-stimulated CK-2 activity was several-fold greater in the Mono Q-eluted fractions. The degree of CK-2 activation, measured in the gradient fractions, varied between 2–6-fold in a series of four totally independent stimulation experiments. Differences in the level of activation of CK-2 are for instance seen in the experiments illustrated (see Figs. 3*D* and 5). In general it was observed that when cells of later passages were used in similar experiments or when the subculture schedule was not strictly followed, basal levels of CK-2 activity were enhanced several-fold and stimulation by bombesin greatly reduced. This would suggest that partially transformed Swiss 3T3 cells already have the CK-2 enzyme in an activated form.

Late Responses (40 Min of Bombesin Treatment)—As illustrated in Fig. 4, prolonged incubation (40 min) of Swiss 3T3 cells with bombesin resulted in a decrease in the activation level of all kinases. The activities measured in the Mono Q profiles reflected very much the respective kinase levels measured in the cytosolic extracts at the 40-min time points in Fig. 1 (except for the CK-2 activation discrepancy). The most important decrement in S_6 peptide kinase activities (Fig. 4*A*) occurred in the breakthrough fraction (80%) as well as in those peaks eluting very early in the gradient (between 0.12 and 0.17 M NaCl), whereas the last S_6 peptide kinase peak was only slightly affected (about 25%). The MBP kinase activations (Fig. 4*C*) decreased by 80% after 40 min of incubation of cells with bombesin (Fig. 3*C*) similarly to what was observed in the extracts (Fig. 1*C*). The coeluting G peptide and RRREEESEEE peptide kinase activities were also reduced after prolonged bombesin treatment to about 45 and 30%, respectively (Fig. 4*D*).

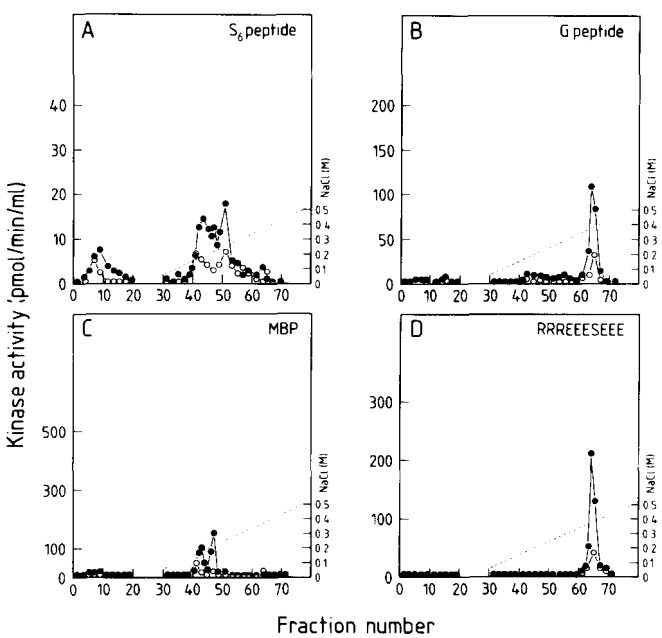

FIG. 4. **Mono Q (FPLC) column chromatography of kinase activities after prolonged bombesin treatment.** Cytosols of cells treated for 40 min with 50 nM bombesin (●) were applied to a Mono Q column and processed as described in Fig. 3.

Implication of CK-2 in the Signaling Pathway of Bombesin

In order to further characterize the RRREEESEEE peptide kinase eluting in the 0.35 M peak on the basis of its site specificity, the GS-1 peptide (34), YRRAAVPPS^{3b}PSLS^{3c}RHSS^{4}PHQS^{5}EDEE, which contains the specific CK-2 phosphorylation site (Ser^{5}) of glycogen synthase, was used to assay kinase activity under similar experimental conditions as in Fig. 3. As shown in Fig. 5*C*, the GS-1 peptide served as a substrate for three kinase activities corresponding to the stimulated MBP kinases I and II (Fig. 5*B*) and the RRREEESEEE peptide kinase (Fig. 5*A*).

Phosphorylation of the GS-1 peptide by kinase F_A/GSK_3, which occurs on sites Ser3b, -3c, and -4, is absolutely dependent upon prior phosphorylation of site 5 by CK-2 (27, 28). Moreover, kinase F_A/GSK_3 activity measured with the GS-1 peptide previously phosphorylated by CK-2 eluted in the breakthrough fraction of the Mono Q column, and its activity was not changed upon bombesin treatment. The GS-1 peptide does not contain phosphorylation sites for the S_6 peptide kinases, so that its increased phosphorylation at the 0.11 and 0.17 M NaCl elution peaks is either due to the activated MBP

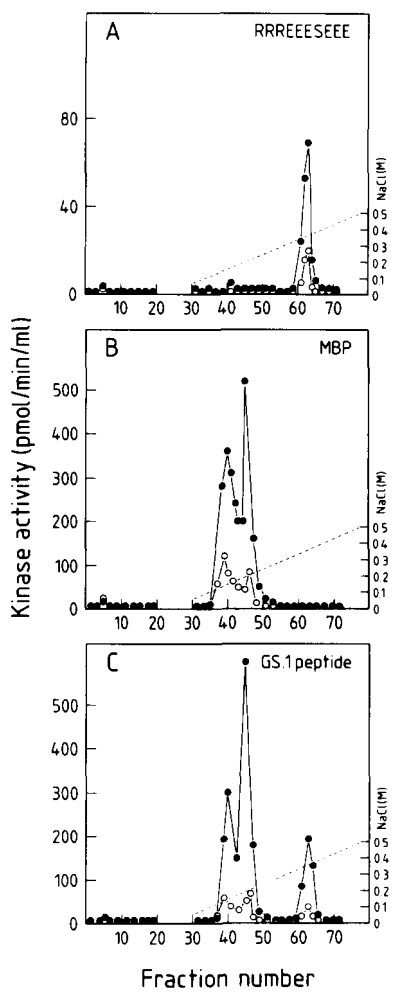

FIG. 5. **Characterization of the bombesin-stimulated CK-2 activity in the Mono Q (FPLC) column elution.** Cytosols of control cells (○) or of cells treated for 5 min with 50 nM bombesin (●) were chromatographed on a Mono Q column as in Fig. 3. Aliquots of the column fractions were assayed for phosphorylation of (*A*), RRREEESEEE peptide; (*B*), MBP and (*C*), GS-1 peptide as described under "Experimental Procedures." Similar profiles were observed in two independent experiments with different cell preparations.

kinases themselves or to still unknown coeluting activities. The rate of phosphorylation of the GS-1 peptide by the two MBP kinases was very similar to that observed with the reference substrate (MBP), indicating that both substrates are recognized equally well by these enzymes (Fig. 5, *B* and *C*). On the contrary, at the 0.35 M NaCl elution place, the GS-1 peptide was phosphorylated more readily than the RRREEESEEE peptide (Fig. 5, *A* and *C*). This observation is consistent with the site specificity studies of CK-2 which demonstrate that this kinase exhibits a very high affinity for the GS-1 peptide (27) and phosphorylates the shorter hexapeptide SEEEEE, reminescent of the glycogen synthase Ser^5 site, extremely well (33). Kinase activities in the 0.35 M NaCl peak fractions, were completely abolished by the addition of heparin in the phosphorylation assay (not shown).

In conclusion, the overlapping activities toward three different peptide substrates (GS-1-, G-, and RRREEESEEE peptides) containing consensus sequences for CK-2, together with the coinciding elution at 0.35 M NaCl in the Mono Q gradient (19) and the inhibition by heparin, are three strong arguments indicating that these phosphorylations are due to CK-2 itself.

Phosphatase Treatment of the Bombesin-activated Kinases

The addition of microcystin, a powerful inhibitor of ATP, Mg-dependent (type 1)- and PCS (type 2A) phosphatases (34), to the column fractions was absolutely required to maintain the activation of the protein kinases. This strongly suggests that phosphorylations are involved in the mechanism of the kinase activations triggered by bombesin. Therefore, fractions containing activated kinases were subjected to the PCS_L (type $2A_2$) phosphatase before addition of microcystin (Fig. 6). After 30 min of phosphatase treatment the MBP I kinase was almost completely inactivated (Fig. 6*B*), whereas the MBP II kinase activity was reduced by 60% (Fig. 6*C*). Since microcystin could prevent MBP kinase inactivation (Fig. 6, *B* and *C*) it can be concluded that the activation of both enzymes depends upon a Ser/Thr phosphorylation reaction. On the contrary, the stimulated CK-2 activity toward the RRREEESEEE peptide was totally unaffected by PCS_L ($PP2A_2$) phosphatase action (Fig. 6*A*). Treatment of the activated CK-2 fractions with alkaline phosphatase bound to agarose did not inactivate the enzyme neither (not shown). Pooled CK-2 fractions from control cytosols were also insensitive to PCS_L ($PP2A_2$) phosphatase action (not shown). This indicates either that the activation of CK-2 is not directly or solely controlled by Ser/Thr phosphorylation or that the phosphorylation sites involved are not accessible to the phosphatases used.

Due to the high instability of the activated S_6 peptide kinases in the absence of microcystin, it was not always possible to obtain a reliable measurement of the residual activities after preincubation of the pooled fractions at 30 °C. Nevertheless, the S_6 peptide kinase activities that were separated by the Mono Q gradient between 0.12 and 0.17 M NaCl were completely abolished by type 2A phosphatase action, whereas the more retarded S_6 peptide kinase peak (eluting at 0.2 M NaCl) did not seem to be affected (not shown).

DISCUSSION

The present study unambiguously shows that treatment of Swiss 3T3 cells with the neuropeptide bombesin results in a rapid increase in the activity of several cytosolic Ser/Thr kinases toward a number of protein- and peptide substrates. In particular, incubation of cells with bombesin (5 min) produces a several-fold activation of multiple S_6 peptide kinases and of two MBP kinases. Interestingly, this mitogen also elevates a kinase activity toward the peptide RRREEESEEE which is only revealed after chromatography of bombesin-stimulated cytosols on a Mono Q FPLC column. This activated kinase was furthermore identified as CK-2.

Quiescent Swiss 3T3 fibroblasts can be induced to proliferate by a variety of peptide growth factors and hormones, seemingly involving different early signal transduction events. However, the activation of certain key enzymes appears to be common to several signaling pathways. This is the case for the S_6 kinases and MBP kinases which are established growth-regulated enzymes (12–14). When comparing our results with published reports it is clear that the signaling pathways of bombesin and EGF involve the activation of similar sets of kinases, both in the early and in the late phases of the cellular response.

A group of five bombesin-stimulated kinases phosphorylate the S_6 peptide, and their time course of activation, substrate specificity, chromatographic behavior, and their sensitivity to phosphatase action relates them to the EGF-activated S_6 peptide kinases described in Ref. (19).

Two distinct peaks of activated MBP kinases are observed in the Mono Q column elution of bombesin-stimulated cytosols. Their chromatographic properties as well as their sensitivity to inactivation by the PCS_L ($PP2A_2$) phosphatase, makes them comparable to the EGF-stimulated MBP kinases 1 and 2 which also recognize microtubule-associated protein 2 (MAP2) as a substrate (19, 35). The MBP/MAP2 kinases have been reported to be growth-stimulated enzymes in various systems (13, 14, 36), and both kinases activate a S_6 peptide kinase from control Swiss 3T3 cells (37). While this work was in progress, Takuwa *et al.* (38) investigated the role of Ca^{2+} influx on the mitogenic response induced by bombesin in Swiss 3T3 cells and reported that bombesin stimulated cytosols incorporated more [^{32}P]phosphate into exogenously added MBP and S_6 protein than control cytosols. This increase in phosphorylation was found to be independent of the extracellular Ca^{2+} concentration, but no attempt was made to characterize the enzymes involved.

Probably the most intriguing finding of the present report is the rapid stimulation of CK-2 following treatment of quiescent Swiss 3T3 fibroblasts with bombesin. This ubiquitous kinase is a very well characterized enzyme as far as its subunit structure, substrate specificity, cellular localization, and sensitivity to inhibitors and activators is concerned, but a full comprehension of its physiological regulation is still missing (for a review see Ref. 39). An interesting but puzzling observation in our study is that a chromatography step is necessary to reveal the bombesin-mediated activation of CK-2. The CK-2 activity of control cytosols is quantitatively recovered in the Mono Q column fractions, indicating that the chromatography step by itself does not produce an artificial increment in the CK-2 activity. Therefore, the increased CK-2 activity measured after the ion exchange chromatography of bombesin-treated cells is likely due to the separation of a factor present in stimulated cytosols, which specifically masks the activated CK-2 enzyme. Since a similar level of CK-2 activity is measured in both control and bombesin-stimulated cytosols it is conceivable that the bombesin-generated CK-2 activity is mobilized from a latent enzyme pool. We have currently no evidence for a direct correlation between the bombesin-induced CK-2 activation and a phosphorylation of the enzyme although clearly more information is required to clarify this point.

As mentioned before, Swiss 3T3 cells of later passages tend to undergo spontaneous transformation, and this is reflected

in an altered cell morphology and an increase in the basal level of CK-2 activity. It is well documented that the CK-2 activity is increased in transformed cells and proliferating tissues (40–44) as well as during cell differentiation (45) and in most cases this correlates with an elevated expression of the enzyme. In addition to this, a transient rise in CK-2 activity has been observed upon stimulation of cells by different growth factors: in W138 human lung fibroblasts by serum (46), in 3T3-L1 adipocytes by insulin or EGF (47), in BALB/c 3T3 fibroblasts by IGF-1 or insulin (48) and in A431 human carcinoma cells by EGF (49). The latter observations have inferred that some short-term modulation of this kinase should also exist, and it has been suggested that a covalent modification of CK-2 underlies its mechanism of regulation (47, 49, 50). The magnitude of CK-2 activation observed with insulin or EGF in 3T3-L1 mouse adipocytes is rather modest and variable (ranging from 30 to 150%) (47) and not always reproduced in similar cell systems (18, 19, 48). It is furthermore interesting to note that the highest levels of CK-2 activation are observed when a combination of different growth factors is used to stimulate the growth of BALB/c 3T3 fibrobasts (48) or when quiescent human W138 fibroblasts are induced to proliferate by whole serum (46). In the case of Swiss 3T3 fibroblasts neither insulin nor EGF alone is able to elicit a mitogenic response and CK-2 does not seem to be activated in these signal transduction events (19). On the contrary, bombesin by itself is a potent mitogen for these cells, and our data clearly shows a drastic stimulation of the CK-2 activity upon treatment of Swiss 3T3 cells with bombesin. It is therefore conceivable that the activation of CK-2 is an intrinsic property of the signal transduction pathway of "sole mitogens" like bombesin as it may well be a compulsory event leading to cell division. This opens the intriguing possibility that CK-2 may also integrate multiple signaling pathways initiated by several different growth factors which work synergistically to make cell proliferation possible. The predominant nuclear localization of CK-2 in actively proliferating cells (51) is one more indication that this kinase may play a fundamental role in the control of cell division, and the observation that a number of nuclear proteins, including the oncogene products Myc, Myb, Fos, p53, E1A, the SV40 T antigen (51), and the serum response factor $p67^{SRF}$ (52) are substrates for CK-2 tends to confirm this.

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REFERENCES

1. Hunter, T., and Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54,** 897–930
2. Yarden, Y., and Ullrich, A. (1988) *Annu. Rev. Biochem.* **57,** 443–478
3. Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G. D.,Panayotatos, N., Radziejewska, E., Ericsson, L., Bratlin, L. R., Cobb, M. H., and Krebs, E. G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6142–6146
4. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64,** 281–302
5. Koch, C. A., Anderson, D., Moran, N. F., Ellis, C., and Panesan, T. (1991) *Science* **252,** 668–674
6. Rozengurt, E., and Sinnett-Smith, J. (1990) *Phil. Trans. R. Soc. Lond. B* **327,** 209–221
7. Cuttitta, F., Carney, D. N., Mulshine, Y., Moody, T. W., Fedorks, Y., Fisher, A., and Minna, Y. D. (1985) *Nature* **316,** 823–826
8. Willey, Y. C., Lechner, Y. F., and Harris, C. C. (1984) *Exp. Cell Res.* **153,** 245–248
9. Battey, Y. F., Way, Y. M., Corjay, M. H., Shapira, H., Kusano, K., Harkins, R., Wu, Y.-M., Sbattery, T., Mann, E., and Feldmann, R. I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 395–399
10. Rozengurt, E. (1986) *Science* **234,** 161–166
11. Zachary, I., Gil, Y., Lehmann, W., Sinnett-Smith, Y., and Rozengurt, E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4577–4591
12. Erikson, R. L. (1991) *J. Biol. Chem.* **266,** 6007–6010
13. Sturgill, T. W., and Wu, J. (1991) *Biochim. Biophys. Acta* **1092,** 350–357
14. Pelech, S. L., Sanghera, J. S., and Caya-Makin, M. (1990) *Biochem. Cell Biol.* **68,** 1297–1330
15. Waelkens, E., Goris, J., and Merlevede, W. (1987) *J. Biol. Chem.* **262,** 1049–1059
16. Baserga, R. (1989) *Cell Growth and Division* (Baserga R., ed) IRL Press, Oxford University, England
17. Krieg, J., Hofsteenge, J., and Thomas, G. (1988) *J. Biol. Chem.* **263,** 11473–11477
18. Haystead, T. A. J., Weiel, J. E., Litchfield, D. W., Tsukitani, Y., Fischer, E. H., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 16571–16580
19. Ahn, N. G., Weiel, J. E., Chan, C. P., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 11487–11494
20. Pelech, S. L., Meijer, L., and Krebs, E. G. (1987) *Biochemistry* **26,** 7960–7968
21. Munro, J., Campbell, D. G., and Leader, D. P. (1990) *Biochim. Biophys. Acta* **1054,** 225–230
22. Lavoinne, A., Erikson, E., Maller, J. L., Price, D. J., Avruch, J., and Cohen, P. (1991) *Eur. J. Biochem.* **199,** 723–728
23. Chessa, G., Borin, G., Marchiori, F., Meggio, F., Brunati, A. M., and Pinna, L. A. (1983) *Eur. J. Biochem.* **135,** 609–614
24. Dent, P., Campbell, D. G., Hubbard, M. J., and Cohen, P. (1989) *FEBS Lett.* **248,** 67–72
25. Fiol, C. J., Haseman, J. H., Wang, Y., Roach, P. J., Roeske, R. W., Kowalczuk, M., and DePaoli-Roach, A. A. (1988) *Arch. Biochem. Biophys.* **267,** 797–802
26. Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P. (1990) *Nature* **348,** 302–308
27. Woodgett, J. R. (1989) *Anal. Biochem.* **180,** 237–241
28. Fiol, C. J., Wang, A., Roeske, R. W., and Roach, P. J. (1990) *J. Biol. Chem.* **265,** 6061–6065
29. Vulliet, P. R., Hall, F. L., Mitchell, J. P., and Hardie, D. G. (1989) *J. Biol. Chem.* **264,** 16292–16298
30. Kuenzel, E. A., Mulligan, J. A., Sommercorn, J., and Krebs, E. G. (1987) *J. Biol. Chem.* **262,** 9136–9140
31. Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M., and Walsh, D. A. (1986) *J. Biol. Chem.* **261,** 989–992
32. Glass, D. B., Masaracchia, R. A., Feramisco, J. R., and Kemp, B. E. (1978) *Anal. Biochem.* **87,** 566–575
33. Marin, O., Meggio, F., Marchiori, F., Borin, G., and Pinna, L. A. (1986) *Eur. J. Biochem.* **160,** 239–244
34. Honkanen, R. E., Zwiller, J., Moore, R. E., Daily, S. L., Khatra, B. S., Dukelow, M., and Boynton, A. L. (1990) *J. Biol. Chem.* **265,** 19401–19404
35. Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K., and Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 4220–4227
36. Haccard, O., Jessus, C., Cayla, X., Goris, J., Merlevede, W., and Ozon, R. (1990) *Eur. J. Biochem.* **192,** 633–642
37. Ahn, N. G., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 11495–11501
38. Takuwa, N., Kumada, M., Yamashita, K., and Takuwa, Y. (1991) *J. Biol. Chem.* **266,** 14237–14243
39. Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054,** 267–284
40. Münstermann, U., Fritz, G., Seitz, G., Yiping, L., Schneider, H. R., and Issinger, O.-G. (1990) *Eur. J. Biochem.* **189,** 251–257
41. Brunati, A. M., Saggioro, D., Chieco-Bianchi, L., and Pinna, L. A. (1986) *FEBS Lett.* **206,** 59–63
42. Seitz, G., Münstermann, U., Schneider, H. R., and Issinger, O.-G. (1989) *Biochem. Biophys. Res. Commun.* **163,** 635–641
43. Schneider, H. R., Reichert, G. H., and Issinger, O.-G. (1986) *Eur. J. Biochem.* **161,** 733–738
44. Pérez, M., Grande, J., and Itarte, E. (1987) *Eur. J. Biochem.* **170,** 493–498
45. Sommercorn, J., and Krebs, E. G. (1987) *J. Biol. Chem.* **262,** 3839–3843

46. Carroll, D., and Marshak, D. R. (1989) *J. Biol. Chem.* **264,** 7345–7348
47. Sommercorn, J., Mulligan, J. A., Lozeman, F. J., and Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8834–8838
48. Klarlund, J. K., and Czech, M. P. (1988) *J. Biol. Chem.* **263,** 15872–15875
49. Ackerman, P., and Osheroff, N. (1989) *J. Biol. Chem.* **264,** 11958–11965
50. Ackerman, P., Glover, C. V. C., and Osheroff, N. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 821–825
51. Yu, I. J., Spector, D. L., Bae, Y.-S., and Marshak, D. R. (1991) *J. Cell Biol.* **114,** 1217–1232
52. Gauthier-Rouvière, C., Basset, M., Blanchard, J.-M., Cavadore, J.-C., Fernandez, A., and Lamb, N. J. C. (1991) *EMBO J.* **10,** 2921–2930

Supplementary Material to "Rapid stimulation of Ser/Thr protein kinases following treatment of Swiss 3T3 cells with bombesin. Involvement of casein kinase-2 in the signaling pathway of bombesin."

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Experimental Procedures

Materials. Swiss mouse 3T3 cells were obtained from American Type Culture Collection (Rockville, USA). Bombesin, aprotinin, pepstatin and leupeptin, were purchased from Boehringer (Germany). The synthetic peptides: RRLSSLR (S_6 peptide), SPQPSRRGSESSEE (G peptide), RRREEESEEE (CK-2 peptide), YRRAAVPPSPSPSLSRHSSPHQSEDEE (GS-1 peptide), LRRASLG (Kemptide) and RRASVA were synthesized with a Milligen 9050, using the 9-fluorenylmethoxycarbonyl (Fmoc) mode and purified using reverse phase HPLC on a Delta-Pack C18 column from Waters. The cAMP-dependent protein kinase (PK-A) inhibitor peptide TTYADFIASGRTGRRNAIHD (PKI peptide) and myelin basic protein (MBP) were obtained from Multiple Peptide System (San Diego, USA) and Sigma (USA) respectively, microcystin-LR from Calbiochem (France) and cell culture media and antibiotics from GIBCO/Life Technologies. The PCS_L ($PP2A_2$) phosphatase was purified from rabbit skeletal muscle as described in (15). The protein phosphatase activity is expressed as units of phosphorylase phosphatase, one unit catalysing the release of 1 nmol [^{32}P] phosphate/min at 30 °C from 10 µM [^{32}P]phosphorylase *a*.

Cell culture and preparation of extracts. In order to minimize the risk of spontaneous transformation, cells cultured for less than five passages, were seeded at an initial density of 5 x 10^4 per 150 mm-dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, 50 U/ml nystatin, 100 U/ml penicillin and 100 µg/ml streptomycin, essentially as described in (16). Cells were trypsinized after 3 days and reached confluency after 11 days. At this stage, cells were harvested, replated at a density of 9 x 10^5 per 150 mm-dish and achieved a density-dependent growth arrest in the following next 7-8 days of culture. Whenever this strict subculture schedule was not followed, we observed a spontaneous transformation of the cells as evidenced by the lack of density-arrested growth and an elongated morphology as also described in (16). The growth factor experiment was performed 48 hr after replacement of the medium with DMEM containing 0.1% heat-inactivated calf serum. Incubation was started by the addition of either 10 µl of bombesin dissolved in 50 mM CH_3COONH_4 pH 7 or 10 µl of the same buffer (controls), to 10 ml of the old medium which is essentially depleted of growth-promoting activity. Incubations were stopped by rinsing twice with 5 ml ice-cold PBS and once with 4 ml ice-cold extraction buffer containing 20 mM Tris/HCl pH 7.4, 10 mM pNPP, 10 mM NaF, 10 mM $MgCl_2$, 20 mM B-glycerophosphate, 2 mM EGTA, 200 µM Na_3VO_4, 10 µM ammonium molybdate, 0.05% triton X-100, 1mM PMSF, 2 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin and 150 mM NaCl (buffer A). Cells were scraped in 0.5 ml of buffer A, homogenized in a Dounce-homogenizer (pestle B) and centrifuged at 100,000 x g for 30 min (4 °C). Supernatants (0.5-0.7 mg protein/ml) were aliquoted and immediately frozen in liquid nitrogen. Except for the initial experiments illustrated in Fig.1, the extraction buffer was also supplemented with 100 nM microcystin.

Chromatography. Anion exchange chromatography was carried out on a *Mono Q HR* 5/5 using a Pharmacia LKB Biotechnology Inc. FPLC system. Cytosols (6-7 mg protein, 12 150-mm dishes) for chromatographic analysis were prepared essentially as described above with the exception that in buffer A, the 150 mM NaCl was omitted and 100 nM microcystin was added. Cytosols were loaded at a rate of 0.5 ml/min and after a 10 ml wash, elution of protein kinases was performed at a rate of 1 ml/min with a 50 ml gradient from 0 to 0.5 M NaCl in a buffer containing: 20 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 20 mM B-glycerophosphate, 10 mM NaF, 200 µM Na_3VO_4, 10 µM ammonium molybdate, 2mM EGTA, 2% glycerol, 1 mM benzamidine, 2 µg/ml aprotinin (buffer B). After withdrawing a 200 µl aliquot for subsequent phosphatase-treatment, 8 µl of a protease-phosphatase inhibitor mixture was added to bring the remaining 800 µl of the fraction to a final concentration of 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 1 mM PMSF and 100 nM microcystin. In the presence of the protease-phosphatase inhibitor mixture, kinase activities were stable for about one week. All chromatographic steps were performed at 4 °C.

Characterization of protein kinases

Rationale of peptide substrates used. The S_6 peptide, RRLSSLRA, which corresponds to a C-terminal amino acid sequence [232-239] of the eukaryotic ribosomal protein S_6 (17), has been used for the detection and characterization of S_6 kinase activities in a variety of cellular systems (18-22). The Kemptide, LRRASLG, as well as the RRASVA peptide, which represents the PK-A phosphorylation site in rat liver pyruvate kinase type L (23), were also used as alternative S_6 peptide kinase substrates. The G peptide, $S^{a}PQPS^{2}RRGS^{1}ES^{+1}SEE$, which corresponds to the primary sequence surrounding the PK-A phosphorylation site 1 (Ser13) in the glycogen binding subunit of the type 1 phosphatase (24), also contains consensus sequences for phosphorylation by kinase F_A/GSK_3 (Ser5- and Ser9-sites) (24,25) and by CK-2 (Ser15-site). Prior phosphorylation of Ser13 is absolutely required for recognition of the peptide by kinase F_A/GSK_3. The Ser13-site is also phosphorylated by an insulin stimulated protein kinase (ISPK) (26) which has been identified as the 90-kDa S_6 kinase-II (22).
The GS-1 peptide (27), $YRRAAVPPS^{3b}PSLS^{3c}RHSS^{4}PHQS^{5}EDEE$, contains the specific CK-2 site (Ser5) of glycogen synthase. Prior phosphorylation of the Ser5 is a prerequisite for the subsequent phosphorylation by kinase F_A/GSK_3 which then phosphorylates Ser4,-3c, and -3b (28). Therefore, the phospho-form of the GS-1 peptide labeled by CK-2, was used to detect kinase F_A/GSK_3 activity. The GS-1 peptide has been reported to serve also as a substrate for a proline-directed protein kinase (29). The RRREEESEEE is a synthetic peptide which is specifically phosphorylated by CK-2 (30).
One should note that in order to prevent contributions of PK-A, protein kinase C and Ca^{2+}/calmodulin-dependent kinases, all phosphorylation assays were performed in the presence of the *PKI* peptide (31), the calcium-chelating agent EGTA and the calmodulin inhibitor calmidazolium.

Kinase Assays. Phosphorylations of the S6 peptide (0.33 mg/ml), G peptide (1 mg/ml), RRREEESEEE peptide (1 mg/ml), RRASVA peptide (0.2 mg/ml), Kemptide (0.33 mg/ml), MBP (0.5 mg/ml), GS-1 peptide (2.4 mg/ml) were performed by incubating 15 µl of cytosolic extracts (7-10 µg of protein) at 30° C with 15 µl of a phosphorylation mixture containing: 50 mM Tris/HCl pH 7.4, 12 mM $MgCl_2$, 8 µM PKI peptide, 10 µM calmidazolium and 200 µM [γ-^{32}P]ATP (specific activity 1000-2000 cpm/pmol). After 20 min the reaction was stopped by spotting 25 µl of the phosphorylation mixture onto *P81* phosphocellulose (Whatman) filter paper (32). The [γ-^{32}P]ATP was separated from the labeled substrates by washing the filter papers 5 times for 10 min in 0.5% phosphoric acid. Papers were then dried and the radioactivity counted. Mono Q column fractions (15 µl) were assayed for kinase activities as described above, except that 125 µM [γ-^{32}P]ATP (specific activity 500-1000 cpm/pmol) was used.

Phosphatase treatment of the activated kinases separated by Mono Q FPLC column chromatography. Aliquots (200 µl) of the Mono Q column fractions containing the peak of the activated kinases were withdrawn before the addition of the phosphatase-protease inhibitor mixture. The microcystin present in the cytosols eluted in the breakthrough of the Mono Q column, so that only the kinases in the gradient fractions could be treated with phosphatases. The dephosphorylation was initiated by incubating the activated kinase pool at 30 °C in the presence of either control buffer C (Tris/HCl, pH 7.4, bovine serum albumin 1 mg/ml, dithiothreitol 0.5 mM), buffer C plus 1 µM microcystin, buffer C plus 50-100 U/ml phosphatase, or buffer C plus microcystin (1 µM) as well as phosphatase. At zero time and after 30 min, an aliquot (10 µl) was withdrawn and mixed with phosphorylation buffer (10 µl) containing the substrate together with microcystin (1 µM) in order to block subsequent phosphatase action. The kinase reaction was then carried out for 20 min, terminated by spotting 15 µl onto P81 papers and processed as described in the previous section.

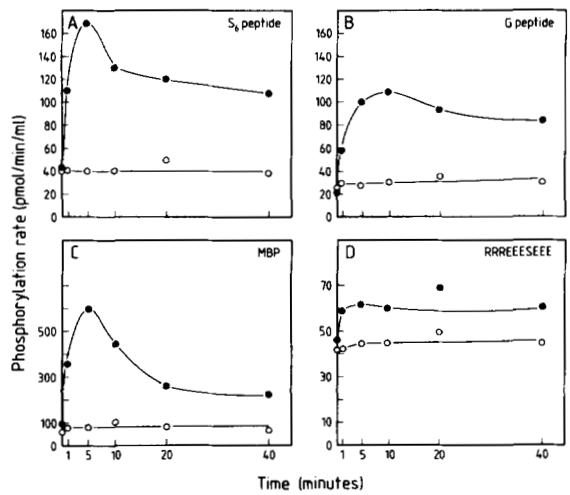

Figure 1. Time-dependence of the activation by bombesin of Ser/Thr kinases in Swiss 3T3 cells. Cells were incubated for the indicated lenghts of time with either 50 nM bombesin (●) dissolved in 50 mM CH_3COONH_4 pH 7 or with buffer alone (○). High speed cytosols were prepared and kinase activities measured toward (A), S_6 peptide; (B), G peptide; (C), MBP; (D), RRREEESEEE peptide as described in "Experimental Procedures". Values represent the means of three independent experiments with different cell preparations.

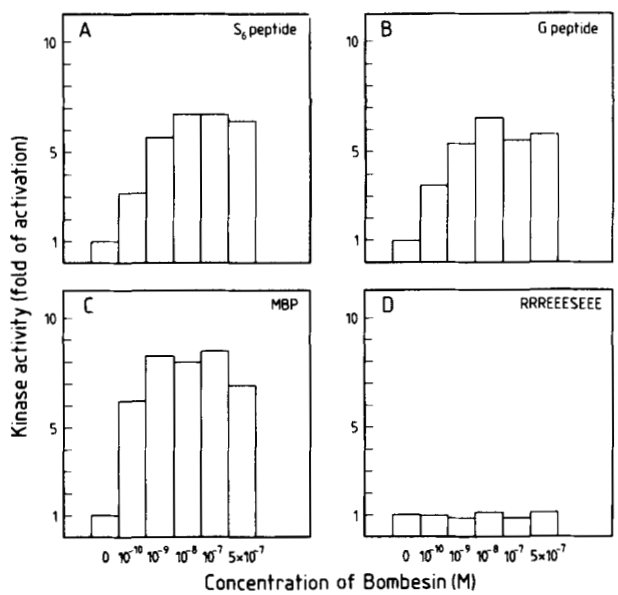

Figure 2. Dose-dependence of the protein kinase activation induced by bombesin in Swiss 3T3 cells.
Cells were treated with the indicated concentrations of bombesin for 5 min and processed as described in "Experimental Procedures". Protein kinase activities in the cytosols were measured as in Fig. 1. Values are the means of two independent experiments with different cell preparations.

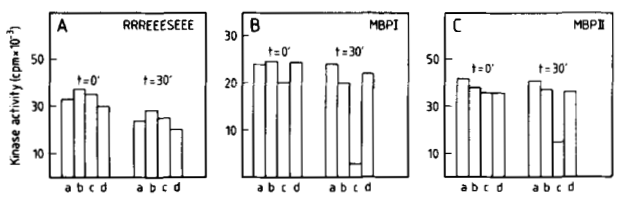

Figure 6. Effect of PCS_L ($PP2A_2$) phosphatase on bombesin- stimulated MBP- and RRREEESEEE peptide kinase activities.
15 µl of pooled fractions of the Mono Q chromatography column containing bombesin-stimulated MBP- or *RRREEESEEE* peptide kinase activities were mixed with 15 µl of (a), control buffer; (b), control buffer plus 1 µM microcystin; (c), 50 U/ml PCS_L phosphatase; (d), 50 U/ml PCS_L phosphatase plus 1 µM microcystin. Immediately after the addition (t=0') or after 30 min of preincubation at 30 °C, a 10 µl aliquot of the mixture was assayed for kinase activities as described in "Experimental procedures". Phosphorylations of RRREEESEEE peptide (A), MBP kinase peak I (B) and MBP kinase peak II (C) were carried out in the presence of 1 µM microcystin.