Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3604–3608, June 1987
Biochemistry

Phosphorylation and proteolytic modification of specific cytoskeletal proteins in human neutrophils stimulated by phorbol 12-myristate 13-acetate

(myosin light chains/signal-directed proteolysis/actin/48-kDa protein/cytoskeletal reorganization)

SANDRO PONTREMOLI*, EDON MELLONI*, MAURO MICHETTI*, BIANCA SPARATORE*, FRANCA SALAMINO*, OLIVIERO SACCO*, AND B. L. HORECKER†

*Institute of Biological Chemistry, University of Genoa, Genoa, Italy; and †Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

Contributed by B. L. Horecker, February 9, 1987

ABSTRACT **Stimulation of intact human neutrophils with phorbol 12-myristate 13-acetate results in the selective phosphorylation of two cytoskeletal protein components with molecular masses of 20 and 48 kDa. After phosphorylation the 48-kDa protein is no longer recovered as a component of the cytoskeletal fraction but is present as a fully soluble phosphoprotein. Phosphorylation of the 20-kDa protein (probably myosin light chains) signals a proteolytic conversion, catalyzed by calpain, to a smaller species having a molecular mass of approximately 15 kDa. Phosphorylation of both the 48- and 20-kDa proteins is related to the conversion of protein kinase C, also catalyzed by calpain, to the soluble fully active form. Leupeptin, an inhibitor of calpain, blocks both the phosphorylation of the target proteins and the proteolytic modification of the 20-kDa polypeptide. Thus, phosphorylation of cytoskeletal proteins and signal-directed proteolysis appear to be related processes that follow stimulation of human neutrophils by phorbol esters. The resulting changes in cytoskeletal organization may be involved in the expression of some neutrophil functions, such as exocytosis of specific granules.**

Selective protein phosphorylation involving protein kinase C is now generally accepted as one mechanism whereby external signals are transduced to generate specific intracellular messages that evoke distinct cell responses (1, 2). In a variety of cell types, including neutrophils (3–8), platelets (9–11), erythrocytes (12), adipocytes (13), 3T3 mouse cells (14), cells of the PC12 pheochromocytoma line (15), melanoma cells (16), and neuronal cells (17), an increase in the number of proteins phosphorylated and in the extent of ^{32}P incorporation into both membrane and intracellular proteins appears to be related to responses to external signals. However, at the present time, neither the specific proteins phosphorylated nor their relation to a well-defined functional response has been clearly established. We have previously shown that in human neutrophils stimulated by phorbol 12-myristate 13-acetate (PMA), the phosphorylation of membrane proteins is due to the intercalation of protein kinase C into the plasma membrane (8, 18) and is responsible for the release of a membrane-bound neutral proteinase and the production of superoxide anion (19). On the other hand, the degranulation responses induced by PMA or fMet-Leu-Phe are mediated by a different form of protein kinase C, the form (PKC-M) that is generated by digestion with a Ca^{2+}-activated proteinase (calpain) (20, 21) and expresses full catalytic activity in the absence of Ca^{2+} and phospholipids (20–25) and no longer binds to the cell membrane (21, 22).

We have recently reported (26) that incubation of the cytoskeletal fraction prepared from human neutrophils with PKC-M results in the phosphorylation of a 20-kDa component of the cytoskeleton, probably representing myosin light chains. Phosphorylation of this polypeptide serves as a specific signal for its digestion by calpain, the enzyme that is also responsible for conversion of protein kinase C to PKC-M (26). We report here that both of these modifications are also observed in intact neutrophils in response to stimulation by PMA. Cytoskeletal reorganization involving primarily the 20- and 48-kDa components appears to be associated with stimuli that evoke the degranulation response.

MATERIALS AND METHODS

Materials. Protein kinase C and PKC-M were partially purified from human neutrophils and assayed with type III-S histone as previously described (22). The specific activities of these two kinase preparations were 10×10^4 and 6×10^4 units/mg of protein, respectively. PMA, phenylmethylsulfonyl fluoride, ATP, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), and the protein standards for $NaDodSO_4$/polyacrylamide gel electrophoresis were from Sigma and [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) and $H_3{}^{32}PO_4$ (10 mCi/ml) were from Amersham International. Triton X-100 was purchased from Rohm and Haas.

Methods. Human neutrophils (5×10^6 cells per ml) prepared as previously described (22) were incubated at 37°C in 10 mM Hepes, pH 7.4, containing 0.14 M NaCl, 5 mM KCl, and 5 mM glucose, with or without PMA at 10–100 ng/ml, for 10 min. The cells were then cooled at 0°C and collected by centrifugation at $600 \times g$ for 5 min.

The cytoskeletal fraction was isolated by a modification (26) of the procedure reported by Yassin *et al.* (27), which is basically an isotonic extraction in 2% Triton X-100 containing EGTA. For phosphorylation the cytoskeletal fraction containing 0.1 mg of protein was incubated with continuous shaking at 30°C in 0.25 ml of 50 mM sodium borate buffer, pH 7.5, containing 5 mM $MgCl_2$ and 5 μM [γ-^{32}P]ATP (0.25 mCi/mmol) in the presence of 1.25 units of either protein kinase C or PKC-M. When native protein kinase C was used the incubation mixture was supplemented with Ca^{2+} and phospholipids (22); for experiments with PKC-M, 1 mM EDTA was added (22). After 10 min, 10% trichloroacetic acid (final concentration) was added and the precipitates were collected on a glass-fiber filter and washed with 15 ml of 5% trichloroacetic acid. Characterization of the phosphorylated polypeptides was carried out by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography as described (26).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PKC-M, proteolytically modified protein kinase C; PMA, phorbol 12-myristate 13-acetate.

The same gels and autoradiographs were scanned with a Cellosystem densitometer (Ciampolini).

RESULTS

Changes in the Cytoskeletal Protein Composition of Human Neutrophils After Stimulation with PMA. The cytoskeletal fraction isolated from human neutrophils contains eight major proteins, having molecular masses ranging from 20 to 80 kDa (Fig. 1*A*). Exposure of human neutrophils to a low concentration of PMA (10 ng/ml) resulted in several modifications in the polypeptide composition of the cytoskeletal protein (Fig. 1*B*). Specifically, the 48-kDa protein was no longer recovered in the cytoskeletal fraction, while the quantity of the 40-kDa protein (probably actin) associated with this fraction was increased by 2- to 3-fold, as previously observed by others (27, 28).

Phosphorylation of the Cytoskeletal Fraction by PKC-M *in Vitro*. To investigate the molecular mechanism responsible for the loss of the 48-kDa cytoskeletal protein, the isolated cytoskeletal fraction prepared from resting neutrophils was phosphorylated with PKC-M in the presence of [γ-^{32}P]ATP. After incubation the insoluble fraction was recovered and both this and the supernatant fraction were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2).

Scanning of the gels stained with Coomassie blue showed that after phosphorylation by PKC-M the 48-kDa protein dissociated from the insoluble cytoskeletal fraction (Fig. 2*A*) and was recovered in the supernatant fraction (Fig. 2*B*). Autoradiographic analysis of the same samples revealed that the major substrates for phosphorylation in the cytoskeletal fraction were the 48-kDa (Fig. 2*D*) and 20-kDa polypeptides (Fig. 2*C*), with smaller quantities of radioactivity detected in polypeptides with molecular masses of 62, 29, and 25 kDa. In contrast to the phosphorylated 48-kDa polypeptide, the other phosphorylated polypeptides remained associated with the insoluble cytoskeletal fraction (Fig. 2*C*).

The above results permit two important conclusions. First, that phosphorylation of the cytoskeletal fraction by protein kinase C is a selective process, involving primarily two of the eight major protein components of the preparation conventionally defined as the cytoskeletal fraction. Second, that phosphorylation of the 48-kDa protein is correlated with its dissociation from the cytoskeletal network.

Effect of Leupeptin on the Cytoskeletal Modification After Activation of Intact Neutrophils by PMA. Intact human neutrophils loaded with ^{32}P were stimulated with PMA at concentrations ranging from 10 to 100 ng/ml. The cells were collected and lysed, and the isolated cytoskeletal fraction was analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 3). With the lowest concentration of PMA the incorporation of label was greatest in the 20- and 48-kDa polypeptides (Fig. 3*A*). The extent of labeling of the 48-kDa component

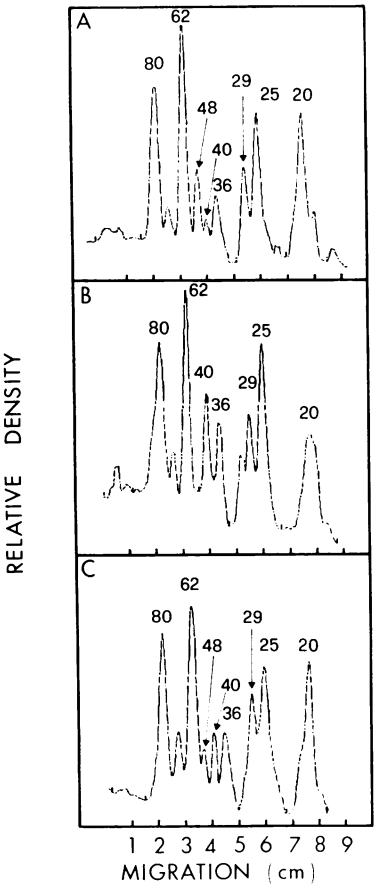

FIG. 1. Changes in cytoskeletal protein composition of human neutrophils stimulated with PMA. (*A*) The cytoskeletal fraction was prepared from unstimulated neutrophils (5 × 10^7 cells, see *Methods*), and an aliquot (80 μg of protein) was solubilized in 0.06 M Tris·HCl, pH 6.8, containing 1% NaDodSO$_4$, 10% (vol/vol) glycerol, and 2% (vol/vol) 2-mercaptoethanol. The samples were boiled for 2 min, subjected to polyacrylamide gel electrophoresis, and stained with Coomassie blue, and the stained gels were scanned with a densitometer. (*B*) Human neutrophils (5 × 10^7 cells in 10 ml) were activated with PMA at 10 ng/ml (see *Methods*) and the cytoskeletal fraction was prepared and analyzed. (*C*) Human neutrophils (5 × 10^6 cells in 1 ml) were incubated at 0°C 30 min with 1 mM leupeptin. The cells were then diluted with 10 vol of fresh medium and PMA was added to a final concentration of 10 ng/ml. After 10 min of incubation the cells were recovered by centrifugation and treated as for *A*.

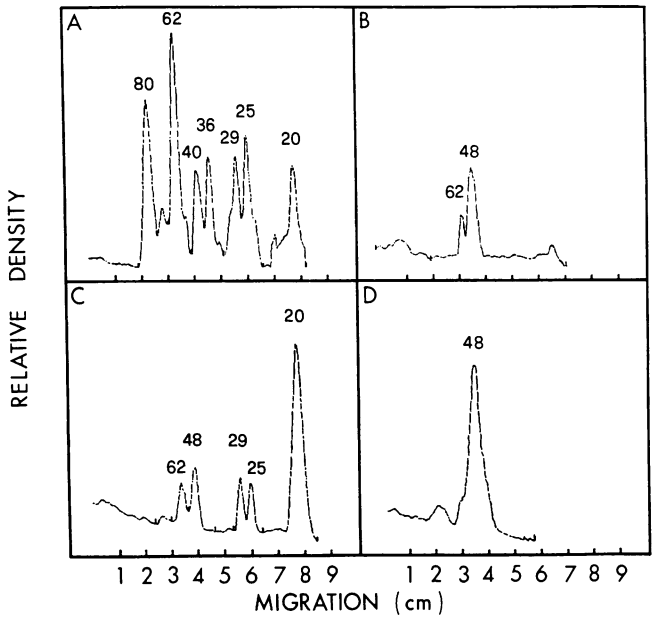

FIG. 2. Effect of phosphorylation on the neutrophil cytoskeletal organization. The cytoskeletal fraction (0.1 mg of protein) was prepared and incubated with PKC-M. After 10 min the incubation mixture was centrifuged at 10,000 × *g* for 10 min and the cytoskeletal (pellet) and supernatant fractions were collected separately. The supernatant fraction was precipitated with trichloroacetic acid (final concentration = 7%), and the precipitate was washed with acetone, dried under nitrogen, and dissolved in 0.1 ml of 0.06 M Tris·HCl, pH 6.8, containing 1% NaDodSO$_4$, 10% glycerol, and 2% 2-mercaptoethanol. The cytoskeletal pellet was suspended in the same buffer mixture. Each fraction was then boiled for 2 min, cooled, and analyzed by polyacrylamide gel electrophoresis. The dried gels were stained with Coomassie blue (*A* and *B*) and also submitted to autoradiography (*C* and *D*). The densitometer results obtained with the recovered cytoskeletal pellet are shown in *A* and *C*; the polypeptides recovered in the supernatant fraction are shown in *B* and *D*.

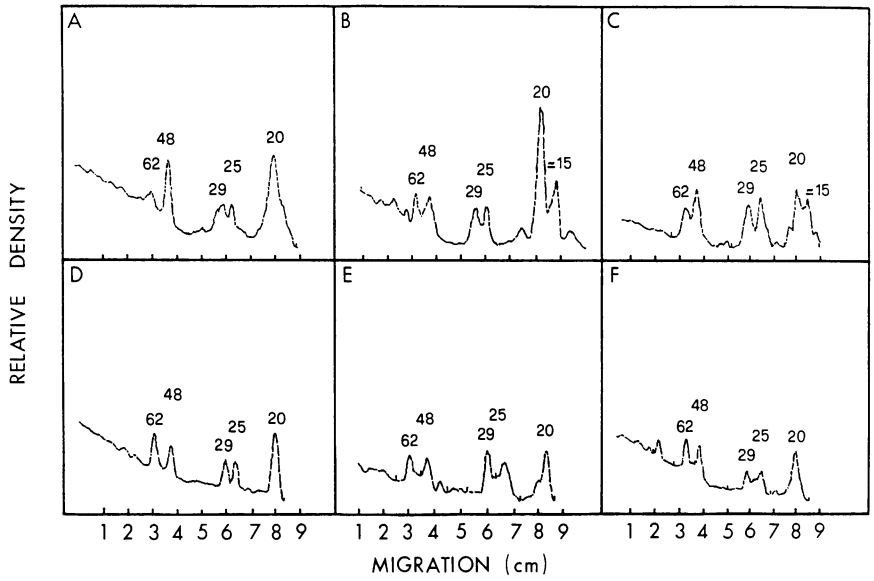

FIG. 3. Effect of leupeptin on ^{32}P incorporation into cytoskeletal proteins of human neutrophils stimulated with PMA. Neutrophils (5×10^7 cells in 10 ml) were incubated for 30 min with 0.5 mCi of $H_3{}^{32}PO_4$. The cells were collected by centrifugation, washed with 10 ml of fresh medium, and resuspended in the same volume of medium. PMA was then added at a final concentration of 10 ng/ml (*A* and *D*), 30 ng/ml (*B* and *E*), or 100 ng/ml (*C* and *F*), and the suspension was incubated for 10 min at 37°C. In *D*, *E*, and *F*, before loading with ^{32}P the cells were treated with leupeptin as described in the legend to Fig. 1. The cytoskeletal fraction was prepared and subjected to polyacrylamide gel electrophoresis. Low levels of phosphorylation were observed in the cytoskeletal fraction recovered from cells loaded with ^{32}P and incubated in the absence of PMA. The estimation of molecular masses lower than 20 kDa was uncertain with the electrophoresis procedure employed.

was much greater than is apparent from the result shown in Fig. 3*A* because most of the phosphorylated form of this protein was released (see preceding section). With increasing concentrations of PMA the major changes were observed in the 20-kDa component; first an increase in total radioactivity incorporated and then a decrease, concomitant with the appearance of a new phosphoprotein with a molecular mass of approximately 15 kDa (Fig. 3 *B* and *C*).

When the exposure to PMA was carried out in the presence of leupeptin both ^{32}P incorporation and conversion of the 20-kDa polypeptide to the 15-kDa product were greatly decreased (Fig. 3 *D*, *E*, and *F*). Leupeptin also diminished the loss of the 48-kDa protein from the cytoskeletal fraction of intact neutrophils treated with PMA (see Fig. 1*C*).

Identification of the Form of Protein Kinase C Involved in the Phosphorylation of the Cytoskeletal Proteins. To confirm that it is indeed PKC-M that is responsible for the observed phosphorylations we tested both forms of the kinase for their activity with the cytoskeletal fraction isolated from untreated human neutrophils. We found incorporation of ^{32}P into the cytoskeletal proteins to be 6-fold greater when PKC-M was employed, as compared to results obtained in experiments with native protein kinase C (Table 1). The results strongly support our previous observations (8, 18, 19), which indicate that phosphorylation of membrane proteins is catalyzed by the native membrane-associated form of protein kinase C, in contrast to cytosolic proteins, whose phosphorylation is correlated with the formation of the soluble PKC-M form. Of particular interest is the low activity observed with the fully intercalated membrane-associated form of protein kinase C, which is formed when neutrophils are treated with PMA and which does not require the addition of Ca^{2+} or phospholipid effectors for full activity.

Table 1. Phosphorylation of the cytoskeletal fraction by different forms of protein kinase C

Protein kinase C	^{32}P incorporation, pmol per sample
Native	166
Membrane-bound	205
Proteolytically modified (PKC-M)	1250

Neutrophil membranes containing native protein kinase C were prepared from cells incubated with PMA as previously described (24, 25). Cytoskeletal fraction (0.1 mg of protein) was incubated with 0.25 unit of the different forms of protein kinase C. After 10 min, 10% trichloroacetic acid was added, the precipitates were collected on glass-fiber filters and washed with 15 ml of 5% trichloroacetic acid, and the radioactivities were measured in a model 300C Packard scintillation counter.

DISCUSSION

Cytoskeletal protein components have been reported to undergo two significant types of posttranslational modification: phosphorylation and proteolytic degradation (for references see ref. 29). These observations have been made mostly with cell-free systems or with the isolated proteins, and neither the relation between these modifications nor their physiological role has been established. Phosphorylation by protein kinase C has been reported for the following proteins: erythrocyte membrane skeleton components 4.1 and 4.2 (12), vinculin (30–32), filamin (31), caldesmon (33), vimentin (34), talin (35), muscle heavy meromyosin (36, 37), and myosin light chain (38, 39). A number of cytoskeletal proteins, including α-actinin (40), actin-binding protein (41), P-235 protein (41), talin (42), calmodulin-binding proteins (43), neurofilament- and microtubule-associated proteins (44–46), and erythrocyte membrane skeletal proteins 2.1 and 4.1 (47, 48) show various degrees of susceptibility to digestion by calpain. In the case of the 20-kDa myosin light chains in human neutrophils, phosphorylation by protein kinase C serves as a specific signal for degradation by calpain (ref. 26 and the present work). These results provide evidence *with intact cells* for phosphorylation serving as a signal for proteolysis. They thus confirm our previous findings with the isolated cytoskeletal fraction, in which we had observed a similar signal-directed proteolysis of the 20-kDa polypeptide by calpain after phosphorylation by PKC-M (26). The inhibitory effects of leupeptin, which prevents the calpain-cata-

lyzed conversion of protein kinase C to PKC-M, support the conclusion that phosphorylation of the cytoskeletal components in PMA-stimulated neutrophils is catalyzed by PKC-M.

We have also shown here that after activation with PMA a second cytoskeletal protein, approximately 48 kDa in size, is also phosphorylated, and that in this case phosphorylation serves as a signal for its release from the cytoskeleton. The modified protein does not appear to be degraded but is recovered apparently quantitatively in the cytosolic fraction prepared from PMA-stimulated neutrophils.

With respect to the 20-kDa polypeptide, it is clear from the results reported here that in intact PMA-stimulated neutrophils phosphorylation precedes proteolytic conversion, as was observed earlier in the experiments carried out with the isolated cytoskeletal fraction (26). The overall process is dose dependent, since at low concentrations of PMA (10 ng/ml) only phosphorylation is observed, whereas proteolytic degradation of the phosphorylated 20-kDa polypeptide is detected with PMA at 30 ng/ml and is substantially increased with PMA at 100 ng/ml. At these highest concentrations of phorbol ester, exocytosis of granule contents has been found to be significantly increased (25, 49). Release of granule contents may thus be related to a remodelling of the cytoskeleton, a process in which phosphorylation and signal-directed proteolysis play important roles. This hypothesis is consistent with the observation that loading the cells with leupeptin greatly reduces the extent of phosphorylation of these cytoskeletal components (Fig. 3) and also the subsequent changes in composition of the cytoskeletal fraction (Fig. 1). These effects of leupeptin can be related to its inhibitory effects on the secretory response (25).

The fact that leupeptin blocks both phosphorylation and subsequent proteolytic modification suggests that calpain plays a dual role in the secretory response elicited by stimuli such as PMA or fMet-Leu-Phe. Calpain is involved in both the formation of PKC-M and the digestion of the phosphorylated 20-kDa polypeptide. The requirement for proteolytically modified protein kinase C is supported by the observation that the isolated Triton X-100 cytoskeletal preparation is a poor substrate for phosphorylation by native protein kinase C but an excellent substrate for PKC-M. These and earlier observations (25) suggest that certain physiological responses evoked in neutrophils by external stimuli, particularly those that require reorganization of the cytoskeletal network (50–52), are mediated through the coordinate and integrated activation of two cytosolic enzymes, namely protein kinase C and calpain, that are normally quiescent under resting conditions.

Note. While this manuscript was in preparation we became aware of a report describing the phosphorylation of 20- and 47-kDa platelet protein by calpain-activated myosin light chain kinase (53).

This work was supported in part by grants from the Consiglio Nazionale delle Ricerche, Progetto Finalizzato Oncologia, Sottoprogetti Biologia Cellulare e Molecolare and from the Association Italiana Ricerche Cancro.

1. Nishizuka, Y. (1984) *Nature (London)* **308,** 693–698.
2. Ashendel, C. L. (1985) *Biochim. Biophys. Acta* **822,** 219–242.
3. Schneider, C., Zanetti, M. & Romeo, D. (1981) *FEBS Lett.* **127,** 4–8.
4. Andrews, P. L. & Babior, B. M. (1984) *Blood* **64,** 883–890.
5. White, J. R., Huang, C. K., Jr., Hill, J. M., Naccache, P. F., Becker, E. L. & Sha'afi, R. I. (1984) *J. Biol. Chem.* **259,** 8605–8611.
6. Irita, K., Takeshige, K. & Minakami, S. (1984) *Biochim. Biophys. Acta* **805,** 44–52.
7. Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P. C. & Babior, B. M. (1986) *J. Biol. Chem.* **261,** 9109–9115.
8. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M., Sacco, O. & Horecker, B. L. (1986) *Arch. Biochem. Biophys.* **250,** 23–29.
9. Lyons, R. M., Standford, N. & Majerus, P. W. (1975) *J. Clin. Invest.* **56,** 924–936.
10. Lyons, R. M. & Shaw, J. O. (1980) *J. Clin. Invest.* **65,** 242–255.
11. Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258,** 6701–6704.
12. Horne, W. C., Leto, T. L. & Marchesi, V. T. (1985) *J. Biol. Chem.* **260,** 9073–9076.
13. Graves, C. B. & McDonald, J. M. (1985) *J. Biol. Chem.* **260,** 11286–11292.
14. Rozengurt, E., Rodrigues-Pena, M. & Smith, K. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7244–7248.
15. Hama, T., Huang, K. P. & Guroff, G. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2353–2357.
16. Bosman, F., Opdenakker, G., Damme, J. V. & Billiau, A. (1986) *Biochem. Biophys. Res. Commun.* **137,** 263–272.
17. LeVine, H., III, & Sahyoun, N. E. (1986) *Biochem. Biophys. Res. Commun.* **138,** 59–65.
18. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M., Sacco, O. & Horecker, B. L. (1986) *Biochem. Biophys. Res. Commun.* **140,** 1121–1126.
19. Melloni, E., Pontremoli, S., Salamino, F., Sparatore, B., Michetti, M., Sacco, O. & Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 11437–11439.
20. Inoue, M., Kishimoto, A., Takai, Y. & Nishizuka, Y. (1977) *J. Biol. Chem.* **252,** 7610–7616.
21. Kishimoto, A., Kajikawa, N., Shiota, M. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258,** 1156–1164.
22. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. & Horecker, B. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6435–6439.
23. Pontremoli, S., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. & Melloni, E. (1985) *Biochem. Int.* **11,** 35–44.
24. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B. & Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 4101–4105.
25. Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B. & Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 8309–8313.
26. Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Sacco, O. & Horecker, B. L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 398–401.
27. Yassin, R., Shefcyk, J., White, J. R., Tao, W., Volpi, M., Molski, T. F. P., Naccache, P. H., Feinstein, M. B. & Sha'afi, R. I. (1985) *J. Cell Biol.* **101,** 182–188.
28. White, J. R., Naccache, P. H. & Sha'afi, R. I. (1983) *J. Biol. Chem.* **258,** 14041–14047.
29. Traub, P. (1986) *Intermediate Filaments, a Review* (Springer, Berlin).
30. Werth, D. K., Niedel, J. E. & Pastan, I. (1983) *J. Biol. Chem.* **258,** 11423–11426.
31. Kawamoto, S. & Hidaka, H. (1984) *Biochem. Biophys. Res. Commun.* **118,** 736–742.
32. Werth, D. K. & Pastan, I. (1984) *J. Biol. Chem.* **259,** 5264–5270.
33. Umekawa, H. & Hidaka, H. (1985) *Biochem. Biophys. Res. Commun.* **132,** 56–62.
34. Huang, C. K., Jr., Hill, J. M., Bormann, B. J., Mackin, W. M. & Becker, E. L. (1984) *J. Biol. Chem.* **259,** 1386–1389.
35. Litchfield, D. W. & Ball, E. H. (1986) *Biochem. Biophys. Res. Commun.* **134,** 1276–1283.
36. Nishikawa, M., Hidaka, H. & Adelstein, R. S. (1983) *J. Biol. Chem.* **258,** 14069–14072.
37. Nishikawa, M., Sellers, J. R., Adelstein, R. S. & Hidaka, H. (1984) *J. Biol. Chem.* **259,** 8808–8814.
38. Endo, T., Naka, M. & Hidaka, H. (1982) *Biochem. Biophys. Res. Commun.* **105,** 942–948.
39. Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) *Nature (London)* **306,** 490–492.
40. Gache, Y., Landon, F., Touitou, H. & Olomucki, A. (1984) *Biochem. Biophys. Res. Commun.* **124,** 877–881.
41. Fox, J. E. B., Goll, D. E., Reynolds, C. C. & Philipids, D. R. (1985) *J. Biol. Chem.* **260,** 1060–1066.
42. O'Holloran, T., Beckerle, M. C. & Burridge, K. (1985) *Nature (London)* **317,** 449–451.

43. Kosaki, G., Tsujinaka, T., Kambayashi, J., Moritomo, K., Yamamoto, K., Yamagami, K., Sobue, K. & Kakiuchi, S. (1983) *Biochem. Int.* **6,** 767–775.
44. Malik, M. N., Fenko, M. D., Iqbal, K. & Wisniewski, H. M. (1983) *J. Biol. Chem.* **258,** 8955–8962.
45. Zimmerman, U. J. P. & Schlaepfer, W. W. (1984) *J. Biol. Chem.* **259,** 3210–3218.
46. Ishizaki, Y., Tashiro, T. & Kurokawa, M. (1983) *Eur. J. Biochem.* **131,** 41–45.
47. Pontremoli, S., Sparatore, B., Melloni, E., Michetti, M. & Horecker, B. L. (1984) *Biochem. Biophys. Res. Commun.* **123,** 331–337.
48. Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M. & Horecker, B. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6714–6717.
49. Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Damiani, G. & Horecker, B. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1685–1689.
50. Pho, D. B., Desbruyères, E., Der Terrossian, E. & Olomucki, A. (1986) *FEBS Lett.* **202,** 117–121.
51. Kruskal, B. A., Shak, S. & Maxfield, F. R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2919–2923.
52. Southwich, F. S. & Stossel, T. P. (1983) *Semin. Hematol.* **20,** 305–321.
53. Kambayashi, J., Kajiwara, Y., Sakon, M., Ohshiro, T. & Mori, T. (1986) *Biochem. Int.* **13,** 571–578.