BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 16–20 (1998)
ARTICLE NO. RC977899

Up-Regulation of Phosphorylation of Focal Adhesion Kinase and Paxillin by Combination of Substance P and IGF-1 in SV-40 Transformed Human Corneal Epithelial Cells

Masatsugu Nakamura, Takashi Nagano, Tai-ichiro Chikama, and Teruo Nishida[1]
Department of Ophthalmology, Yamaguchi University School of Medicine, 1144 Kogushi, Ube City, Yamaguchi 755, Japan

Received November 26, 1997

We previously reported that substance P (SP) and insulin-like growth factor-1 (IGF-1) synergistically facilitate corneal epithelial migration *in vitro* and *in vivo*. We wanted to determine whether proteins responsible for cellular attachment are activated in corneal epithelial cells. To do this, we examined changes in tyrosine phosphorylation in focal adhesion kinase (FAK) and paxillin in cultured SV-40 transformed human corneal epithelial cells (HCE cells). HCE cells were cultured in the absence or presence of either SP (2×10^{-5} M) or IGF-1 (10 ng/ml) or both SP and IGF-1. Treatment of HCE cells by either SP or IGF-1 alone did not alter tyrosine phosphorylation in either FAK or paxillin. However, the combination of SP and IGF-1 significantly increased tyrosine phosphorylation in both FAK and paxillin. In contrast, the combination of SP and IGF-1 was not observed to produce synergistic effects on the activation of mitogen-activated protein kinase in HCE. These results show that the synergistic effects of SP and IGF-1 on corneal epithelial wound healing were expressed through activation of the integrin, FAK, and paxillin system.

Integrins are classically considered a family of cell surface receptors for various extracellular matrix proteins (1, 2). Recent investigations have shown, however, that their functions mediate various effects through signal transduction pathways, and tyrosine phosphorylation plays a critical role in signal transduction via integrins (3, 4). Integrin-mediated cell attachment to an extracellular matrix induced tyrosine phosphorylation of focal adhesion kinase (FAK) (5–9), and the focal adhesion protein, paxillin, was tyrosine phosphorylated by FAK as a substrate (10, 11). Furthermore, mitogen-activated protein (MAP) kinase was also activated by the integrin-mediated cell attachment (12–15). Therefore, several signal pathways mediate the interaction of integrin and extracellular matrix proteins at the adhesion complexes.

Because the integrity of the epithelium is so important for maintaining the internal environment of the body, we must try to understand the mechanisms of epithelial wound healing. Epithelial cell migration is an early and essential process for epithelial wound healing. In this process, migrating epithelial cells attach to a provisional fibronectin matrix over the newly created wound area and are expressed as cell surface receptors for fibronectin (integrin $\alpha 5\beta 1$), thereby increasing their responsiveness to fibronectin (16–18). The fibronectin-integrin system thus plays an important role in the process of epithelial migration.

Using an organ culture of the cornea, we recently found that substance P (SP) and insulin-like growth factor-1 (IGF-1) synergistically stimulate corneal epithelial migration (19). The addition of either SP or IGF-1 alone did not affect epithelial migration, but the combination of SP and IGF-1 stimulated epithelial migration significantly. In addition, SP and IGF-1 synergistically stimulated integrin activity in corneal epithelial cells. This action of SP was specific among various kinds of neurotransmitters and tachykinins. Furthermore, SP and IGF-1 synergistically facilitated corneal epithelial wound closure *in vivo* (20).

To understand the mechanisms of this synergistic action of SP and IGF-1 on epithelial migration, in this study we investigated whether the combination of SP and IGF-1 stimulates the tyrosine phosphorylation of integrin-related proteins, FAK, and paxillin and the activation of MAP kinase in cultured human corneal epithelial cells.

MATERIALS AND METHODS

Human corneal epithelial cells. A human corneal epithelial (HCE) cell line was used. Dr. K. Araki-Sasaki kindly donated an

[1] Corresponding author. Fax: +81-836-22-2334. E-mail: nishida1@po.cc.yamaguchi-u.ac.jp.

0006-291X/98 $25.00

HCE cell line, which had been transformed by an SV40-adenovirus recombinant vector, as described in the literature (21). HCE cells were cultured in supplemented hormone epithelial medium (SHEM) consisting of 1 vol of Dulbecco-modified Eagle's medium and 1 vol of Ham's nutrient mixture F-12 (DMEM/HamF-12, BIBCO, Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Flow Laboratories, North Ryde, Australia), 5 μg/ml insulin, 0.1 μg/ml choleratoxin, 10 ng/ml human epidermal growth factor, 0.5% dimethyl-sulfoxide, and 40 μg/ml gentamicin.

Tyrosine phosphorylation of FAK and paxillin. HCE cells were cultured in SHEM until 70–80% confluency and then transferred to another medium, unsupplemented DMEM/HamF-12, for another 3 days. The cells were then incubated with SP (2×10^{-5} M) and/or IGF-1 (10 ng/ml) for 5, 10, or 30 min. After incubation, the cells were washed with PBS containing 1 mM Na_3VO_4 and lysed with lysis buffer (10 mM Tris-HCl, pH8.0, containing 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, 1% Triton-X-100). After centrifugation, the supernatants were immunoprecipitated with monoclonal antibody against FAK (anti-$p125^{FAK}$, Upstate Biotechnology, Lake Placid, NY) or against paxillin (Transduction Laboratories, Lexington, KY) antibody. The immunoprecipitated samples were subjected to electrophoresis on 4/20% SDS-polyacrylamide gels and electrophoretically transferred to transfer membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were probed with anti-phosphotyrosine antibody (RC20, Transduction Laboratories, Lexington, KY). The same membranes were reprobed with anti-FAK or anti-paxillin antibody to confirm that equal amounts of FAK or paxillin were precipitated.

Activation of mitogen-activated protein (MAP) kinase. To examine the phosphorylation of MAP kinase, HCE cells were cultured in SHEM until 70–80% confluency and then transferred to unsupplemented DMEM/HamF-12 medium for another 3 days. The cells were then incubated with SP (2×10^{-5} M) and/or IGF-1 (10 ng/ml) for 5 min. After incubation, the cells were washed with PBS containing 1 mM Na_3VO_4 and lysed with lysis buffer (1% Triton X-100, 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerol phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethanesulphonyl fluoride, pH 7.4). After centrifugation, the supernatants were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels and electrophoretically transferred to transfer membranes. The membranes were probed with rabbit anti-phospho-MAP kinase antibody (New England Biolabs, Beverly, MA), which detects p44 and p42 MAP kinase.

To examine the translocation of MAP kinase, HCE cells were cultured in SHEM until 70– 80% confluency and then transferred to unsupplemented DMEM/HamF-12 medium for another 3 days. The cells were incubated with SP (2×10^{-5} M) and/or IGF-1 (10 ng/ml) for 30 min. After incubation, the cells were fixed with 3% formaldehyde in PBS for 5 min. After being washed with PBS three times, HCE cells were quenched with 0.15 M glycine in PBS and permeabilized with 0.1% SDS for 1 min. The specimen was washed and blocked with blocking buffer (5% normal goat serum and 0.05% Tween-20 in PBS) for 1 h at 23°C, incubated with rabbit anti-MAP kinase antibody (Sigma, St. Louis, MO) for 1 h, and then incubated with fluoresceine isothiocynate-conjugated goat anti-rabbit IgG (Cappel Organon Teknika Co., Durham, NC) for 30 min. Samples were observed with a laser confocal microscope (FLUOVIEW, Olympus, Tokyo, Japan).

RESULTS AND DISCUSSION

We first tried to determine whether the combination of SP and IGF-1 stimulates tyrosine phosphorylation of FAK and paxillin, which are cytoskeletal proteins associated with integrins. When the cells were treated with SP alone or IGF alone, no changes were observed in the staining-band density of tyrosine phosphorylation of FAK. When the cells were treated with a combination of SP and IGF-1, however, the staining-band density of tyrosine phosphorylation of FAK clearly greater than that of cells treated with the control, SP alone, or IGF-1 alone (Fig. 1A). In densitometric analysis, the combination of SP and IGF-1 showed a 5-fold increase compared with the control. Stimulation of tyrosine phosphorylation of FAK by the combination of SP and IGF-1 was transient, reaching a maximum 5 or 10 min after the addition of SP and IGF-1 and returning to the basal level after 30 min, irrespective of the continuous presence of SP and IGF-1 in the medium (Fig. 1B).

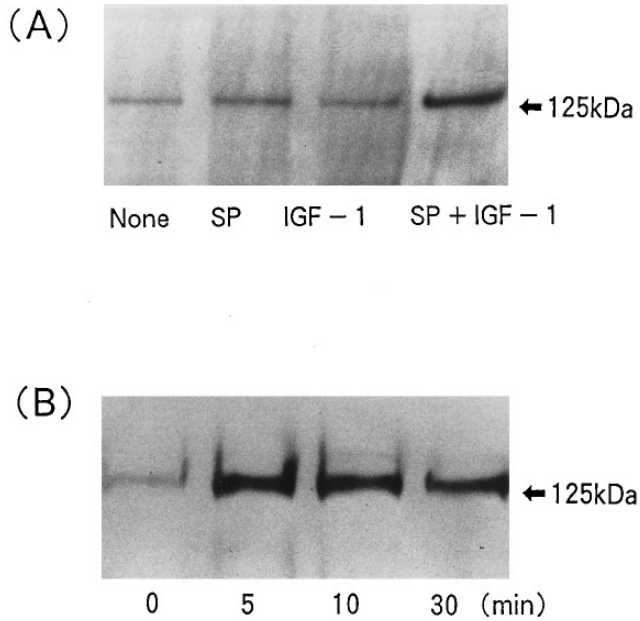

FIG. 1. Tyrosine phosphorylation of FAK in HCE cells treated with SP and IGF-1. (A) HCE cells were treated with DMEM/HamF-12 containing SP (2×10^{-5} M) and/or IGF-1 (10 ng/ml) for 10 min. (B) HCE cells were treated with DMEM/HamF-12 containing SP (2×10^{-5} M) and IGF-1 (10 ng/ml) for 0, 5, 10, or 30 min. After treatments, the cells were lysed and immunoprecipitated with anti-FAK antibody and then immunoblotted with anti-phosphotyrosine antibody.

Nearly identical results were obtained when we examined the tyrosine phosphorylation of paxillin. Although neither SP nor IGF-1 alone affected the staining-band density of tyrosine phosphorylation of paxillin, the combination of SP and IGF-1 caused a dramatic increase (Fig. 2A). In densitometric analysis, the combination of SP and IGF-1 showed a 7-fold increase compared with the control. The maximum response time of tyrosine phosphorylation of paxillin differed from that of FAK, reaching a maximum 30 min after the addition of SP and IGF-1 (Fig. 2B). These results demonstrated that SP and IGF-1 synergistically activated tyrosine phosphorylation of FAK and paxillin.

We next investigated whether the combination of SP and IGF-1 stimulates the activation of MAP kinase.

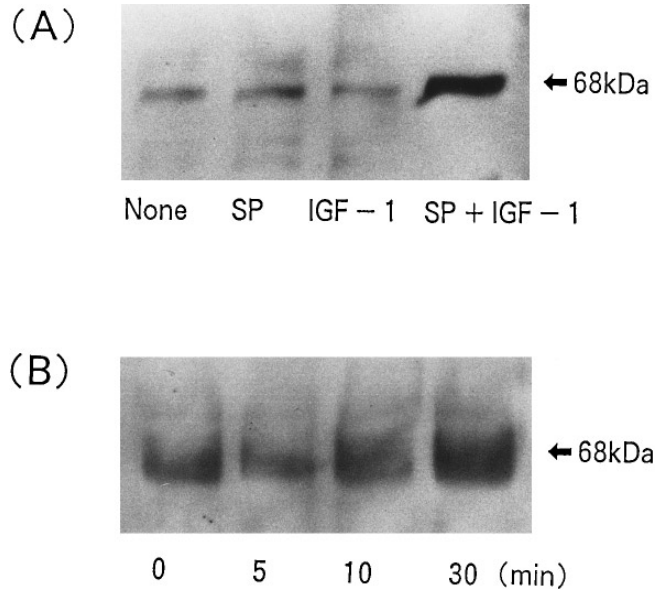

FIG. 2. Tyrosine phosphorylation of paxillin in HCE cells treated with SP and IGF-1. (A) HCE cells were treated with DMEM/HamF-12 containing SP (2×10^{-5} M) and/or IGF-1 (10 ng/ml) for 30 min. (B) HCE cells were treated with DMEM/HamF-12 containing SP (2×10^{-5} M) and IGF-1 (10 ng/ml) for 0, 5, 10, or 30 min. After treatment, the cells were lysed and immunoprecipitated with anti-paxillin antibody and then immunoblotted with anti-phosphotyrosine antibody.

When the cells were treated with either SP alone or IGF-1 alone, the staining-band density of both p44 and p42 phosphorylated-MAP kinase (activated MAP kinase) increased compared with that of the cells treated with unsupplemented DMEM/HamF-12 (control). However, although the combination of SP and IGF-1 activated MAP kinase compared with the control, we observed no synergistic effects of SP and IGF-1 on the activation of MAP kinase (Fig. 3).

Nearly identical results were observed when we examined the translocation of MAP kinase in place of phosphorylation of MAP kinase. When cells were treated with unsupplemented DMEM/HamF-12, the specific immunofluorescence of MAP kinase was observed only in the cytoplasma but not in the nucleus. In contrast, when cells were treated with SP and/or IGF-1, MAP kinase was translocated into the nucleus, and immunofluorescence of MAP kinase was observed in whole cells, including the nucleus. However, no synergistic effects of SP and IGF-1 on the translocation of MAP kinase were observed (Fig. 4).

We recently demonstrated that, acting alone, the neuropeptide SP does not influence the healing of corneal epithelial wounds, but in the presence of IGF-1, SP synergistically stimulates such healing (19, 20). We hypothesized that the synergistic effect of SP and IGF-1 might be mediated by activation of the integrin, FAK, and paxillin system. The present results clearly demonstrate that SP and IGF-1 synergistically stimulate the tyrosine phosphorylation of FAK and paxillin in HCE cells. These results suggest that the synergistic effects of SP and IGF-1 on corneal epithelial wound healing might be expressed through activation of the integrin, FAK, and paxillin system.

Integrins are not only a family of cell surface receptors for various extracellular matrix proteins, but they also transduce a series of signals after binding ligands (1–4). Integrin-mediated cell attachment to extracellular matrix proteins leads to enhanced tyrosine phosphorylation of FAK at the focal contacts and to further tyrosine phosphorylation of FAK with several focal adhesion proteins, such as paxillin (5–7, 10, 11). In the present study, it is interesting that neither SP nor IGF-1 by itself affected tyrosine phosphorylation of FAK or paxillin, but the combination of SP and IGF-1 synergistically stimulated tyrosine phosphorylation of FAK and paxillin. Although we cannot explain the mechanisms of the synergistic effect of SP and IGF-1, this is the first demonstration that the neural regulator itself has no direct effect on the tyrosine phosphorylation of FAK and paxillin but has a synergistic effect in the presence of a humoral regulator. Attachment to a fibronectin matrix stimulates the tyrosine phosphorylation of FAK (5, 8, 9, 22, 23). The level of tyrosine phosphorylation of FAK is related to the level of expression of $\beta 1$ integrin at the focal contacts (24). Therefore, the synergistic effects of SP with IGF-1 on tyrosine phosphorylation of FAK and paxillin may be mediated by increased cell attachment to the fibronectin. Further studies on the regulatory mechanisms of the synergistic effect of SP and IGF-1 on tyrosine phosphorylation of FAK and paxillin are needed.

MAP kinase is a serine/threonine protein kinase, which is activated by phosphorylation on its threonine and tyrosine residues. There are two isoforms of MAP kinase, extracellular regulated protein kinase (ERK)-1 (p44 MAP kinase) and ERK-2 (p42 MAP kinase). MAP kinase seems to play an important role in conveying signals from the cytoplasm to the nucleus (translocation) and regulates several cellular functions, such as cell proliferation and

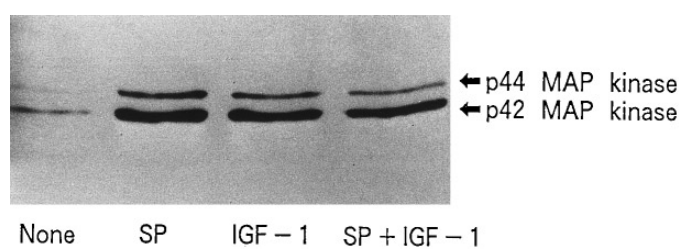

FIG. 3. Phosphorylation of MAP kinase in HCE cells treated with SP and IGF-1. HCE cells were treated with DMEM/HamF-12 containing SP (2×10^{-5} M) and/or IGF-1 (10 ng/ml) for 5 min. After treatment, the cells were lysed and immunoblotted with anti-phospho-MAP kinase antibody.

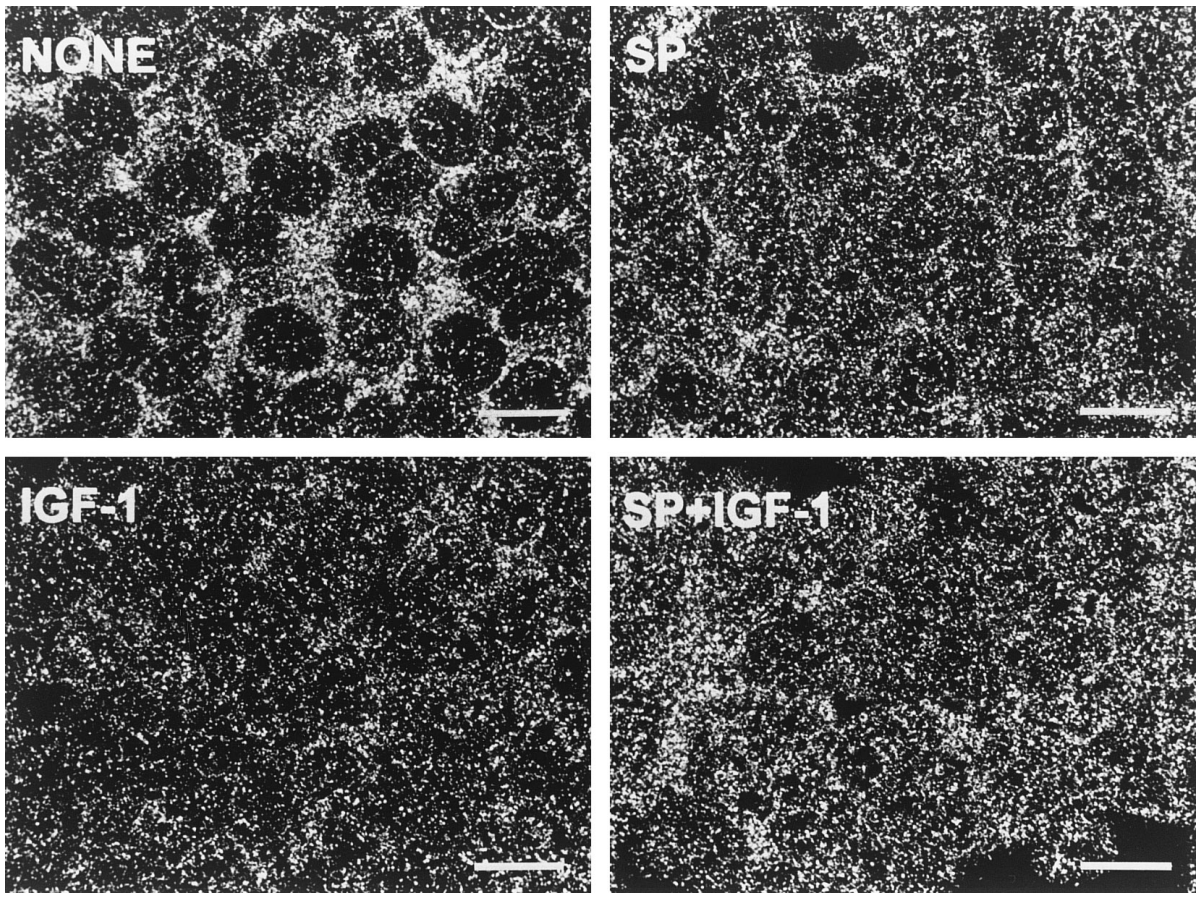

FIG. 4. Translocation of MAP kinase in HCE cells treated with SP and IGF-1. HCE cells were treated with DMEM/HamF-12 containing SP (2×10^{-5} M) and/or IGF-1 (10 ng/ml) for 15 min. After treatment, the cells were fixed and immunostained with anti-MAP kinase antibody. Bar shows 20 μm.

differentiation, by phosphorylating and activating nuclear transcriptional factors (25–27). Recently, MAP kinase has been activated by integrin-mediated cell attachment, possibly as a downstream consequence of phosphorylation of FAK (12–15). In the present study, SP and IGF-1 were more effective than a control in stimulating the phosphorylation and translocation of MAP kinase. Although synergistic effects of SP and IGF-1 on the phosphorylation of FAK were observed, no such effects on the activation of MAP kinase were noted. Furthermore, we previously reported that SP alone and IGF-1 alone did not affect cellular proliferation in corneal epithelial cells, and we observed no synergistic effects of SP and IGF-1 on cell proliferation (19). These results suggest that synergistic effects of SP and IGF-1 on corneal epithelial wound healing might not mediate the MAP kinase cascade.

In the process of corneal epithelial wound healing, attachment of the epithelial cells to the underlying provisional extracellular matrix is required for spreading and migration, which constitute the first phase of corneal wound healing (17, 28, 29). Fibronectin and fibronectin receptors (integrins) play an important role in this process. When the corneal epithelium is defective, fibronectin appears on the surface of the bare stroma and serves as a temporary matrix for epithelial migration (30–32). In contrast, in an intact cornea, fibronectin receptors are limited to the basal epithelial cell layers. After corneal wounding, however, fibronectin receptors are observed at the surface of actively migrating epithelial cells and show increased sensitivity to fibronectin (32, 33). In addition, antibodies against fibronectin, integrin $\alpha 5\beta 1$, or RGD peptide inhibit corneal epithelial migration (34, 35). These results demonstrate that the fibronectin-integrin system plays a central role in the first phase of epithelial wound healing. We previously reported that SP and IGF-1 synergistically stimulate corneal epithelial migration (19). Furthermore, we reported here that SP and IGF-1 synergistically stimulate the activation of FAK and paxillin. Therefore, the synergistic effects of SP and IGF-1 on corneal epithelium may result from their effect on the fibronectin-integrin system in the first phase of wound healing.

ACKNOWLEDGMENTS

The authors thank Dr. K. Araki-Sasaki (Division of Ophthalmology, Toyonaka Municipal Hospital, Osaka, Japan) for her kind gift of HCE cells. We also thank Miss Michiyo Suetomi for her secretarial assistance during the preparation of the manuscript. The technical assistance of Miss Kiyoe Takeuchi is acknowledged. This research was supported in part by a grant from the Ministry of Education, Culture, Sports, and Science of Japan (09470381) and by a grant from International Lions Club District 336-D.

REFERENCES

1. Ruoslahti, E. (1991) *J. Clin. Invest.* **87,** 1–5.
2. Hynes, R. O. (1992) *Cell* **69,** 11–25.
3. Clark, E. A., and Brugge, J. S. (1995) *Science* **268,** 233–239.
4. Yamada, K. M., and Geiger, B. (1997) *Curr. Opin. Cell Biol.* **9,** 76–85.
5. Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991) *Cell Regul.* **2,** 951–964.
6. Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8392–8396.
7. Guan, J. L., and Shalloway, D. (1992) *Nature* **358,** 690–692.
8. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) *J. Biol. Chem.* **267,** 23439–23442.
9. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5192–5196.
10. Pavalko, F. M., and Otey, C. A. (1994) *Proc. Soc. Exp. Biol. Med.* **205,** 282–293.
11. Turner, C. E. (1994) *Bioessays* **16,** 47–52.
12. Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K., and Juliano, R. L. (1994) *J. Biol. Chem.* **269,** 26602–26605.
13. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) *Nature* **372,** 786–791.
14. Morino, N., Mimura, T., Hamasaki, K., Tobe, K., Ueki, K., Kikuchi, K., Takehara, K., Kadowaki, T., Yazaki, Y., and Nojima, Y. (1995) *J. Biol. Chem.* **270,** 269–273.
15. Zhu, X., and Assoian, R. K. (1995) *Mol. Biol. Cell* **6,** 273–282.
16. Juhasz, I., Murphy, G. F., Yan, H. C., Herlyn, M., and Albelda, S. M. (1993) *Am. J. Pathol.* **143,** 1458–1469.
17. Nishida, T. (1993) *Curr. Opin. Ophthalmol.* **4,** 4–13.
18. Yamada, K. M., Gailit, J., and Clark, R. A. F. (1996) *in* The Molecular and Cellular Biology of Wound Repair (Clark, R. A. F., Ed.), pp. 311–338, Plenum Press, New York.
19. Nishida, T., Nakamura, M., Ofuji, K., Reid, T. W., Mannis, M. J., and Murphy, C. J. (1996) *J. Cell. Physiol.* **169,** 159–166.
20. Nakamura, M., Ofuji, K., Chikama, T., and Nishida, T. (1997) *Curr. Eye Res.* **16,** 275–278.
21. Araki-Sasaki, K., Ohashi, Y., Sasabe, T., Hayashi, K., Watanabe, H., Tano, Y., and Handa, H. (1995) *Invest. Ophthalmol. Vis. Sci.* **36,** 614–621.
22. Burridge, K., Turner, C. E., and Romer, L. H. (1992) *J. Cell Biol.* **119,** 893–903.
23. Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8487–8491.
24. Byers, H. R., Etoh, T., Vink, J., Franklin, N., Gattoni-Celli, S., and Mihm, M. C., Jr. (1992) *J. Dermatol.* **19,** 847–852.
25. Blenis, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5889–5892.
26. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556.
27. Seger, R., and Krebs, E. G. (1995) *FASEB J.* **9,** 726–735.
28. Dua, H. S., Gomes, J. A. P., and Singh, A. (1994) *Br. J. Ophthalmol.* **78,** 401–408.
29. Gipson, I. K., and Inatomo, T. (1995) *Curr. Opin. Ophthalmol.* **6,** 3–10.
30. Fujikawa, L. S., Foster, C. S., Harrist, T. J., Lanigan, J. M., and Colvin, R. B. (1981) *Lab. Invest.* **45,** 120–129.
31. Suda, T., Nishida, T., Ohashi, Y., Nakagawa, S., and Manabe, R. (1981) *Curr. Eye Res.* **1,** 553–556.
32. Murakami, J., Nishida, T., and Otori, T. (1992) *J. Lab. Clin. Med.* **120,** 86–93.
33. Grushkin-Lerner, L. S., and Trinkaus-Randall, V. (1991) *Curr. Eye Res.* **10,** 75–85.
34. Nishida, T., Nakagawa, S., Awata, T., Ohashi, Y., Watanabe, K., and Manabe, R. (1983) *J. Cell Biol.* **97,** 1653–1657.
35. Nakamura, M., and Nishida, T. (1994) *Jpn. J. Ophthalmol.* **38,** 246–251.