Age-dependent Decline in Mitogenic Stimulation of Hepatocytes

REduced Association Between Shc and the Epidermal Growth Factor Receptor Is Coupled to Decreased Activation of Raf and Extracellular Signal-Regulated Kinases

(Received for publication, October 6, 1998, and in revised form, February 8, 1999)

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The proliferative potential of the liver has been well documented to decline with age. However, the molecular mechanism of this phenomenon is not well understood. Cellular proliferation is the result of growth factor-receptor binding and activation of cellular signaling pathways to regulate specific gene transcription. To determine the mechanism of the age-related difference in proliferation, we evaluated extracellular signal-regulated kinase-mitogen-activated protein kinase activation and events upstream in the signaling pathway in epidermal growth factor (EGF)-stimulated hepatocytes isolated from young and old rats. We confirm the age-associated decrease in extracellular signal-regulated kinase-mitogen-activated protein kinase activation in response to EGF that has been previously reported. We also find that the activity of the upstream kinase, Raf kinase, is decreased in hepatocytes from old compared with young rats. An early age-related difference in the EGF-stimulated pathway is shown to be the decreased ability of the adapter protein, Shc, to associate with the EGF receptor through the Shc phosphotyrosine binding domain. To address the mechanism of decreased Shc/EGF receptor interaction, we examined the phosphorylation of the EGF receptor at tyrosine 1173, a site recognized by the Shc phosphotyrosine binding domain. Tyrosine 1173 of the EGF receptor is underphosphorylated in the hepatocytes from old animals compared with young in a Western blot analysis using a phosphospecific antibody that recognizes phosphotyrosine 1173 of the EGF receptor. These data suggest that a molecular mechanism underlying the age-associated decrease in hepatocyte proliferation involves an age-dependent regulation of site-specific tyrosine residue phosphorylation on the EGF receptor.

The proliferative potential of several tissues, including the liver, declines with age (1, 2). The ability to proliferate or regenerate hepatocytes is particularly important in the elderly, who, due to drug metabolism and other environmental exposures, need to replace cells that are destroyed due to toxic reactions. Thus, an age-related decline in the ability to proliferate may limit the ability of the elderly to recover from toxic exposures. The molecular mechanism underlying the age-related decrease in proliferative response is not well understood. Cellular proliferation is activated by a cascade of signals initiated at the cell membrane in response to growth factor binding to its receptor. In response to the mitogen, epidermal growth factor (EGF), hepatocytes from old rats compared with young rats have diminished induction of DNA synthesis (2). However, hepatocyte EGF receptor number and binding to the receptor are similar with age (3). Such data suggest that age-related differences in proliferation may involve differences in the signal transduction pathways stimulated by growth factors.

The binding of EGF to its receptor initiates a series of signaling events to induce DNA synthesis and cell division. In response to ligand binding, the EGF receptor is phosphorylated, which leads to membrane recruitment of adapter and exchange proteins for activation of Ras and subsequent Raf kinase activation (4–6). Downstream of Raf kinase, activation of a series of kinases proceeds to activate primarily ERK-MAP kinase in response to EGF (7, 8). The MAP kinase family of proteins are activated by dual phosphorylation on Thr-X-Tyr residues (reviewed in Refs. 9 and 10). Other members of the MAP kinase family include JNK and p38 MAP kinases, which are primarily activated by proinflammatory cytokines and cellular stress (reviewed in Ref. 11). Upstream activators of these kinases can function both specifically and with cross-specificity, resulting in activation of more than one MAP kinase. For example, in response to EGF, ERK-MAP kinase is activated as much as 20-fold, while JNK-MAP kinase is activated up to 6-fold (12–14). Activation of ERK-MAP kinase results in phosphorylation of a number of target proteins including Elk-1 of the ternary complex factor. Elk-1 mediates transcriptional activation of many genes, including c-fos and, consequently, AP-1 target genes (15–19).

We have used a model of primary hepatocytes obtained from young (4–6-month) or old (>32-month) rats to investigate the mechanism for the age-related differences in signal transduction pathways that are activated in response to EGF. ERK-MAP kinase activity was previously reported to be substantially reduced in hepatocytes from old compared with young

*This work was supported by National Institutes of Health Grant RO1 DK50442 (to K. E. P.) and by the Center for Gastroenterology Research on Absorptive and Secretory Processes, NEMCH (NIDDK, NIH, Grant P30 DK34928). This project was also funded in part with federal funds from the U. S. Department of Agriculture Agricultural Research Service under contract 53-3K06-01 (to K. E. P.). The contents of this publication do not necessarily reflect the views or policies of the USDA nor does mention of trade names, commercial products, or organisms imply endorsement by the U. S. government. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: EGF, epidermal growth factor; MAP, mitogen-activated protein; GST, glutathione S-transferase; MRK, mitogen-activated protein kinase phosphatase; PTB, phosphotyrosine binding; SH2, Src homology 2; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

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rats (20). In addition to decreased ERK-MAP kinase activation by EGF, we found that activity of Raf kinase, an upstream signal transducer, was also substantially decreased without a change in Raf protein level in hepatocytes from old rats. The tyrosine phosphorylation of the EGF receptor was similar in hepatocytes from young and old after EGF stimulation, suggesting that the age-related difference is after receptor activation and before Raf kinase activation. Therefore, we examined the ability of the adapter protein, Shc, to associate with the EGF receptor. Using both direct immunoprecipitation and GST fusion protein capture assays, we observed a decrease in the association of the activated EGF receptor with Shc in hepatocytes from old animals compared with young. Furthermore, we show by Western blot analysis that site-specific phosphorylation at tyrosine 1173 of the EGF receptor, a Shc interaction site, is reduced in hepatocytes from old compared with the young. These results indicate that the reduced ability of the EGF receptor to associate with Shc is an early step in the EGF signal transduction pathway that is altered with age.

EXPERIMENTAL PROCEDURES

Hepatocyte Isolation—Hepatocytes were isolated from young (4–6-month-old, >30-month-old) male F1 F344 X BN rats by perfusion of the liver with collagenase, type 2 ( Worthington), according to the procedure of Seglen (21) with the modification of using a HEPES (10 mM)-based buffer (22). Cell viability (>85%) was determined by trypan blue exclusion. The cells (5.5 × 10^9/100-mm dish) were plated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) on tissue culture dishes precoated with Matrigel (1.2 mg/100-mm dish; Collaborative Research, Lexington, MA). Matrigel supports the differentiated state of hepatocytes similar to that in the native liver (22). Two hours after plating, the medium was removed, cells were washed gently two times with Dulbecco’s modified Eagle’s medium alone, and treatment medium was added (Dulbecco’s modified Eagle’s medium supplemented with 0.5% fetal bovine serum plus glucose, 5 mM; insulin, 0.14 μg/ml; hydrocortisone, 5 μg/ml; sodium selenite, 0.2 μg/ml; and transferrin, 1 μg/ml).

EGF Treatment and Cell Harvest—Following plating of the cells (24 h), human EGF (50 ng/ml; Life Technologies, Inc.) was added to the medium. Cells were harvested by washing with ice-cold phosphate-buffered saline (twice), scraped into phosphate-buffered saline, and pelleted by centrifugation, and protein extracts were prepared by extraction into a whole cell extract buffer containing HEPES, pH 7.5, 0.3 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM orthovanadate, 0.5 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), and pepstatin (1 μg/ml) for 30 min at 4 °C. Extracted cells were centrifuged at 12,000 × g for 15 min. Supernatants were frozen in aliquots at −70 °C.

Immunoprecipitation and Western Blot Analysis—Specific antibody immunoprecipitation and Western blotting were performed using an immune complex assay (23). Briefly, whole cell extract (50 μg of protein) was immunoprecipitated using specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) bound to protein A-Sepharose beads (Repligen Corp., Needham, MA). Myelin basic protein (Sigma) was used as the substrate for the p44 ERK kinase assay and the N-terminal c-Jun obtained from a GST fusion protein purified on glutathione-agarose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as substrate for p46 JNK kinase. Purified MEK protein (Santa Cruz Biotechnology) was used as substrate for Raf kinase. Activity was visualized by autoradiography of the dried 10% SDS-polyacrylamide gel and quantitation was assessed by PhosphorImager analysis using Molecular Dynamics phosphor imaging equipment.

RESULTS

Age-related Differences in MAP Kinase Activation—In the signal transduction cascade to activate transcription of genes involved in proliferation, ERK-MAP kinase is the final regulatory step that directly interacts with transcriptional regulators. Therefore, we first measured activation of ERK-MAP kinase in response to EGF in hepatocytes isolated from young and old rats. In hepatocytes isolated from young rats, ERK-MAP kinase was maximally activated 15-fold (15 min, Fig. 1). Activation of ERK-MAP kinase was only 5-fold in hepatocytes from old animals (Fig. 1). The activity returned to near base-
Ras GTPase is induced into the active GTP-bound form measured the activity of Raf kinase. Raf kinase is activated by differences in upstream activation of the kinase pathway, we age-dependent decline in ERK-MAP kinase activity was due to not involve MKP-1. The protein level of MKP-1 in both the liver (4.5-fold) and cultured hepatocytes (3-fold) with age. This suggests that the mechanism for MAP kinase activation in response to EGF (13) was previously suggested to be involved in the mechanism for the decreased ERK-MAP kinase activation in response to EGF (13). We measured the protein level of MKP-1 in whole cell extracts from hepatocytes of young and old rats as well as the level in the native liver. Fig. 3 shows that there is actually a decline in the amount of MKP-1 protein in both the liver (4.5-fold) and cultured hepatocytes (3-fold) with age. This suggests that the mechanism for the age-related difference in ERK-MAP kinase activation does not involve MKP-1.

EGF-stimulated Raf Kinase Activity—To determine if the age-dependent decline in ERK-MAP kinase activity was due to differences in upstream activation of the kinase pathway, we measured the activity of Raf kinase. Raf kinase is activated by Ras GTPase (6). Ras is induced into the active GTP-bound form following the coupling of Ras to the EGF receptor via the SOS-GRB2-SHC complex (5). Subsequent to Raf kinase activation, a series of kinases including MEK1 and -2 are activated by phosphorylation leading to activation of ERK-MAP kinase. Raf kinase activity was measured using an immune complex kinase assay with purified MEK as substrate. Raf kinase activity was significantly reduced at 15 min in EGF-stimulated hepatocytes from old animals compared with those from young animals (Fig. 4). Importantly, Raf kinase activity was greater in the young than the old without a change in the protein level, indicating that specific activity of Raf kinase declined with age. These data suggest that age-related changes in the activation of the ERK-MAP kinase pathway may be upstream of Raf kinase.

Total Tyrosine Phosphorylation of the EGF Receptor—Upon activation of the EGF receptor, autophosphorylation of specific receptor tyrosines initiate the signaling cascade by creating phosphotyrosine sites for binding of SH2 domain-containing proteins that are critical for the recruitment of SHC, GRB2, and SOS proteins to the membrane. To establish if age-related differences in Raf kinase activity were due to differences in signaling initiated at the membrane, we determined the amount of tyrosine-phosphorylated EGF receptor by Western blot analysis. As shown in Fig. 5, no age-related differences in the amount of EGF receptor protein nor in the phosphorylation of the receptor upon EGF stimulation were found. Although no age-related changes in total phosphorylation of the EGF receptor were detected, we could not conclude that membrane signaling events are not different with age, since phosphorylation levels at specific sites were not determined by these methods. Therefore, as a functional measure of membrane membrane-coupled signaling, we examined the adapter protein Shc, in response to EGF.

Tyrosine Phosphorylation of Shc and Association with the EGF Receptor—The adapter protein Shc associates with phosphotyrosine residues at positions 1148 and 1173 of the EGF receptor (28, 29). These Shc phosphotyrosine residues create binding sites for the SH2 domain of the adapter protein, Grb2 (30). To determine differences in the age-associated ability to phosphorylate Shc, extracts from hepatocytes isolated from young and old rats were immunoprecipitated with Shc antibody and immunoblotted with a phosphotyrosine antibody. The age-dependent ability to phosphorylate Shc in response to EGF was not different (Fig. 6A), nor was there any age-associated difference in

**Fig. 2.** JNK-MAP kinase activation in response to EGF in hepatocytes from young and old rats. Top panel, representative activity (ACT.) of JNK-MAP kinase after 15, 60, or 120 min of EGF treatment (50 ng/ml) of hepatocytes isolated from young and old rats. The activity was determined by an immune complex kinase assay using the GST fusion protein of N-terminal c-Jun. Middle panel, effect of EGF on JNK-MAP kinase protein levels in primary hepatocytes as detected by Western blot analysis using a JNK-1 antibody. Bottom panel, graphic results of JNK-MAP kinase activity in response to EGF treatment of hepatocytes from three pairs of animals. Results are expressed as the mean ± S.E. and normalized to the average activity of the young controls.

**Fig. 3.** MKP-1 protein levels in hepatocytes and liver from young and old rats. MKP-1 protein was assayed by Western blot analysis of whole cell extracts from hepatocytes or native liver of young or old rats.
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**Fig. 4.** Raf kinase activity in response to EGF treatment in hepatocytes from young and old rats. Top panel, representative activity (ACT.) of Raf kinase after 15 or 60 min of EGF treatment (50 ng/ml) of hepatocytes isolated from young and old rats. The activity was determined by an immune complex kinase assay using MEK-1 as a substrate. Middle panel, effect of EGF on Raf kinase protein levels in primary hepatocytes as detected by Western blot analysis using a Raf-1 antibody. Bottom panel, graphic results of Raf kinase activity in response to EGF treatment of hepatocytes from four pairs of animals. Results are expressed as the mean ± S.E. and normalized to the young control.

**Fig. 5.** Total amount and tyrosine phosphorylation of the EGF receptor in response to EGF in hepatocytes from young and old rats. A, total tyrosine phosphorylation of the EGF receptor was determined by immunoprecipitating the whole cell extracts with the EGF receptor antibody and immunoblotted with an antibody specific for phosphotyrosine. The results shown here are representative of analysis done on three pairs of animals. B, the amount of EGF receptor was analyzed by Western blot using the human EGF receptor antibody. The results shown here are representative of analysis done on three pairs of animals.

Using bacterial fusion proteins of GST-Shc domains was performed. Three domains (shown in Fig. 7A) have been identified in the Shc protein. The N-terminal phosphotyrosine binding (PTB) domain and the C-terminal SH2 domain recognize phosphotyrosine residues of the activated EGF receptor (27, 28). The internal collagen homology domain contains tyrosine residues that are phosphorylated upon activation and create the binding sites for the SH2 domain of Grb2 (29, 30). As shown in Fig. 7B, the amount of EGF receptor “captured” from the extracts of the hepatocytes from old animals is decreased in comparison with the young when using the bacterially expressed PTB domain of the Shc protein. The amount of decreased association between the proteins ranged from 2- to 3-fold in three independent experiments. Tyrosine 1148 has been identified to be a recognition site for binding of the Shc PTB domain (27, 32), and tyrosine 1173 has been reported to be recognized by the Shc-SH2 domain (26, 32) as well as the PTB domain (32). Therefore, it is possible that the EGF receptor may be less phosphorylated specifically at tyrosine 1148 and/or 1173 with age. A difference in the phosphorylation state at these positions could exist and would not have been detectable by Western blot analysis for total phosphotyrosine in the EGF receptor-immunoprecipitated extracts (Fig. 5) due to the abundance of tyrosine phosphorylation on multiple tyrosines on the EGF receptor. Furthermore, due to the low levels of EGF receptor in primary hepatocytes, we could not directly map the amount of phosphotyrosine residues at these specific sites.

**Phosphorylation Level of Tyrosine 1173 on the EGF Receptor**

To determine whether the age-related difference in the ability of Shc to associate with the EGF receptor is due to a difference in level of phosphorylation at a specific site on the EGF receptor, we determined the amount of EGF receptor phosphorylation at tyrosine 1173 by Western blot analysis. Whole cell extracts were immunoprecipitated with the EGF receptor antibody and immunoblotted with an antibody specific for the epitope containing phosphotyrosine 1173 of the EGF receptor. We found decreased levels of EGF receptor phosphotyrosine 1173 in response to EGF in hepatocytes isolated from...
old rats compared with young (Fig. 8).

The results with both direct Shc immunoprecipitation and the GST-Shc capture assay clearly suggest that a major cause of the age-related decrease in ERK-MAP kinase signaling pathway is due to an alteration in the EGF receptor coupling to the membrane signaling machinery. Furthermore, the results of the GST capture experiment (Fig. 7), using the PTB domain of Shc, indicate that the age-related difference is in the activated EGF receptor protein. Western blotting for phosphotyrosine 1173 (Fig. 8) confirms that the age-related difference in the ability of the EGF receptor to associate with Shc is due to a site-specific difference in tyrosine phosphorylation of the EGF receptor.

**DISCUSSION**

The molecular basis of the age-related decrease in the capacity of some tissues to proliferate is not well understood. Cellular proliferation is initiated by growth factor binding to specific receptors to initiate signal transduction. Two models of age-related responses to proliferative stimuli have shown differences in either receptor number and/or receptor phosphorylation (33–35). However, such differences are not found in the hepatocyte model for the liver (3). In a human skin fibroblast model of aging, the EGF receptor number and phosphorylation kinetics were reduced in old compared with young (33, 34). However, the comparison was done in cells derived from different locations i.e. foreskin (young) and forearm (old). This may reflect two very different sources of cells that are influenced by differences in environmental exposures as well as aging. Age-related differences were also reported in tyrosine phosphorylation of the CD3ζ chain of the T-cell receptor (35). Additionally, these age-related differences suggest cell type differences in the mechanism of responses to proliferative stimuli with age. Our results agree with those of Ishigami (3) and show that EGF receptor protein levels are similar, and in addition that total tyrosine phosphorylation of the receptor is similar in hepatocytes from young or old rats. Thus, the age-related differences in EGF-stimulated hepatocyte proliferation do not seem likely to be caused by gross overall differences in receptor number or phosphorylation. These data suggested that the mechanistic age-related differences in the response to proliferative stimuli in hepatocytes may be downstream in the signal transduction pathway.

MAP kinases are key regulators of transcription, since these are the catalysts for phosphorylation of transcription factors regulating proliferation. Blocking the ERK-MAP kinase pathway has been shown to down-regulate the mitogenic response (36). Reduced activity of ERK-MAP kinase in response to EGF was previously identified in hepatocytes isolated from old rats compared with young. The mechanism of this decreased activation was suggested to be due to an increase in the basal RNA expression of the dual specificity phosphatase, MKP-1 (13). MKP-1 functions as a dual site phosphatase to inactivate the dually phosphorylated ERK-MAP kinase (24, 25) and inhibits cell division (37). Our data also show ERK-MAP kinase activity to be diminished in hepatocytes from old animals in response to EGF (Fig. 1). However, rather than measuring MKP-1 RNA expression, we determined the amount of MKP-1 protein and found the levels to be decreased in hepatocytes as well as in the native livers from old rats (Fig. 3), suggesting that MKP-1 is not regulating the age-related decrease in ERK-MAP kinase activity. Further support for the lack of a role for MKP-1 in regulating the decrease in ERK-MAP kinase comes from the work of Shapiro and Ahn (38). They reported that MKP-1 has a positive effect on Raf kinase activity independent of the basal inhibition of ERK-MAP kinase activity. These results are consistent with our own, where we observed a decrease in Raf kinase activation in hepatocytes from old compared with young (Fig. 4), not an increase as would be expected if MKP-1 were increased.

Our results indicate that the age-related difference in the proliferative response to EGF is upstream from ERK-MAP kinase activation. Since Raf kinase activation also declined with age (Fig. 4), we examined the coupling of the EGF receptor to the ERK-MAP kinase signaling pathway. Age-related differences in the membrane complexes involved in signaling have previously been reported in T-lymphocytes (35, 39, 40) and B-cells (41). We have identified an early step in the EGF receptor signal transduction pathway to be a decrease in the association of the adapter protein, Shc, with the activated EGF receptor in hepatocytes from old compared with young (Fig. 6). Furthermore, the results of the GST capture experiment (Fig. 7B), which employed bacterially expressed GST fusion protein containing the PTB domain of Shc, indicate that the age-related difference in hepatocytes is in the activated EGF receptor protein. The PTB domain recognizes the tyrosine phosphoryl-
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Understanding the molecular mechanisms for the age-related differences in the proliferative response to EGF will require more information about the subtle mechanisms that modulate the EGF receptor-ERK signaling pathway. There are certain to be newly discovered regulators that will play a role, and understanding the molecular mechanisms for the age-related differences in response to proliferative stimuli in different tissues will require a more in-depth knowledge of the EGF signaling pathway.

Acknowledgments—We thank Drs. Trudy Kokkonen, Doug Jefferson, and David Johnston for information about hepatocyte isolation and culture conditions; Dr. Ben Neel for helpful discussion of the data; K. Ravichandran for GST-Shc domain constructs; and Drs. Brent Cochran and Larry Feig for reviewing the manuscript.

REFERENCES

1. Bucher, N. L. R., Swaffield, M. N., and DiTroia, J. F. (1984) Cancer Res. 44, 509–512
2. Beyer, H. S., Sherman, R., and Zieve, L. (1991) J. Lab. Clin. Med. 117, 101–108
3. Ishigami, A., Reed, T. D., and Roth, G. S. (1993) Biochem. Biophys. Res. Commun. 196, 181–186
4. Brawley, L., and Downward, J. (1993) Cell 73, 611–620
5. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45–51
6. Arruacho, J., Zhang, X., and Kyriakis, J. M. (1994) Trends Biochem. Sci. 19, 279–283
7. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556
8. Minden, A., Lin, A., Smeal, T., Derijard, B., Cobb, M. D., Davis, J. R., and Karin, M. (1994) Mol. Cell. Biol. 14, 6883–6888
9. Hunter, T. (1995) Cell 80, 225–236
10. Robinson, M. J., and Cobb, M. H. (1997) Curr. Opin. Cell Biol. 9, 180–186
11. Kyriakis, J. M., and Arruacho, J. (1996) J. Biol. Chem. 271, 24313–24316
12. Minden, A., Lin, A., Malagon, M., Lange-Carter, C., Derijard, B., Davis, J. R., Johnson, G. L., and Kain, M. (1994) Science 266, 1719–1723
13. Liu, Y., Guyton, K. Z., Gorospe, M., Xu, Q., Kokkonen, G. C., Mock, Y. D., Roth, P. G., and Holbrook, N. J. (1995) J. Cell. Biol. 127, 6048–6067
14. Logan, S. K., Falasca, M., Hu, P., and Schlessinger, J. (1996) Mol. Cell. Biol. 17, 5784–5790
15. Karin, M. (1995) J. Biol. Chem. 270, 16483–16491
16. Marshall, C. J. (1995) Cell 80, 179–185
17. Hill, C. S., and Treisman, R. (1995) Cell 80, 199–211
18. Whitmarsh, A. J., Shure, P., Sharrocks, A. D., and Davis, R. J. (1995) J. Biol. Chem. 270, 405–407
19. Whitmarsh, A. J., Yang, S., Su, M. S.-S., Sharrocks, A. D., and Davis, R. J. (1997) Mol. Cell. Biol. 17, 2360–2371
20. Liu, Y., Guyton, K. Z., Gorospe, M., Xu, Q., Lee, J. C., and Holbrook, N. J. (1999) Free Radical Biol. Med. 27, 711–718
21. Pagano, M., and Marchio, R. (1996) Methods Cell Biol. 53, 29–83
22. Rana, B., Mischoulon, D., Xie, Y., Bucher, N. L. R., and Farmer, S. R. (1994) Mol. Cell. Biol. 14, 5858–5869
23. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) J. Biol. Chem. 270, 7420–7426
24. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1995) Cell 75, 487–493
25. Chu, Y., Solaki, P. A., Khosravi-Far, B., Der, C. J., and Kelly, K. J. (1996) J. Biol. Chem. 271, 6497–6501
26. Batzer, A. G., Rotin, D., Urena, J. M., Skolkiny, E. Y., and Schlessinger, J. (1994) Mol. Cell. Biol. 14, 5192–5203
27. Batzer, A. G., Blaikie, P., Nelson, K., Schlessinger, J., and Margolis, B. (1995) Mol. Cell. Biol. 15, 4403–4409
28. Pelici, G., Lanfrancone, L., Grigioni, F., McGlade, J., Cavalli, F., Forini, G., Nicoletti, I., Grigioni, F., Pawson, T., and Pelicci, P. G. (1996) Cell 68, 93–104
29. Salcini, A. E., McGlade, J., Pelici, G., Nicoletti, I., Pawson, T., and Pelicci, P. G. (1994) Oncogene 9, 2527–2536
30. Gotto, N., Toyoda, M., and Shibuya, M. (1997) Mol. Cell. Biol. 17, 1824–1831
31. Ricketts, W. A., Rose, D. W., Shoelson, S., and Olefsky, J. M. (1996) J. Biol. Chem. 271, 26165–26169
32. Sakaguchi, K., Okahayashi, Y., Kido, Y., Kimura, S., Maeta, J., and Inushima, K. (1998) Mol. Endocrinol. 12, 536–543
33. Reenstra, W. R., Yaar, M., and Gilchrest, B. A. (1993) Exp. Cell Res. 209, 113–122
34. Reenstra, W. R., Yaar, M., and Gilchrest, B. A. (1996) Exp. Cell Res. 227, 282–295
35. Garcia, G. G., and Miller, R. A. (1997) J. Biol. Chem. 272, 16271–16276
36. Sale, E. M., Atkinson, P. G., and Sale, G. J. (1995) EMBO J. 15, 674–684
37. Brondello, J. M., McKenzie, F. R., Sun, H., Tenks, N. K., and Pousseger, J. (1995) Oncogene 10, 1885–1894
38. Shapira, P. S., and Ahi, N. G. (1998) J. Biol. Chem. 273, 1788–1793
39. Ghosh, J., and Miller, R. A. (1995) Mech. Ageing Dev. 80, 171–187
40. Pahlavani, M. A., Harris, M. D., and Richardson, A. (1998) Cancer Res. 58, 101–108
41. Tridakapani, S., Chacko, G. W., Brocklyn, J. R., and Coggleshall, K. M. (1997) J. Immunol. 158, 1125–1132
42. Gotto, N., Tojo, A., Muruya, K., Hashimoto, Y., Hattori, S., Nakamura, S., Takenawa, T., Yazaki, Y., and Shibuya, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 167–171
43. Sokol, C., Alvarez, C. V., Beguinot, L., and Carpenter, G. (1994) Oncogene 9, 2507–2215
44. Li, N., Schlessinger, J., and Maroglia, B. (1994) Oncogene 9, 3457–3465
45. Huang, G. C., Ouyang, X., and Epstein B. J. (1998) Biochem. J. 331, 113–119
46. Kim, H. H., Vijapurkar, U., Hellyer, N. J., Bravo, D., and Roland, J. G. (1998) Biochem. J. 334, 189–195
47. Alvarez, C. V., Shon, J. K., Milo, M., and Beguinot, L. (1995) J. Biol. Chem. 270, 16271–16276
48. Gallo, R., Provenzano, C., Carbone, R., Di Fiore, P. P., Castellani, L., Falcone, M. E., and Milanese, S. (1997) J. Biol. Chem. 272, 15985–15990
49. Ishigami, A., Reed, T. D., and Roth, G. S. (1993) Biochem. Biophys. Res. Commun. 196, 181–186
Age-dependent Decline in Shc-EGF Receptor Association

49. Xiao, S., Rose, D. W., Sasaoka, T., Maegawa, H., Burke, T. R., Jr., Roller, P. P., Shoelson, S. E., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 21244–21248

50. Shi, Z. Q., Lu, W., and Feng, G. S. (1996) J. Biol. Chem. 273, 4904–4908

51. Bennett, A. M., Hausdorff, S. F., O’Reilly, A. M., Freeman, R. M., Jr., and Neel, B. G. (1996) Mol. Cell Biol. 16, 1189–1202

52. Keilhack, H., Tenev, T., Nyakatura, E., Godovac-Zimmermann, J., Nielsen, L., Seedorf, K., and Bohmer, F.-D. (1998) J. Biol. Chem. 273, 24839–24846

53. Lim, Y. P., Low, B. C., Ong, S. H., and Guy, G. R. (1997) J. Biol. Chem. 272, 29892–29898

54. Therrien, M., Michaud, N. R., Rubin, G. M., and Morrison, D. K. (1996) Genes Dev. 10, 2684–2695

55. Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X.-H., Basu, S., McGinley, M., Chan-Hui, P.-Y., Lichenstein, H., and Kolesnick, R. (1997) Cell 89, 63–72

56. Michaud, N. R., Therrien, M., Cacace, A., Edsall, L. C., Spiegel, S., Rubin, G. M., and Morrison, D. K. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 12792–12796

57. Okabayashi, Y., Kido, Y., Okutani, T., Sugimoto, Y., Sakaguchi, K., and Kasuga, M. (1994) J. Biol. Chem. 269, 19674–19678