REFERENCES

Abo, A. et al. 1991. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. Nature (London). 353: 668-670.

Adamson, P. et al. 1999. Lymphocytes migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a rho-dependent pathway. J. Immunol. 162: 2964-2973.

Akasaki, T. et al. 1999. Phosphoinositide 3-kinase-dependent and –independent activation of the small GTPase Rac2 in human neutrophils. J. Biol. Chem. 274: 18055-18059.

Ahmad, I., and Suhall, M. 1993. Erythrocyte membrane stabilization by indomethacin. Indian J. Pharmacol. 25: 173.

Allan, V.J. 2000. Protein Localization by Fluorescence Microscopy: A Practical Approach. New York: Oxford University Press. pp. 1-26.

Allen, W.E. et al. 1998. A role for Cdc42 in macrophage chemotaxis. J. Cell Biol. 141: 1147-1157.

Amano, M. et al. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. 271: 20246-20249.

Anand-Apte, B. et al. 1997. Platelet-derived growth factor and fibronectin-stimulated migration are differentially regulated by the Rac and extracellular signal-regulated kinase pathways. J. Biol. Chem. 272: 30688-30692.

Aposhian, H.V. 1989. Biochemical Toxicology of Arsenic. New York, Elsevier Science. pp. 1-50.

Arber, S. et al. 1998. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature (London). 393: 805-809.

Aspenstrom, P. 1999. Effectors for the Rho GTPases. Curr. Opin. Cell Biol. 11: 95-102.

Atfi, A. et al. 1997. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth-factor beta-mediated signaling. J. Biol. Chem. 272: 1429-1432.

Auer, K.L. et al. 1998. The Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade is a key pathway by which agonist stimulate DNA synthesis in primary cultures of rat hepatocytes.

Mol. Biol. Cell. 9: 561-573.

Bae, Y.S. et al. 1997. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. J. Biol. Chem. 272: 217-221.

Bagrodia, S. et al. 1995. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J. Biol. Chem. 270: 27995-27998.

Bagrodia, S. et al. 1995. Identification of a mouse p21Cdc42/Rac activated kinase. J. Biol. Chem. 270: 22731-22737.

Bamburg, J.R. et al. 1999. Putting a new twist on actin: ADF/cofilins modulate actin dynamics. Trends Cell Biol. 9: 364-370.

Barchowsky, A. et al. 1996. Arsenic induces oxidant stress & NF-B activation in cultured aortic endothelial cells. Free Radical Biol. Med. 21: 783-790.

Bauhl, A.M. et al. 1995. G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly. J. Biol. Chem. 270: 24631-24634.

Baxley, M.N. et al. 1981. Prenatal toxicity of orally administered sodium arsenite in mice. Bull. Environ. Contam. Toxicol. 26: 749-756.

Bellot, F. et al. 1990. High-affinity epidermal growth factor binding is specifically reduced by a monoclonal antibody, and appears necessary for early responses. J. Cell Biol. 110: 491-502.

Benard, V. et al. 1999. Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J. Biol. Chem. 274: 13198-13204.

Bencko, V. et al. 1978. Effects of the combined action of selenium & arsenic on mice versus suspension culture of mice fibroblasts. Excerpta. Medica. 1: 312-316.

Bertoledo, F. et al. 1987. Cellular uptake and metabolic reduction of pentavalent to trivalent arsenic as determinants of cytotoxicity and morphological transformation. Carcinogenesis. 8: 803-808.

Biscardi, J.S., et al. 1999. s-Src-mediated phosphorylation of the epidermal growth factor on Tyr 845 and Tyr 1101 is associated with modulation of receptor function. J.Biol.Chem. 274: 8335-8343.

Biscardi, J.S., Tice, D.A., and Parsons, S.J. 1999. c-Src, receptor tyrosine kinase, and human cancer. Adv. Cancer Res. 76: 61-119.

Bishop, A.L., and Hall, A. 2000. Rho GTPases and their effector proteins. Biochem. J. 348: 241-255.

Blaikie, P. et al. 1994. A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptor. J. Biol. Chem. 269: 32031-32034.

Blankley, B.R. et al. 1980. The effect of methylmercury, tetraethyl lead, and sodium arsenite on the humoral immune response in mice. Toxicol. Appl. Pharmacol. 52: 245-254.

Boonstra, J. et al. 1995. The epidermal growth factor. Cell Biol. Int. 19: 413-430.

Bresnick, A.R. 1999. Molecular mechanism of nonmuscle myosin-II regulation. Curr. Opin. Cell Biol. 11: 26-33.

Bretscher, A. 1999. Regulation of cortical structure by the ezrin-radixin-moesin protein family. Curr. Opin. Cell Biol. 11: 109-116.

Brown, J.L. et al. 1996. Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. Curr. Biol. 6: 598-605.

Burgaud, J.L., and Baserga, R. 1996. Intracellular transactivation of the insulin-like growth factor I receptor by an epidermal growth factor receptor. Exp. Cell Res. 223: 412-419.

Burke, C.L., and Stern, D.F. 1998. Activation of Neu (Erb-2) mediated by disulfide bond-induced dimerization reveals a receptor tyrosine kinase dimer interface. Mol. Cell Biol. 18: 5371-5379.

Burleson, F.G. et al. 1996. Dermatotoxic chemical stimulate of c-jun and c-fos transcription and AP-1 binding in human keratinocytes. Res. Commun. Molec. Pathol. Pharmacol. 93: 131-48.

Burstamante, J. et al. 1997. The semiconductor arsenic and indium induce apoptosis in rat thymocytes. Toxicology. 118: 129-136.

Bustelo, X.R. 1996. The VAV family of signal transduction molecules. Crit. Rev. Oncog. 7: 65-88.

Calautti, E. et al. 2002. Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocytes cell-cell adhesion. J. Cell Biol. 156: 137-148.

Campion, S.R. et al. 1990. Biochemical properties of site-directed mutants of human epidermal growth factor: importance of solvent-exposed hydrophobic residues

of the amino-terminal domain in receptor binding. Biochem. 29: 9988-9993.

Cantrell, D. 1998. Lymphocyte signaling: A coordinating role of Vav? Curr. Biol. 8: R535-R538.

Carmignami, M. et al. 1985. Metabolic fate and cardiovascular effects of arsenic in rats and rabbits chronically exposed to trivalent and pentavalent arsenic. Arch. Toxicol. Suppl. 8: 452-455.

Carpenter, S.J. 1987. Developmental analysis of cephalic axial dysraphic disorders. Anat. Embryo. 176: 345-365.

Castrillon, D.H., and Wasserman, S.A. et al. 1994. Diaphanous is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the limb deformity gene. Development. 120: 3367-3377.

Cebrian, M.E. et al. 1988. Assessment of arsenic effects on cytosolic heme status using tryptophan pyrrolase as an index. J. Biochem. Toxicol. 3: 77-86.

Centeno, J.A. et al. 2002. Pathology related to chronic arsenic exposure. Environ. Health Perspect. 110 Suppl 5: 883-886.

Geuens, G. et al. 1986. Ultrastructural colocalization of tyrosinated and detyrosinated alpha-tubulin in interphase and mitotic cells. J. Cell Biol. 103: 1883-1893.

Cell Signaling Technology. 2003. Immunoblotting. New York: Cell Signaling Technology, Inc.

Chakravortty, D., and Nanda Kumar, K.S. 2000. Bacterial lipopolysaccharide induces cytoskeletal rearrangement in small intestinal lamina propria fibroblasts: actin assembly is essential for lipopolysaccharide signaling. Biochim. Biophys. Acta. 1500: 125-136.

Chan, D.C. et al. 1996. Formin binding proteins bear WWP/WW domains that bind proline-rich peptides and functionally resemble SH3 domains. EMBO J. 15: 1045-1054.

Chang, W.C. et al. 1991. Cytoprotective effect of reduced glutathione in arsenical-induced endothelial cell injury. Toxicology. 69: 101-110.

Chanock, S.J. et al. 1994. The respiratory burst oxidase. J. Biol. Chem. 269: 24519-24522.

Chapman, E.R. et al. 1996. A novel function for the second C2 domain of synaptotagmin, Ca^{2+} -triggered dimerization. J. Biol. Chem. 271. 5844-5849.

Cheatham, B. et al. 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70S6 kinase, DNA synthesis, and glucose transporter translocation. Mol. Cell. Biol. 14: 4902-4911.

Chen, K. et al. 2001. c-Jun N-Terminal kinase activation by hydrogen peroxide in endothelial cells involves Src-dependent epithelial growth factor receptor transactivation. J. Biol. Chem. 276: 16045-16050.

Chen, L.Y. et al. 2002. IL-1 receptor-associated kinase and low molecular weight GTPase RhoA signal molecules are required for bacterial lipopolysaccharide -induced cytokine gene transcription. J. Immunol. 169: 3934-3939.

Chong, L.D. et al. 1994. The small GTP-binding protein Rho regulates a phosphatidyl-inositol 4-phosphate 5-kinase in mammalian cells. Cell. 79: 507-513.

Cohen, S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new born animal. J. Biol. Chem. 237: 1555-1562.

Collins, L.R. et al. 1996. Galpha12 stimulates c-Jun NH2-terminal kinase through the small G proteins Ras and Rac. J. Biol. Chem. 271: 17349-17353.

Cold Spring Harbor Laboratory. 1999. Cells: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Press.

Cooper, J.A. et al. 1986. Tyr 527 is phosphorylated in pp60c-Src: implications for regulation. Science. 231: 1431-1434.

Coso, O.A. et al. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell. 81: 1137-1146.

Crespo, P. et al. 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature. 385: 169-172.

Damer, C.K., and Cruetz, C.E. 1994. Synergistic membrane interactions of the two C2 domains of synaptotagmin. J. Biol. Chem. 269. 31115-31123.

Daniels, R.H. et al. 1998. Membrane targeting of p21-activated kinase (PAK1) induces neurite outgrowth from PC12 cells. EMBO J. 17: 754-764.

Davis, W. et al. 1999. Synergistic activation of JNK/SAPK by interleukin-1 and

platelet-derived growth factor is independent of Rac and Cdc42. Biochem. J. 338: 387-392.

Das, T. et al. 1993. Modification of clastogenicity of three known clastogens by garlic extract in mice in vivo. Environ. Mol. Mutagen. 21: 383-388.

Daub, H. et al. 1996. Role of transactivation of the EGF receptor in signaling by G-protein-coupled receptors. Nature. 379: 557-560.

Daub, H. et al. 1997. Signal characteristics of G protein-transactivated EGF receptor. EMBO J. 16: 7032-7044.

Deknudt, G. et al. 1986. In vivo studies in male mice on the mutagenic effects of inorganic arsenic. Mutagenesis. 1: 33-34.

Delfize, L.H.K. et al. 1989. Signal transduction by epidermal growth factor occurs through the subclass of high affinity receptors. J. Cell Biol. 109: 2495-2507.

Delnomdedieu, M. et al. 1993. Transfer of arsenite from glutathione to dithiols: a model of interaction. Chem. Res. Toxicol. 6: 598-602.

Delnomdedieu, M. et al. 1994a. Reduction of binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study. Chem. Biol. Interact. 90: 139-155.

Delnomdedieu, M. et al. 1994b. Complexation of arsenic species in rabbit erythrocytes. Chem. Res. Toxicol. 7: 621-627.

Delta Rocca, G.T. et al. 1997. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. J. Biol. Chem. 272: 19125-19132.

Den Hartigh, J.C. et al. 1992. The EGF receptor is an actin-binding protein. J. Cell. Biol. 119: 349-355.

Desrivieres, S. et al. 1998. MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in *Saccharomyces cerevisea*. J. Biol. Chem. 273: 15787-15793.

Devarajan, P. et al. 1994. Ankyrin binds to two distinct cytoplasmic domains of Na,K-ATPase alpha subunit. Proc. Natl. Acad. Sci. USA. 91: 2965-2969.

Dharmawardhane, S. et al. 1997. Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. J. Cell Biol. 138: 1265-1278.

Diekmann, D. et al. 1994. Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. Science. 265: 531-533.

Done, A.K., and Peart, A.J. 1971. Acute toxicities of arsenical herbicides. Clin. Toxicol. 4: 343-355.

Drechsel, D.N. et al. 1997. A requirement for Rho and Cdc42 during cytokinesis in *Xenopus* embryos. Curr. Biol. 7: 12-23.

Dröge, W. 2002. Free radicals in the physiological control of cell finction. Physiol. Rev. 82: 47-95.

Edwards, D.C. et al. 1999. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signaling to actin cytoskeletal dynamics. Nat. Cell Biol. 1: 253-259.

Eguchi, S. et al. 2001. Activation of MAP kinase by angiotensin II in vascular smooth muscle cells: metaloproteinase-dependent EGF receptor activation is required for activation of ERK and p38 MAP kinase, but not for JNK. J. Biol. Chem. 276: 7957-7962.

Egugh, S. et al. 1998. Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. J. Biol. Chem. 273: 8890-8896.

Engler, D.A. 1988. Cloning of authentic human epidermal growth factor as a bacterial secretory protein and its initial structure-function analysis by site-directed mutagenesis. J. Biol. Chem. 263: 12384-12390.

Engler, D.A. 1990. Human epidermal growth factor, Distinct roles of tyrosine 37 and arginine 41 in receptor binding as determined by site-directed mutagenesis and nuclear magnetic resonance spectroscopy. FEBS Lett. 271: 47-50.

Fan, W.T. et al. 1998. The exchange factor Ras-GRF2 activates Ras-dependent and Rac-dependent mitogen-activated protein kinase pathways. Curr. Biol. 8: 935-938.

Fanger, G.R. et al. 1997. MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. EMBO J. 16: 4961-4972.

Feng, S. et al. 1994. Two binding orientations for peptides to the Src SH3 domain: Development of a general model for SH3-ligand interactions. Science. 266: 1241-1247.

Fischer, K.D. et al. 1998. Vav is a regular of cytoskeletal reorganization mediated by the T-cell receptor. Curr. Biol. 8: 554-562.

Flanagan, J.G., and Vanderhaeghen, P. 1998. The ephrins and Eph receptors in neural development. Annu. Rev. Neurosci. 21: 309-345.

Fleming, I.N. et al. 1999. Ca2+/calmodulin-dependent protein kinase II regulated Tiam1 by reversible protein phosphorylation. J. Biol. Chem. 274: 12753-12758.

Franke, K.W., and Moxon, A.L. 1936. A comparison of the minimum fatal doses of selenium, tellurium, arsenic and vanadium. J. Pharm. Exp. Ther. 58: 454-459.

Fukata, et al. 1999. Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. J. Cell Biol. 145: 347-361.

Ganju, P. et al. 2001. p38 stress-activated protein kinase inhibitor reverses bradykinin B(1) receptor-mediated component of inflammatory hyperalgesia. Eur. J. Pharmacol. 421: 191-199.

Garcia-Vargas, G. et al. 1995. Time-dependent porphyric response in mice subchronically exposed to arsenic. Hum. Exp. Toxicol. 14: 475-483.

Germolec, D.R. et al. 1997. Arsenic can mediate skin neoplasia by chronic stimulation of keratinocyte-derived growth factors. Mutat Res. 386: 209-281.

Gilmore, A.P., and Burridge, K. et al. 1996. Regulation of vinculin binding to talin and Actin by phosphatidylinositol 4,5-bisphosphate. Nature (London). 381: 531-535.

Glise, B., and Noselli, S. 1997. Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in Drosophila morphogenesis. Genes Dev. 11: 1738-1747.

Gohla, A. et al. 1998. The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor to Rho. J. Biol. Chem. 273: 4653-4659.

Gohla, A. et al. 1999. Differential involvement of Galpha12 and Galpha13 in receptor-Mediated stress fiber formation. J. Biol. Chem. 274: 17901-17907.

Griendling, K.K. et al. 2000. NAD(P)H oxidase. Role in cardiovascular biology and disease. Circ. Res. 86: 494-501.

Groves, R.W. et al. 1992. Abnormal expression of epidermal growth factor receptor in cutaneous epithelial tumours. J. Cutan. Pathol. 19: 66-72.

Hackel, P.O. et al. 1999. Epidermal growth factor receptors: critical mediators of multiple receptors pathways. Curr. Opin. Cell Biol. 11: 184-189.

Han, J. et al. 1997. Lck regulates Vav activation of members of the Rho family of GTPases. Mol. Cell. Biol. 17: 1346-1353.

Han, J. et al. 1998. Role of substrates and products of PI3-K in regulating activation of Rac-related guanosine triphosphatases by Vav. Science. 279: 558-560.

Harlan, J.E. et al. 1994. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. Nature. 371: 168-170.

Hart, M.J. et al. 1998. Direct stimulation of the guanine nucleotide exchange activity of P115 RhoGEF by Galpha13. Science. 280: 2112-2114.

Hawkins, P.T. et al. 1995. PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. Curr. Biol. 5: 393-403.

Heeneman, S. et al. 2000. Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor β receptor. J Biol. Chem. 275: 15926- 15932.

Heinrich, P.C. et al. 1998. Interleukin-6-type cytokine signaling through the gp130/Jak/STAT pathway. Biochem. J. 334: 297-314.

Heldin, C.H. 1995. Dimerization of cell surface receptors in signal transduction. Cell. 80: 213-223.

Hill, C.S. et al. 1995. The Rho family GTPases RhoA, Rac1, and Cdc42Hs regulate transcriptional activity by SRF. Cell. 81: 1159-1170.

Hirata, K. et al. 1992. Involvement of rho p21 in the GTP-enhanced calcium ion Sensitivity of smooth muscle contraction. J. Biol. Chem. 267: 8719-8722.

Holgado-Madruga, M. et al. 1996. A Grb2-associated docking protein in EGF- and insulin-receptor signaling. Nature. 379: 560-564.

Holland, S.J. et al. 1998. Cell-contact-dependent signaling in axon growth and guidance: Eph receptor protein tyrosine phoaphatase beta. Curr. Opin. Neurobio. 8: 117-127.

Holsinger, L.J. et al. 1998. Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocytes signal transduction. Curr. Biol. 8: 563-572.

Honda, K. et al. 1992. Heat shock proteins in human and mouse embryonic cells after exposure to heat shock or teratogenic agents. Teratogen Carcinog. Mutagen. 11: 235-244.

Hood, R.D., and Harrison, W.P. 1982. Effects of prenatal arsenite exposure in the hamster. Bull. Environ. Contam. Toxicol. 29: 671-678.

Hooley, R. et al. 1996. G alpha 13 stimulates Na+-H+ exchange through distinct Cdc42-Dependent and RhoA-dependent pathways. J. Biol. Chem. 271: 6152-6158.

Hsuan, J.J., and Tan, S.K. 1997. Growth factor-dependent phosphoinositide signaling. Int. J. Biochem. Cell Biol. 29: 415-435.

Hubbard, S.R., and Till, S.R. 2000. Protein tyrosine kinase structure and function. Annu. Rev. Biochem. 69: 373-398.

Ihle, J.N. et al. 1998. Signaling by the cytokine receptor superfamily. Ann. N.Y. Acad. Sci. 865: 1-9.

Imamura, H. et al. 1997. Bni1p and Bnr1p; downstream targets of the Rho family Small G-proteins with interact with profilin and regulate actin cytoskeleton in Saccharomyces cerevisiae. EMBO J. 16: 2745-2755.

Irvine, R., and Cullen, P. 1996. Inositol phosphates-whither bound. Curr. Biol. 6: 537-540.

Ishizaki, T. et al. 1996. The small GTP-binding protein Rho binds to an activates a 160 KDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 15: 1885-1893.

Ishizaki, T. et al. 1997. p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Lett. 404: 118-124.

Iwamoto, R., and Mekada, E. 2000. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. Cytokine Growth Factor Rev. 11: 335-344.

Janmey, P.A., and Stossel, T.P. 1987. Modulation of gelsolin function by phosphatidylinositol 4,5-biphosphate. Nature (London). 325: 362-364.

Jones, P.L. et al. 1997. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. J Cell Biol. 139: 279-293.

Jost, M. et al. 2001. Epidermal growth factor receptor-dependent control of keratinocyte

survival and Bcl-xL expression through a MEK-dependent pathway. J. Biol. Chem. 276: 6320-6326.

Kalmes, A. et al. 2000. Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. Cir. Res. 87: 92-98.

Kalmes, A. et al. 2001. EGFR transactivation in the regulation of SMC function. Ann. N.Y. Acad. Sci. 947: 42-55.

Kamm, K.E., and Stull, J.T. 2001. Dedicated myosin light chain kinases with diverse cellular functions. J. Biol. Chem. 276: 4527-4530.

Karb, G. 1999. Cell and Molecular Biology. 2nd ed. New York: John Wiley&Sons, Inc. pp. 655-699.

Katoh, H. et al. 1998. Constitutively active Galpha12, Galpha13, and Galphaq induce Rho-dependent neurite retraction through different signaling pathways. J. Biol. Chem. 273: 28700-28707.

Katoh, K. et al. 2001. Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. Am. J. Physiol. Cell Physiol. 280: C1669-C1679.

Katoh, K. et al. 2001. Rho-kinase-mediated contraction of isolated stress fibers. J. Cell Biol. 153: 569-584.

Kato, M. et al. 1987. Altered signal transduction in erb-transformed cells. Implication of enhanced inositol phospholipid metabolism in erbB-induced transformation. J. Biol. Chem. 262: 5696-5704.

Kavanaugh, W.M. et al. 1995. PTB domain binding to signaling proteins through sequence motif containing phosphotyrosine. Science. 268: 1177-1179.

Kavanaugh, W.M., and Williams, L.T. 1994. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science. 266: 1862-1865.

Kawano, Y. et al. 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. J. Cell. Biol. 147: 1023-1038.

Keely, P.J. et al. 1997. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. Nature. 390: 632-636.

Khosravi-Far, R. et al. 1995. Activation of Rac1, RhoA, and mitogen-activated protein kinase is required for Ras transformation. Mol. Cell Biol. 15: 6443-6453.

Kieser, A. et al. 1999. LMP1 signal transduction differs substantially from TNF receptor 1 signaling in the molecular functions of TRADD and TRAF2. EMBO J. 18: 2511-2521.

Kimura, K. et al. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science. 273: 245-248.

Kimura, K. et al. 1998. Regulation of the association of adducin with actin filaments by Rho-associated kinase (Rho-kinase) and myosin phosphatase. J. Biol. Chem. 273: 5542-5548.

Kishi, K. et al. 1993. Regulation of cytoplasmic division of Xenopus embryo by rho p21 and it inhibitory GDP/GTP exchange protein (rho GDI). J. Cell Biol. 120: 1187-1195.

Kiyono, M. et al. 1999. G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25 (Mm). Proc. Natl. Acad. Sci. USA. 96: 4826-4831.

KjØller, L., and Hall, A. et al. 1999. Signaling to Rho GTPases. Exp. Cell Res. 253: 166-179.

Klemke, R.L. et al. 1994. Receptor tyrosine kinase signaling required for integrin alpha v beta 5-directed cell motility but not adhesion on vitronectin J. Cell Biol. 127: 859-866.

Kmiecik, T.E., and Shalloway, D. 1987. Activation and suppression of pp60c-Src transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell. 49: 65-73.

Knaus, U.G. et al. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac2. Science. 254: 1512-1515.

Kochhar, T.C. et al. 1996. Effect of trivalent and pentavalent arsenic in causing chromosome alterations in cultured Chinese hamster ovary (CHO) cells. Toxicol. Lett. 84: 37-42.

Koide, H. et al. 1992a. Recognition of an antiparallel b-sheet structure of human epidermal growth factor by its receptor. FEBS Lett. 302: 39-42.

Koide, H. et al. 1992b. A site-directed mutagenesis study on the role of isoleucine-23 of human growth factor in the receptor binding. Biochim. Biophys. Acta.

1120: 257-261.

Kosako, H. et al. 1997. Phosphorylation of glial fibrillary acidic protein at the same sites by cleavage furrow kinase and Rho-associated kinase. J. Biol.Chem. 272: 10333-10336.

Kozasa, T. et al. 1998. p115RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. Science. 280: 2109-2111.

Kozma, R. et al. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol. Cell. Biol. 15: 1942-1952.

Krasilnikov, M.A. 2000. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. Biochemistry (Mosc). 65: 59-67.

Krauss, G. 2001. Biochemistry of Signal Transduction and Regulation. 2nd ed. New York: John Wiley&Sons, Inc. pp. 187-192.

Kuriyama, J., and Cowburn, D. 1997. Molecular peptide recognition domains in eukaryotic signaling. Annu. Rev. Biophys. Struct. 26: 259-288.

Kuwada, S.K., and Li, X. 2000. Integrin alpha5/beta1 mediates fibronectin-dependent epithelial cell proliferation through epidermal growth factor receptor activation. Mol. Biol. Cell. 11: 2485-2496.

Kyriakis, J.M., and Avruch, J. 2001. Mammalian mitogen-activated protien kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. 81: 807-869.

Larramendy, M.L. et al. 1981. Induction by inorganic metals salts of sister chromatid exchanges and chromosome aberrations in human and Syrian hamster cell strains. Environ. Mutagen. 3: 597-606.

Laudanna, C. et al. 1996. Role of Rho in chemoattractant-activated leukocytes adhesion through integrins. Science. 271: 981-983.

Leberer, E. et al. 1992. The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein βα subunits to downstream signaling components. EMBO J. 11: 4815-4824.

Lee, T.C. et al. 1985. Comparison of arsenic-induced cell transformation, cytotoxicity,

mutation and cytogenetic effects in Syrian hamster embryo cells in culture. Carcinogenesis. 6: 1421-1426.

Lee, T.C. et al. 1986. Differential effects of pre- and posttreatment of sodium arsenite on the genotoxicity of methyl methanesulfonate in Chinese hamster ovary cells. Cancer Res. 46: 1854-1857.

Lemmon, M.A., and Ferguson, K.M. 1998. Pleckstrin homology domains. Curr. Top. Microbio. Immuno. 228: 39-74.

Lerman, S.A. et al. 1983. Arsenic uptake and metabolism by liver cells is dependent on arsenic oxidation state. Chem. Biol. Interactions. 45: 401-406.

Leung, T. et al. 1996. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the organization of the cytoskeleton. Mol. Cell Biol. 16: 5313-5327.

Li, C. et al. 1995. Ca^{2+} -dependent and –independent activities of neural and non-neural synaptotagmins. Nature. 375: 594-599.

Li, J. et al. 1999. Integrin-mediated migration of murine B82L fibroblasts is depended on the expression of an intact epidermal growth factor receptor. J. Biol. Chem. 274: 11209-11219.

Li, Y.M., and Broome, J.D. 1999. Arsenic targets tubulins to induce apoptosis in myeloid leukemia cells. Cancer Res. 59: 776-780.

Lopez, S. et al. 1990. Structually based, selective interaction of arsenite with steroid receptors. J. Biol. Chem. 265: 16039-16042.

Lopez-Ilasaca, M. et al. 1998. Phosphoinositide 3-kinase gamma is a mediator of Gbeta-gamma-dependent Jun kinase activation. J. Biol. Chem. 273: 2505-2508.

Lu, W. et al. 1997. Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. Curr. Biol. 7: 85-94.

Luttrell., L.M. et al. 1997. G-protein-coupled receptors and their regulation: activation of the MAP kinase signaling pathway by G-protein-coupled receptors. Adv. Second Messenger Phosphoprotein Res. 31: 263-277.

Luttrell., L.M. et al. 1997. Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. J. Biol. Chem. 272: 4637-4644.

Ma, A.D. et al. 1998. Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase gamma, a Rac guanosine exchange factor, and Rac. Mol. Cell. Biol. 18: 4744-4751.

Mabuchi, I. et al. 1993. A rho-like protein in the organization of the contractile ring in dividing sand dollar eggs. Zygote. 1: 325-331.

Machesky, L.M., and Insall, R.H. 1999. Signaling to actin dynamics. J. Cell Biol. 146: 267-272.

Madaule, P. et al. 1995. A novel partner for the GTP-bound forms or rho and rac. FEBS Lett. 377: 243-248.

Madaule, P. et al. 1998. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. Nature (London). 394: 491-494.

Maekawa, M. et al. 1999. Signaling from Rho to the actin cytoskeleton through protein kinase ROCK and LIM-kinase. Science. 285: 895-898.

Malcolm, K.C. et al. 1994. Activation of rat liver phospholipase D by the small GTP-binding protein RhoA. J. Biol. Chem. 269: 25951-25954.

Malliri, A. et al. 1998. The transcription factor AP-1 is required for EGF-induced activation of rho-like GTPases, cytoskeletal rerrangements, motility, and in vitro invasion of A431 cells. J. Cell Biol. 143: 1087-1099.

Manser, E. et al. 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature (London). 367: 40-46.

Manser, E. et al. 1995. Molecular cloning of a new member of the p21-Cdc42/Rac activated kinase (PAK) family. J. Biol. Chem. 270: 25070-25078.

Mao, J. et al. 1998. Guanine nucleotide exchange factor GEF115 specifically mediates activation of Rho and serum response factor by the G protein alpha subunit Galpha13. Proc. Natl. Acad. Sci. USA. 95: 12973-12976.

Martinez, G. et al. 1983. Urinary urophyrin as an indicator of arsenic exposure in rats. Proc. West. Pharm. Soc. 26: 171-174.

Matsui, T. et al. 1996. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J. 15: 2208-2216.

Matsui, T. et al. 1998. Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association.

J. Cell Biol. 140: 647-657.

Matsui, T. et al. 1999. Activation of ERM proteins in vivo by Rho involves phosphatidylinositol-4-phosphate 5-kinase and not ROCK kinases. Curr. Biol. 9: 1259-1262.

Michiels, F. et al. 1995. A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature. 375: 338-340.

Michiels, F. et al. 1997. Regulated membrane localization of Tiam1, mediated by the NH2-terminal pleckstrin homology domain, is required for Rac-dependent Membrane ruffling and C-Jun NH2-terminal kinase activation. J. Cell Biol. 137: 387-398.

Minden, A. et al. 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell. 81: 1147-1157.

Missy, K. et al. 1998. Lipid products of phosphoinositide 3-kinase interact with Rac1 GTPase and stimulate GDP dissociation. J. Biol. Chem. 273: 30279-30286.

Mitamura, T. et al. 1995. Diptheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diptheria toxin receptor and inhibits specifically its mitogenic activity. J. Biol. Chem. 270: 1015-1019.

Mohammadi, M. et al. 1993. Aggregation-induced activation of the epidermal growth factor receptor protein tyrosine kinase. Biochemistry. 32: 8742-8748.

Moore, M.M. et al. 1997. Relative genotoxic potency of arsenic and its methylated metabolites. Mutat. Res. 386: 279-290.

Moro, L. et al. 1998. Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. EMBO. J. 17: 6622-6632.

Murasawa, S. et al. 1998. Angiotensin II type I receptor-induced extracellular signal-regulated protein kinase activation is mediated by Ca2+/ calmodulin-dependent transactivation of epidermal growth factor receptor. Circ. Res. 82: 1338-1348.

Muro, A.F. et al. 2000. Mild spherocytic hereditary elliptocytosis and altered levels of α- and γ-adducins in β-adducin-deficient mice. Blood. 95: 3978-3985.

Nada, S. et al. 1991. Cloning of a complementary DNA for a protein-tyrosine kinase that

specifically phosphorylates a negative regulatory site of p60c-Src. Nature. 351: 69-72.

Nakano, K. et al. 1999. Distinct actions and cooperative roles of ROCK and mDia in Rho small G protein-induced reorganization of the actin cytoskeleton in Madin-Darby canine kidney cells. Mol. Biol. Cell. 10: 2481-2491.

Neet, K. and Hunter, T. 1996. Vertebrate non-receptor protein-tyrosine kinase families. Genes Cells. 1: 147-169.

Neiger, R.D., and Osweiler, G.D. 1989. Effect of subacute low level dietary sodium arsenite on dogs. Fundam. Appl. Toxicol. 13: 439-451.

Nimnual, A.S. et al. 1998. Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. Science. 279: 560-563.

Nisimoto, Y. et al. 1997. Rac binding to p67(phox). Structural basis for interactions of Rac1 effector region and insert region with components of the respiratory burst oxidase. J. Biol. Chem. 272: 18834-18841.

Nobes, C.D., and Hall, A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell. 81: 53-62.

Nobes, C.D., and Hall, A. 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. 144: 1235-1244.

Nobes, C.D. et al. 1995. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J. Cell Sci. 108: 225-233.

Norman, R.I., and Lodwick, D. 1999. Medical Cell Biology. 1st ed. New York. Churchill Livingstone. pp. 54-62.

Northwood, I.C., and Davis, R.J. 1988. Activation of the epidermal growth factor receptor tyrosine protein kinase in the absence of receptor oligomerization. J. Biol. Chem. 263: 7450-7453.

Oberly, T.J. et al. 1982. Mutagenicity of metal salts in the L5178Y mouse lymphoma assay. J. Toxicol. Environ. Health. 9: 367-376.

Ochi, T. et al. 1996. Dimethylarsinic acid causes apoptosis in HL-60 cells via interaction with glutathione. Arch. Toxicol. 70: 815-821.

Okada, T. et al. 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced

glucose transport and antilipolysis in rat adipocytes studies with selective inhibitor wortmannin. J. Biol. Chem. 269: 3568-3573.

Okui, T., and Fujiwata, Y. 1986. Inhibition of human excision DNA repair by inorganic arsenic and the co-mutagenic effect in V79 Chinese hamster cells. Mutat. Res. 172: 69-76.

Olson, M.F. et al. 1995. An essential role of Rho, Rac, and Cdc42 GTPases in cell cycle progression through G_1. Science. 269: 1270-1272.

Olson, M.F. et al. 1997. Distinct roles for DH and PH domains in the Lbc oncogene. Oncogene. 15: 2827-2831.

Oshikawa, S. et al. 2001. Long-term changes in severity of arsenical skin lesions following intervention to reduce arsenic exposure. Env. Sci. 5: 435-438.

Pan, Z.K. et al. 1998. Role of the Rho GTPase in bradykinin-stimulated nuclear factor-kappaB activation and IL-1beta gene expression in cultured human epithelial cells. J. Immunol. 160: 3038-3045.

Pawson, T. 1995. Protein modules and signaling networks. Nature. 373: 573-580.

Payrastre, B. et al. 1991. Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: effect of epidermal growth factor. J. Cell Biol. 115: 121-128.

Peppelenbosch, M.P. et al. 1995. Rac mediates growth factor-induced arachidonic acid release. Cell. 81: 849-856.

Peters, R.A. 1995. Biochemistry of some toxic agents. I. Present state of knowledge of biochemical lesions induced by trivalent arsenical poisoning. Bull. Johns Hopkins Hosp. 97: 1-20.

Peter, M. et al. 1996. Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast. EMBO J. 15: 7046-7059.

Pietenpol, J.A. et al. 1990. Transforming growth factor 1 suppression of *c-myc* gene transcription: role in inhibition of keratinocyte proliferation. Proc. Natl. Acad. Sci. USA. 87: 9178-9182.

Pierce Endogen. 2003. Instructions of Rat IL-6 ELISA Kit. Rockford: Pierce Endogen. pp. 1-10.

Plonk, S.G. et al. 1998. The alpha subunit of the heterotrimeric G protein G13 activates a phospholipase D isozyme by a pathway requiring Rho family GTPases. J. Biol. Chem. 273: 4823-4826.

Ponting, C.P., and Parker, P.J. 1996. Extending the C2 domain family: C2s in PKCs delta, epsilon, eta, theta, phospholipases, GAPs and perforin. Protein Sci. 5: 162-166.

Prenzel, N. et al. 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature. 402: 884-888.

Puls, A. et al. 1999. Activation of the small GTPase Cdc42 by the inflammatory cytokines TNF(α) and IL-1, and by the Epstein-Barr virus transforming protein LMP1. J. Cell Sci. 112: 2983-2992.

Qiu, R.G. et al. 1995. A role for Rho in Ras transformation. Proc. Natl. Acad. Sci. USA. 92: 11781-11785.

Quinn, M.T. et al. 1993. Translocation of Rac correlates with NADPH oxidase activation: Evidence for equimolar translocation of oxidase components. J. Biol. Chem. 268: 20983-20987.

Raab, G., and Klagsbrun, M. 1997. Heparin-binding EGF-like growth factor. Biochim. Biophys. Acta. 1333: F179-199.

Rameh, L.E. et al. 1995. Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulate PI 3-kinase association with tyrosine-phosphorylated proteins. Cell. 83: 821-830.

Rameh, L.E. et al. 1997. A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. J. Biol. Chem. 272: 22059-22066.

Ramirez, P. et al. 1997. Disruption of microtubule assembly and spindle formation as a mechanism for the induction of aneuploid cells by sodium arsenite and vanadium pentoxide. Mutat. Res. 386: 291-298.

Rao, G.N. et al. 1995. Thrombin stimulates phosphorylation of insulin-like growth factor-1 receptor, insulin receptor substrate-1, and phospholipase C-gamma 1 in rat aortic smooth muscle cells. J. Biol. Chem. 270: 27871-27875.

Rao, G.N. 1996. Hydrogen peroxide induces complex formation of SHC-Grb2-SOS with receptor tyrosine and activates Ras and extracellular signal-regulated protein

kinase group of mitogen-activated protein kinase. Oncogene. 13: 713-719.

Reichl, F.X. et al. 1988. Effect of arsenic on carbohydrate metabolism after single or repeated injection in guinea pigs. Arch. Toxicol. 62: 473-475.

Ren, X.D. et al. 1996. Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol-4-phosphate 5-kinase in Swiss 3T3 cells. Mol. Biol. Cell. 1996: 435-442.

Repetto, G. et al. 1994. Comparative in vitro effects of sodium arsenite and sodium arsenate on neuroblastoma cells. Toxicology. 92: 143-153.

Richard, S. et al. 1995. Association of p62, a multifunctional SH2- and SH3-domain-binding protein, with src family tyrosine kinases, Grb2, and phospholipase C gamma-1. Mol. Cell. Biol. 15: 186-197.

Ridley, A.J., and Hall, A. 1992. The small GTP-binding proteins rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell. 70: 389-399.

Ridley, A.J. et al. 1995. Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac and Rho in MDCK cells. Mol. Cell Biol. 15: 1110-1122.

Ridley, A.J. et al. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell. 70: 401-410.

Roche. 2000. In Situ Cell Death Detection Kit, Fluorescein. Mannheim: Roche. pp. 1-15.

Rodriguez-Viciana, P. et al. 1997. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell. 89: 457-467.

Rossman, T.G. et al. 1980. Absence of arsenite mutagenicity in *E. coli* and Chinese hamster cells. Environ. Mutagen. 2: 371-379.

Roundabush, F.L. et al. 2000. Transactivation of the EGF receptor mediates IGF-1 Stimulated Shc phosphorylation and ERK1/2 activation in COS-7 cells. J. Biol. Chem. 275: 22583-22589.

Roy, L.M. et al. 1989. Characterization of the epidermal growth factor associated with cytoskeletons of A431 cells. J. Cell Physiol. 140: 295-304.

RoyChoudhury, A. et al. 1996. Dietary garlic extract in modifying clastogenic effects of inorganic arsenic in mice: two-generation studies. Mutat. Res. 359: 165-170.

Sahai, E. et al. 1998. RhoA effector mutants reveal distinct effector pathways for

Cytoskeletal reorganization, SRF activation and transformation. EMBO J. 17: 1350-1361.

Salojin, K.V. et al. 1999. TCR and CD28 are coupled via ZAP-70 to the activation of the Vav/Rac-1/PAK-1/p38 MAPK signaling pathway. J. Immunol. 163: 844-853.

Sanders, L.G. et al. 1999. Inhibition of myosin light chain kinase by p21-activated kinase. Science. 283: 2083-2085.

Saraste, M. M. 1995. Pleckstrin homology domains: a fact file. Curr. Biol. 5: 403-408.

Sato, K. et al. 1995. c-Src phosphorylates epidermal growth factor receptor on tyrosine 845. Biochem. Biophys. Res. Commun. 215: 1078-1087.

Schlessinger, J., and Ullrich, A. 1992. Growth factor signaling by receptor tyrosine kinase. Neuron. 9: 383-391.

Schmalzing, G. et al. 1995. Involvement of the GTP binding protein Rho in constitutive endocytosis in *Xenopus laevis* oocytes. Cell Biol. 130: 1319-1332.

Schuebel, K.E. et al. 1998. Phosphorylation-dependent and constitutive activation of Rho Proteins by wild-type and oncogenic Vav-2. EMBO J. 17: 6608-6621.

Scott, N. et al. 1993. Reactions of arsenic (III) and arsenic (V) species with glutathione. Chem. Res. Toxicol. 6: 102-106.

Seasholtz, T.M. et al. 1999. Rho as a mediator of G protein-coupled receptor signaling. Mol. Pharmacol. 55: 949-956.

Sells, M.A. et al. 1997. Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. Curr. Biol. 7: 202-210.

Shaw, L.M. et al. 1997. Activation of phosphoinositide 3-OH kinases by the alpha6beta4 integrin promotes carcinoma invasion. Cell. 91: 949-960.

Sherrill, J.M. 1997a. Self-phosphorylation of epidermal growth factor receptor is an intermolecular reaction. Biochemistry. 36: 5677-5684.

Sherril, J.M. 1997b. Insufficiency of self-phosphorylation for the activation of epidermal growth factor receptor. Biochemistry. 36: 12890-12896.

Shibasaki, Y. et al. 1997. Massive actin polymerization induced by phosphatidylinositol-4-phosphate 5-kinase *in vivo*. J. Biol. Chem. 272: 7578-7581.

Simeonova, P.P. et al. 2002. c-Src-dependent activation of the epidermal growth factor

receptor and mitogen-activated protein kinase pathway by arsenic: Role in carcinogenesis. J. Biol. Chem. 277: 2945-2950.

Simons, S.S. Jr. et al. 1990. Arsenite and cadmium (II) as probes of glucocorticoid receptor structure and function. J. Biol. Chem. 265: 1938-1945.

Smith, K.R. et al. 2001. Arsenite stimulates plasma membrane NADPH oxidase in Vascular endothelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 280: L442-L449.

Soler, C. et al. 1994. Individual epidermal growth factor receptor autophosphorylation sites do not stringently define association motifs for several SH2-containing proteins. J. Biol. Chem. 269: 12320-12324.

Songyang, Z. et al. 1993. SH2 domains recognize specific phosphopeptide sequences. Cell. 72: 767-778.

Souza, K. et al. 2001. Arsenite activation of PI3K/AKT cell survival pathway is mediated by p38 in cultured human keratinocytes. Mol. Med. 7: 767-772.

Stam, J.C. et al. 1998. Invasion of T-lymphoma cells: Cooperation between Rho family GTPases and lysophospholipid receptor signaling. EMBO J. 17: 4066-4074.

Stein, E. et al. 1998. Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment and assembly responses. Genes Dev. 12: 667-678.

Stover, D.R. et al. 1995. Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and p85 alpha. J. Biol. Chem. 270: 15591-15597.

Su, Y.C. et al. 1998. The Drosophila Ste20-related kinase misshappen is required for embronic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway. Genes Dev. 12: 2371-2380.

Superti-Furga, G., and Courtneidge, S.A. 1995. Structure-function relationships in Src family and related protein tyrosine kinases. BioEssays. 17: 321-330.

Suzuki, M. et al. 1997. Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. J. Biol. Chem. 272: 31730-31737.

Szinicz, L., and Forth, W. 1988. Effects of sodium As_2O_3 on glucogenesis. Arch. Toxicol.

61: 444-449.

Suzuki, K., and Takahashi, K. 2001. Actin filament assembly and actin-myosin contractility are necessary for anchorage- and EGF-dependent activation of phospholipase Cgamma. J. Cell Physiol. 189: 64-71.

Tadaki, D.K., and Niyoki, S.K. 1993. The function importance of hydrophobicity of the tyrosine at position 13 of human epidermal growth factor receptor binding. J. Biol. Chem. 268: 10114-10119.

Takaishi, K. et al. 1995. Translocation of activated Rho from the cytoplasm to membrane ruffling are, cell-cell adhesion sites and cleavage furrows. Oncogene. 11: 39-48.

Thomas, S.R. et al. 2002. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. J. Biol. Chem. 277: 6017-6024.

Timokhina, I. et al. 1998. Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. EMBO J. 17: 6250-6262.

Tinwell, H. et al. 1991. Arsenite as the probable active species in the human carcinogenicity of arsenic: mouse micronucleus assays on Na and K arsenite, orpiment, and Fowler's solution. Environ Health Perspect. 95: 205-210.

Tolias, K.F. et al. 1995. Rho family GTPases bind to phaophoinositide kinases. J. Biol. Chem. 270: 17656-17659.

Tominaga, T. et al. 1993. Inhibition of PMA-induced, LFA-1-weight GTP binding Protein, rho. J. Cell Biol. 120: 1529-1537.

Tominaga, T, and Barber, D.L. 1998. Na-H exchange acts dowstream of Rho to regulate integrin-induced cell adhesion and spreading. Mol. Biol. Cell. 9: 2287-2303.

Tominaga, T. et al. 1998. p160ROCK mediates RhoA activation of Na-H exchanger. EMBO J. 17: 4712-4722.

Tonks, N.K., and Neel, B.G. 1996. From form to function: signaling by protein tyrosine phosphatases. Cell. 87: 365-368.

Totsukawa, G. et al. 2000. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal

adhesions in 3T3 fibroblasts. J. Cell Biol. 150: 797-806.

Tsai, W. et al. 1997. The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. EMBO. J. 16: 4597-4606.

Uehata, M. et al. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature (London). 389: 990-994.

Ullrich, A., and Schlessinger, J. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell. 61: 203-212.

U.S. Environment Protection Agency (US EPA). 1992. Second draft for the drinking water criteria document on arsenic. Washington DC: US EPA.

Ushio-Fukai, M. et al. 1999. Role of reactive oxygen species in angiotensin II-induced transactivation of epidermal growth factor receptor in vascular smooth muscle cells. Circulation .100 Suppl: I-263.

Van Belzen, N. et al. 1990. Interaction of the epidermal growth factor receptor with the cytoskeleton is related to receptor clustering. J. Cell Physiol. 145: 365-375.

Van Bergen en Henegouwen, P.M.P. et al. 1992. The epidermal growth factor is associated with actin filaments. Exp. Cell Res. 199: 90-97.

Vandekerckhove, J.S. et al. 1989. Acanthamoeba actin and profilin can be cross-linked between glutamic acid 364 of actin and lysin 115 of profilin. J. Cell Biol. 109: 619-626.

Van Delft, S. et al. 1995. Epidermal growth factor induces serine phosphorylation of actin. FEBS Lett. 357: 251-254.

Van Leeuwen, F.N. et al. 1999. Rac regulates phosphorylation of the myosin-II heavy chain, actinomyosin disassembly and cell spreading. Nat. Cell. Bio. 1: 242-248.

Venkatakrishnan, G. et al. 2000. Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. J Biol. Chem. 275: 6868-6875.

Vexler, Z.S. et al. 1996. Activation of Na+/H+ exchange is necessary for RhoA-induced Stress fiber formation. J. Biol.Chem. 271: 22281-22284.

Wahlberg, J.E., and Borman, A. 1986. Contact sensitivity to arsenical compounds. Dermatosen. 34: 10-12.

Wan, Y.S. et al. 2001. EGF receptor crosstalk with cytokine receptors leading to the

activation of c-Jun kinase in response to UV irradiation in human keratinocytes. Cell Signal. 13: 139-144.

Wang, D. et al. 2000. Distinct effects of N-acetylcysteine and nitric oxide on angiotensin II-induced epidermal growth factor receptor phosphorylation and intracellular Ca^{2+} levels. J. Biol. Chem. 275: 12223-12230.

Wang, Z. et al. 1996. Efflux mediated resistance to arsenicals in arsenic resistant and hypersensitive Chinese hamster cells. Toxicol. Appl. Pharmacol. 137: 112-119.

Wasserman, S. 1998. FH proteins as cytoskeletal organizers. Trends Cell Biol. 8: 111-115.

Watanabe, N. et al., 1997. p140mDia, a mammalian homolog of *Drosophila diaphanous*, is a target protein for Rho small GTPase and is a ligand for profilin. EMBO J. 16: 3044-3056.

Watanabe, N. et al. 1999. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat. Cell Biol. 1: 136-143.

White, M.F. 1998. The IRS-signaling system: a network of docking proteins that mediate insulin action. Mol. Cell. Biochem. 182: 3-11.

Wiegant, F.A.C. et al. 1986. Epidermal growth factor receptors associated to cytoskeletal elements of epidermoid carcinoma (A431) cells. J. Cell Biol. 103: 87-94.

Wojciak-Stothard, B. et al. 1998. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. J. Cell Physiol. 176: 150-165.

World Health Organization (WHO). 2001. Environmental Health Criteria for Arsenic and Arsenic Compounds. Geneva: WHO.

Wu, J. et al. 1997. The Vav binding site (Y315) in ZAP-70 is critical for antigen receptor-mediated signal transduction. J. Exp. Med. 185: 1877-1882.

Yang, N. et al. 1998. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature (London). 393: 809-812.

Yamauchi, T. et al. 1997. Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. Nature. 390: 91-96.

Yancopoulos, G.D., Klagsbrun, M., and Folkman, J. 1998. Vasculogenesis, angiogenesis, and growth factor: ephrins enter the fray at the border. Cell. 93: 661-664.

Yang, L.J. et al. 1994. Epidermal growth factor-induced activation and translocation of phospholipase C-γ1 to the cytoskeleton in rat hepatocytes. J. Biol. Chem. 269: 7156-7162.

Yasui, Y. et al. 1998. Roles of Rho-associated kinase in cytokinesis; mutations in Rho-associated kinase phosphorylation sites impair cytokinetic segregation of glial filaments. J. Cell. Biol. 143: 1249-1258.

Yen, H.T. et al. 1996. Arsenic induces interleukin-8 expression in cultured keratinocytes. Arch. Derm. Res. 288: 716-717.

Yu, X. et al. 2000. Integrin –dependent EGF receptor activation at cell-cell contact sites. J. Cell Sci. 113: 2139-2147.

Zeigler, M.E. et al. 1999. Role of ERK and JNK pathways in regulating cell motility and matrix metalloproteinase 9 production in growth factor-stimulayed human epidermal keratinocytes. J. Cell Physiol. 180: 271-284.

Zhang, B. et al. 1998. Interaction of Rac1 with GTPase-activating proteins and putative effectors, A comparison with Cdc42 and RhoA. J. Biol. Chem. 373: 8776-8782.

Zhang, J. et al. 1993. Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. J. Biol. Chem. 268: 22251-22254.

Zhou, S., and Cantley, L.C. 1995. Recognition and specificity in protein tyrosine kinase-mediated signaling. Trends Biochem. Sci. 20: 470-475.

Zhang, S. et al. 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J. Biol. Chem. 270: 23934-23936.

Zheng, Y. et al. 1994. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J. Biol. Chem. 269: 18727-18730.

APPENDICES

APPENDIX A

Reagents for Microtubule Staining (Geuens *et al.*, 1986)

Buffer A

0.1M NaMES

1 mM $MgCl_2$

1 mM EGTA

0.05 mM EDTA

4% PEG-8000

pH 6.75

MgPBS

150 mM NaCl

30 mM NaH_2PO_4-Na_2HPO_4

1 mM $MgCl_2$

pH 7.4

APPENDIX B

Immunoblotting Reagents

Note: Prepare solutions with Milli-Q or equivalently purified water

Transfer Buffer

25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)

SDS Sample Buffer (1X)

62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% W/V bromophenol blue or phenol red

Blocking Buffer

1XTBS, 0.1% Tween-20 with 5% W/V nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml, add 0.15 ml Tween-20 (100%)

10X TBS (Tris-buffered saline)

To prepare 1 liter of 10X TBS; 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).

Primary Antibody Dilution Buffer

1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%). If background is high, a better signal to noise ratio can be obtained by diluting the primary antibody in 5% milk (in place of 5% BSA) in 1XTBS/T

Phototope ® -HRP Western Blot Detection

Biotinylated protein marker, secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO chemiluminescent reagent, peroxide

Wash Buffer TBS/T

1X TBS, 0.1% Tween-20

APPENDIX C

Rat IL-6 ELISA Kit

(Pierce Endogen, 2003)

INSTRUCTIONS

Rat IL-6 ELISA Kit

PIERCE
ENDOGEN
brands of PERBIO
3747 N. Meridian Road
P.O. Box 117
Rockford, IL 61105

ER2IL6 ER2IL65

1368w

Number	Description
ER2IL6	**Rat Interleukin-6 (IL-6) ELISA Kit,** sufficient reagents for 96 determinations
	Kit Contents:
	Anti-Rat IL-6 Precoated 96-well Strip Plate, 1 each
	Lyophilized Recombinant Rat IL-6 Standard, 2 vials
	Sample Diluent, 12 ml, contains 0.1% sodium azide
	Biotinylated Antibody Reagent, 12 ml, contains 0.1% sodium azide
	30X Wash Buffer, 50 ml
	Streptavidin-HRP Concentrate, 75 µl
	Streptavidin-HRP Dilution Buffer, 13 ml
	Premixed TMB Substrate Solution, 12 ml
	Stop Solution, 13 ml, contains 0.18 M sulfuric acid
	Adhesive Plate Covers, 6 each
ER2IL65	**Rat Interleukin-6 ELISA Kit,** sufficient reagents for 5 x 96 determinations
	Kit Contents:
	Anti-Rat IL-6 Precoated 96-well Strip Plate, 5 each
	Lyophilized Recombinant Rat IL-6 Standard, 5 vials
	Sample Diluent, 75 ml, contains 0.1% sodium azide
	Biotinylated Antibody Reagent, 55 ml, contains 0.1% sodium azide
	30X Wash Buffer, 200 ml
	Streptavidin-HRP Concentrate, 250 µl
	Streptavidin-HRP Dilution Buffer, 70 ml
	Premixed TMB Substrate Solution, 5 x 13 ml
	Stop Solution, 55 ml, contains 0.18 M sulfuric acid
	Adhesive Plate Covers, 30 each

For research use only. Not for use in diagnostic procedures.

Storage: Upon receipt store all reagents at 2-8°C. Do not freeze reagents. Product shipped with ice pack.

Refer to the expiration date stamped on the kit box. Do not use kit beyond the stated expiration date.

Introduction

The Pierce Endogen Rat Interleukin-6 (IL-6) ELISA is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of rat IL-6 in culture supernatants, EDTA plasma, sodium citrate plasma, heparin plasma and serum.

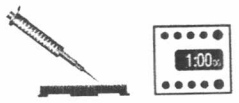
Table of Contents

Procedure Summary

1. Add 50 µl of Sample Diluent to each well. Add 50 µl of standards or samples to each well in duplicate.

2. Cover plate and incubate at room temperature (20-25°C) for 2 hours.

3. Wash plate THREE times.



4. Add 100 µl of Biotinylated Antibody Reagent to each well. Cover plate and incubate at room temperature for 1 hour.

5. Wash plate THREE times.

6. Add 100 µl of prepared Streptavidin-HRP Solution to each well.

7. Cover plate and incubate at room temperature for 30 minutes.

8. Wash plate THREE times.

9. Add 100 µl of Premixed TMB Substrate Solution to each well.

10. Develop plate in the dark at room temperature for 30 minutes.

11. Stop reaction by adding 100 µl of Stop Solution to each well.

12. Measure absorbance on a plate reader at 450 nm minus 550 nm. Calculate results using graph paper or curve-fitting statistical software.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1,000 µl
- Plastic pipettes to deliver 5-15 ml
- Ultrapure water
- A glass or plastic two-liter container to prepare Wash Buffer
- A squirt wash bottle or an automated 96-well plate washer
- 1.5 ml polypropylene or polyethylene tubes to prepare standards — do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs
- 15 ml plastic tube to prepare Streptavidin-HRP Solution
- Microcentrifuge to prepare Streptavidin-HRP Solution
- A standard ELISA reader for measuring absorbance at 450 nm and 550 nm. If a 550 nm filter is not available, the absorbance can be measured at 450 nm only. Refer to the instruction manual supplied with the instrument being used.
- Graph paper or a computerized curve-fitting statistical software package

Precautions

- **All samples and reagents must be at room temperature (20-25°C) before use in the ELISA.**
- Review the instruction booklet carefully and verify all components against the Kit Contents list (page 1) before beginning the assay.
- Do not use water baths to thaw samples. Thaw at room temperature.
- When preparing standard curve and sample dilutions in culture medium, use the same medium used to culture the cells. For example, if RPMI with 10% fetal calf serum (FCS) was used to culture cells, then use RPMI with 10% FCS to dilute the standard and samples. Do NOT use RPMI without serum supplement.
- If using a multichannel pipettor, always use a new disposable reagent reservoir.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Once reagents have been added to the plate, take care NOT to let plate DRY at any time during the assay.
- Avoid microbial contamination of reagents.
- Vigorous plate washing is essential.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Discard unused kit components. Do not mix reagents from different kit lots.
- Do not use glass pipettes to measure TMB Substrate Solution. Take care not to contaminate the Solution. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.
- Some components of this kit contain sodium azide. Please dispose of reagents according to local regulations.

Additional Precautions for the 5-plate Kit

- Dispense only reagent volumes required for the number of plates being used. Do not combine leftover reagents with those reserved for additional plates.
- Use only one bottle of the TMB Substrate Solution per 96-well plate. Do not combine leftover substrate with that reserved for other plates.
- Equilibrate to room temperature only the reagent volumes required for the number of plates being used.
- Use only one vial of standard per 96-well plate.

Sample Preparation

Sample Handling

- Serum; EDTA, heparin and sodium citrate plasma; or culture supernatants may be tested in this ELISA.
- 50 µl per well of serum, plasma or culture supernatant are required.
- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C.
- Avoid repeat freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate each time the ELISA is performed.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use heated water baths to thaw or warm samples.
- Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret results with caution.

Sample Dilution

- If the rat IL-6 concentration possibly exceeds the highest point of the standard curve (i.e., 2,000 pg/ml), prepare one or more five-fold dilutions of the test sample using the Sample Diluent provided. For example, a five-fold dilution is prepared by adding 0.05 ml (50 µl) of test sample to 0.2 ml (200 µl) of Sample Diluent. Mix thoroughly between dilutions before assaying.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout this instruction booklet.

Note: When using the 5-plate kit, only one standard per plate is supplied. Therefore, partial plates cannot be used.

Wash Buffer

1. Label a clean glass or plastic two-liter container "Wash Buffer." The 30X Wash Buffer may have a cloudy appearance.
2. Add the entire contents of the 30X Wash Buffer bottle (50 ml) to the two-liter container and dilute to a final volume of 1.5 liters with ultrapure water. Mix thoroughly.

   (PP) When using partial plates, store the reconstituted Wash Buffer at 2-8°C.

   Note: Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer if it becomes visibly contaminated during storage.

   Note: If using a 5-plate kit, add 30 ml Wash Buffer to 870 ml water for each plate used.

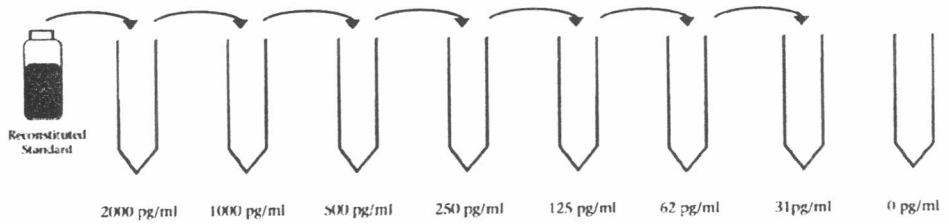
Standards

- **(PP)** Reconstitute and use one vial of the lyophilized standard per partial plate.
- Prepare standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.

1. Reconstitute standard with ultrapure water. Reconstitution volume is stated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the Sample Diluent provided to prepare standard curve serial dilutions.
2. Label eight tubes, one for each standard curve point: 2,000, 1,000, 500, 250, 125, 62, 31, and 0 pg/ml, then prepare 1:2 serial dilutions for the standard curve as follows:
3. Pipette 150 µl of Sample Diluent into each tube.

4. Pipette 150 µl of the reconstituted standard into the first tube (i.e., 2,000 pg/ml) and mix.
5. Pipette 150 µl of this dilution into the second tube (i.e., 1,000 pg/ml) and mix.
6. Repeat the serial dilutions (using 150 µl) five more times to complete the standard curve points. These concentrations, 2,000 pg/ml, 1,000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62 pg/ml, 31 pg/ml, and 0 pg/ml are the standard curve.

Serial Dilutions using 150 µl



Assay Procedure

A. Sample Incubation

- **(PP)** Determine the number of strips required. Leave these strips in the plate frame. Place remaining unused strips in the provided foil pouch with desiccant. Store reserved strips at 2-8°C. Make sure foil pouch is sealed tightly. After completing assay, retain plate frame for second partial plate. When using the second partial plate, place strips securely in the plate frame.
- Use the Data Template provided to record the locations of the zero standard (blank or negative control), rat IL-6 standards and test samples. Perform seven standard points and one blank in duplicate with each series of unknown samples.

1. Add 50 µl of Sample Diluent to each well.
2. Add 50 µl of reconstituted standards or test samples in duplicate to each well. Mix well by gently tapping the plate several times.

   Note: If the rat IL-6 concentration in any sample possibly exceeds the highest point on the standard curve, 2,000 pg/ml, see Sample Preparation-Sample Dilution section.
3. Carefully cover plate with a new adhesive plate cover. Ensure all edges and strips are sealed tightly by running your thumb over edges and down each strip. Incubate for two (2) hours at room temperature, 20-25°C.
4. Carefully remove adhesive plate cover. Wash plate THREE times with Wash Buffer as described in the Plate Washing section (section B).

B. Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips securely remain in the frame.
2. Empty plate contents. Use a squirt bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

   Note: For automated washing, aspirate all wells and wash THREE times with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material.

C. Biotinylated Antibody Reagent Incubation

- If using a multichannel pipettor, **use a new reagent reservoir and pipette tips** when adding the Biotinylated Antibody Reagent. Remove from the vial only the amount required for the number of strips being used. Take care not to touch the samples in wells with the pipette tip when adding the Biotinylated Antibody Reagent.

1. Add 100 µl of Biotinylated Antibody Reagent to each well containing sample or standard.
2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for one (1) hour at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times as described in the Plate Washing section (section B).

D. Streptavidin-HRP Solution Preparation and Incubation

- Prepare Streptavidin-HRP Solution **immediately before use.** Do not prepare more Streptavidin-HRP Solution than required.
- Do not store prepared Streptavidin-HRP Solution.
- Use a 15 ml plastic tube to prepare Streptavidin-HRP Solution.
- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the prepared Streptavidin-HRP Solution.

1. Briefly centrifuge the Streptavidin-HRP Concentrate to force entire vial contents to the bottom.
2. **(PP)** Use only the Streptavidin-HRP Solution amount required for the number of strips being used. For each strip, mix 2.5 µl of Streptavidin-HRP Concentrate with 1 ml of Streptavidin-HRP Dilution Buffer. Store Streptavidin-HRP Concentrate reserved for additional strips at 2-8°C.

   For one complete 96-well plate, add 30 µl of Streptavidin-HRP Concentrate to 12 ml of Streptavidin-HRP Dilution Buffer and mix gently.
3. Add 100 µl of prepared Streptavidin-HRP Solution to each well.
4. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 30 minutes at room temperature, 20-25°C.
5. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times as described in the Plate Washing section (section B).

E. Substrate Incubation and Stop Step

- Use new disposable reagent reservoirs when adding TMB Substrate Solution and Stop Solution.
- Dispense from bottle ONLY amount required, 100 µl per well, for the number of wells being used. Do not use a glass pipette to measure the TMB Substrate Solution.
- **(PP)** Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate remaining TMB Substrate Solution.

1. Pipette 100 µl of TMB Substrate Solution into each well.
2. Allow enzymatic color reaction to develop at room temperature in the dark for 30 minutes. Do not cover plate with aluminum foil or a plate sealer. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
3. After 30 minutes, stop the reaction by adding 100 µl of Stop Solution to each well.

F. Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

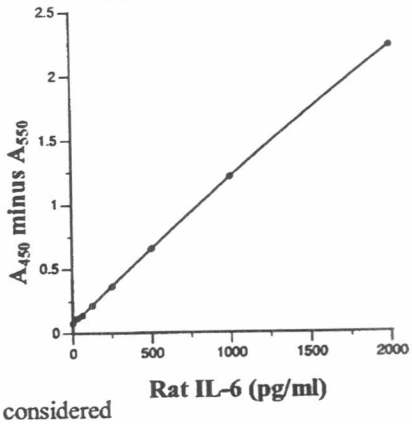
Measure the absorbance on an ELISA plate reader set at 450 nm and 550 nm. Subtract 550 nm values from 450 nm values to correct for optical imperfections in the microplate. If an absorbance at 550 nm is not available, measure the absorbance at 450 nm only.

Note: When the 550 nm measurement is omitted, absorbance values will be higher.

G. Calculation of Results

- The standard curve is used to determine rat IL-6 amount in an unknown sample. Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding rat IL-6 concentration (pg/ml) on the horizontal (X) axis.
- Calculate results using graph paper or curve-fitting statistical software. The rat IL-6 amount in each sample is determined by interpolating from the absorbance value (Y axis) to rat IL-6 concentration (X axis) using the standard curve.
- If the test sample was diluted, multiply the interpolated value obtained from the standard curve by the dilution factor to calculate pg/ml of rat IL-6 in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Duplicate values that differ from the mean by greater than 10% should be considered suspect and repeated.

Standard Curve Example



Performance Characteristics

Sensitivity: <16 pg/ml
The sensitivity or Lower Limit of Detection (LLD)[1] is determined by assaying replicates of zero and the standard curve. The mean signal of zero + 2 standard deviations read in dose from the standard curve is the LLD. This value is the smallest dose that is not zero with 95% confidence.

Assay Range: 31-2,000 pg/ml
Suggested standard curve points are 2,000, 1,000, 500, 250, 125, 62, 31, and 0 pg/ml.

Reproducibility:
Reproducibility of this assay is evaluated in each sample matrix. To determine the intra-assay precision, 20 replicates of samples containing two levels of recombinant rat IL-6 are assayed on a single plate. To evaluate inter-assay precision, samples are tested by three operators who perform at least three separate assays on more than one day. Twelve duplicate sample values are used to calculate inter-assay precision data for each level of IL-6. Data are indicated in the table below:

		Intra-assay Precision			Inter-assay Precision		
Sample	**Level**	**Mean (pg/ml)**	**SD (pg/ml)**	**CV (pg/ml)**	**Mean (pg/ml)**	**SD (pg/ml)**	**CV (pg/ml)**
Serum	1	1,032.3	85.4	8.3	970.6	76.0	7.8
	2	201.9	18.5	9.2	203.5	14.0	6.9
EDTA Plasma	1	1,059.9	111.1	10.5	1,005.3	70.8	7.0
	2	272.2	22.4	8.2	343.7	31.1	9.1
Citrate Plasma	1	901.4	84.7	9.4	943.6	64.0	6.8
	2	112.1	10.0	8.9	102.7	8.3	8.1
Heparin Plasma	1	978.9	89.7	9.2	1,067.5	116.4	10.9
	2	225.5	14.8	6.6	227.0	13.8	6.1
Cell Culture Supernatant	1	1,139.4	123.4	10.8	1,341.5	107.6	8.0
	2	247.9	17.1	6.9	327.1	34.3	10.5

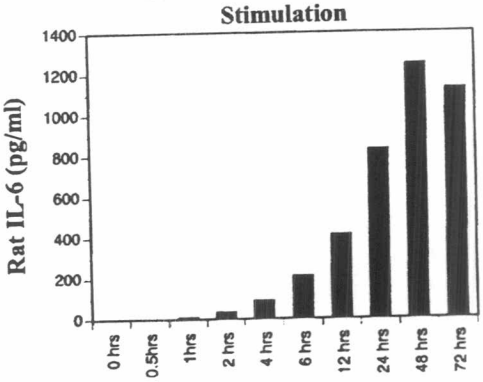
Specificity:
This ELISA is specific for the measurement of natural and recombinant rat IL-6. This ELISA does not cross-react with the following cytokines: rat GCSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-10, IL-18, Eotaxin, MIP-1α, RANTES and TNFα; or human, mouse and pig IL-6.

Calibration:
The standard in this ELISA is calibrated to a Pierce Endogen rat IL-6 reference standard.

Expected Values:
For supernatant from stimulated rat splenocytes:
Rat spleens are harvested and splenocytes are isolated. Isolated splenocytes (2×10^5 cells/ml) are cultured with LPS (10 µg/ml). Supernatants are collected at various timepoints and assayed for rat IL-6.

For normal serum and plasma:
Twenty pooled serum and EDTA, heparin, and sodium citrate plasma samples collected from apparently healthy Wistar rats are evaluated in this assay. IL-6 levels in 77 of the 80 samples tested are below the detection limit of the assay.

Rat IL-6 Levels after LPS Stimulation



Spike and Recovery:
Recovery of rat IL-6 is evaluated using the Pierce Endogen Rat IL-6 ELISA Kit. Pooled serum and plasma samples from Wistar rats and sample diluent controls are spiked with recombinant rat IL-6. Endogenous IL-6 levels are determined by evaluating unspiked samples along with spiked aliquots of the same samples in the ELISA. Expected values are calculated by adding endogenous IL-6 levels to those of spiked diluent controls. Percent (%) recovery is found by dividing observed by expected values. Results for representative individual samples and populations are shown below:

		Representative Sample			Sample Population			
Sample	Level	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)	n
Serum	1	1,770	1,554	88	1,678	1,479	88	6
	2	470	464	99	411	403	98	6
EDTA Plasma	1	1,801	1,542	86	1,801	1,552	86	6
	2	239	232	97	277	267	96	6
Citrate Plasma	1	1,858	1,666	90	1,761	1,584	90	6
	2	215	204	95	238	230	97	6
Heparin Plasma	1	1,147	1,041	91	1,136	1,014	89	6
	2	230	237	103	253	247	98	6

Linearity of Dilution:
Pooled serum and plasma samples from Wistar rats are spiked with recombinant rat IL-6, serially diluted in Sample Diluent, and evaluated in the Pierce Endogen Rat IL-6 ELISA. Linearity of Dilution for plasma is assessed for each anticoagulant. Representative data from EDTA plasma samples are shown below. Results for heparin and citrate plasma are similar to those shown for EDTA plasma. Observed values are compared to the expected values to calculate % recovery and demonstrate the Linearity of Dilution of the assay.

Sample	Dilution	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
Serum 1	Neat	660.8	660.8	-
	1:2	330.4	336.4	101.8
	1:4	165.2	178.7	108.2
	1:8	82.6	86.6	104.9
	1:16	41.3	43.9	106.3
EDTA Plasma	Neat	1,306.5	1,306.5	-
	1:2	653.2	656.9	100.6
	1:4	326.6	361.6	110.7
	1:8	163.3	170.1	104.1
	1:16	81.7	73.6	90.1
Cell Culture Medium	Neat	772.3	772.3	-
	1:2	386.2	377.1	97.7
	1:4	193.1	200.9	104.1
	1:8	96.5	100.3	103.9

Reference

1. *Immunoassay: A Practical Guide,* Chan and Perlstein, Eds., 1987, Academic Press: New York, p71.

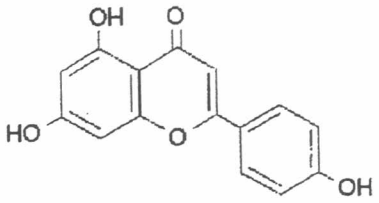
Data Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

APPENDIX D

Apigenin



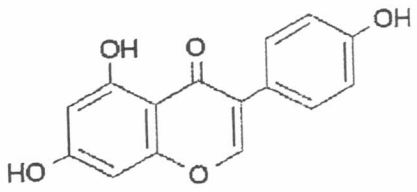
Appearance:	yellow powder
Molecular Weight:	270.2
Molecular Formula:	$C_{15}H_{10}O_5$
Synonyms:	4',5, 7-Trihydroxyflavone
Solubility:	soluble in DMSO at 27 mg/ml or in 1 M KOH at 50 mg/ml

Apigenin is a plant flavone that has been found to inhibit cell proliferation by arresting the cell cycle at the G2/M phase. This effect was found to be dose-dependent and reversible when apigenin was removed from the culture medium. Inhibitory effects on tumor promotion may also be due to inhibition of kinase activity and the resulting suppression of oncogene expression. At a fairy low concentration (12.5 μM), apigenin induced the reversion of transformed phenotypes of v-H-ras transformed NIH 3T3 cells. Studies indicate that this is due to an inhibition of mitogen activated protein kinase (MAPK). It was found that 25 μM apigenin greatly inhibited with 30 minutes of incubation with the transformed cells, and this inhibition persisted for more than 4 hours. Downstream oncogenes may also be involved in the reversion since this same treatment was found to significantly reduce the expression of c-jun and c-fos. In contrasts, no effect on the level of the ras protein or its mRNA was observed.

Similarly, apigenin was shown to inhibit protein kinase C (PKC) by competing with ATP (IC_{50} = 10±0.5 μM). Other kinases affected by apigenin include the tyrosine kinases fibroblast growth factor (FGF) receptor (IC_{50} = 20 μM) and pp60v-src (IC_{50} > 200 μM).

Apigenin has also been reported to inhibit topoisomerase I catalyzed DNA relegation and enhance gap junctional intercellular communication.

Genistein



Molecular Formula:	$C_{15}H_{10}O_5$
Molecular Weight:	270.2
Synonyms:	5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one; 4',5,7-Trihydroxyisoflavone
Solubility:	soluble at 100 mM (27 mg/ml) in DMSO, practically insoluble in water and soluble in dilute alkalies producing a yellow color

Genistein is reported to be a specific inhibitor of tyrosine-specific protein kinases, i.e., the EGF receptor kinase, pp60 v-src kinases from Rous sarcoma virus and pp110 kinase from Gardner-Arnstein feline sarcoma virus. Genistein did not inhibit the activity of serine and threonine-specific kinases such as cAMP-dependent protein kinase, protein kinase C, and phosphorylase kinase.

APPENDIX E

Protein Tyrosine Kinases

One of the fundamental mechanisms by which cells in multicellular organisms communicate is the binding of polypeptide ligands to cell surface receptors that possess tyrosine kinase catalytic activity. Receptor tyrosine kinase (RTKs) are transmembrane glycoproteins that are activated by the binding of their cognate ligands, and they transduce the extracellular signal to the cytoplasm by phosphorylating tyrosine residues on the receptor themselves (autophosphorylation) and on downstream signaling proteins. RTKs activate numerous pathways within cells, leading to cell proliferation, differentiation, migration, or metabolic changes (Schlessinger and Ullrich, 1992). The RTK family includes the receptors for insulin and for many growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF). In addition to the RTKs, there exists a large family of nonreceptor tyrosine kinase (NRTKs), which includes Src, the Janus kinases (JAKs), and Abl, among others. The NRTKs are integral components of the signaling cascades triggered by RTKs and by other cell surface receptors such as G protein-coupled receptors and receptors of the immune system. The specific reaction catalyzed by PTKs is the transfer of the γ phosphate of ATP to the hydroxyl group of a tyrosine in a protein substrate.

Protein Tyrosine Kinases in Cellular Signaling

Several examples are cites to illustrate the importance of PTKs in embryonic development, metabolism, and immune system function. The development of the vascular system relies on the concerted action of several subfamilies of RTKs and their cognate ligands (Yancopoulos, Klagsbrun, and Folkman, 1998). The vascular system is formed in a two-step process. In the first step, referred to as vasculogenesis, endothelial cells differentiate to form a crude network of interconnected vessels. In the second step, termed angiogenesis, the vessels are remodeled and extended, and nonendothelial support cells are recruited to the maturing vasculature. Vasculogenesis requires the growth factor VEGF and one of the RTKs through which it acts, KDR. Angiogenesis requires another VEGF receptor, Flt1, as well as the angiogenic factor angiopoietin 1, which is a ligand for the RTK Tie2.

The effects of the hormone insulin are mediated by the insulin receptor, an RTK family member. Insulin binding to its receptor results in receptor activation and the recruitment of a family of downstream signaling molecules, the IRS proteins, to the activated receptor (White, 1998). The IRS proteins are adaptor proteins, i.e. they have no identifiable catalytic function, which are phosphorylated on multiple tyrosine residues by the insulin receptor. Activation of phosphoinositide 3-kinase (PI-3K) through binding to phosphorylated IRS is a critical step in translocation of glucose transporters to the cell membrane to facilitate glucose uptake (Cheatham *et al.*, 1994; Okada *et al.*, 1994).

The largest subfamily of NTRKs, with nine members, is the Src family members participate in a variety of signaling processes, including mitogenesis, T- and B-cell activation, and cytoskeleton restructuring. Multiple in vivo substrates have been decribed for Src and include, among others, the PDGF and EGF receptors; the NRTK focal adhesion kinase (FAK); p130Cas, an adapter protein involved in integrin- and growth factor-mediated signaling; and cortactin, an actin-binding protein important for the proper formation of cell matrix contact sites (Biscardi, Tice, and Parsons, 1999). Src has also been implicated in several human carcinomas, including breast, lung, and colon cancer (Biscardi, Tice, and Parsons, 1999).

Protein Tyrosine Kinase Architecture

RTKs consist of an extracellular portion that binds polypeptide ligands, a transmembrane helix, and a cytoplasmic portion that possesses tyrosine kinase catalytic activity. The vast majority of RTKs exist as a single polypeptide chain and are monomeric in the absence of ligand. Exceptions include Met and its family members, which comprise a short α chains disulfide-linked to two membrane-spanning β chains. The α chains are also disulfide-linked to one another, forming an $\alpha_2\beta_2$ heterotetramer. Most polypeptide ligands for RTKs are soluble. Exceptions include the ephrins, the ligands for the Eph receptor family, which either span the cell membrane or are tethered to the membrane via a GPI (glycosyl-phosphatidylinositol) linkage (Flanagan and Vanderhaeghen, 1998; Holland *et al.*, 1998).

The extracellular portion of RTKs typically contains a diverse of discrete globular domains such as immunoglobulin (Ig)-like domains, fibronectin type III-like domains, cysteine-rich domains, and EGF-like domains. In contrast, the domain

organization in the cytoplasmic portion of RTKs is simpler, consisting of a juxtamembrane region (just after the transmembrane helix), followed by the tyrosine kinase catalytic domain and a carboxyl-terminal region. Some receptors, most notably members of the PDGF receptor family contain a large insertion of ~ 100 residues in the tyrosine kinase domain. The juxtamembrane and carboxyl-terminal regions vary in length among RTKs. Along with the tyrosine kinase insert, these regions contain tyrosine residues that are autophosphorylated upon ligand binding.

NRTKs lack receptor-like features such as extracellular ligand-binding domain and a transmembrane-spaning region, and most NRTKs are localized in the cytoplasm (Neet and Hunter, 1996). Some NRTKs are anchored to the cell membrane through aminoterminal modification, such as myristoylation or palmitoylation. In addition to a tyrosine kinase domain, NRTKs possess domains that mediate protein-protein, protein-lipid, and protein-DNA interactions. The most commonly found protein-protein interaction domains in NRTKs are the Src homology (SH2) and 3 (SH3) domains (Kuriyan and Cowburn, 1997). The SH2 domain is a compact domain of ~100 residues that binds phosphotyrosine residues in a sequence-specific manner. The smaller SH3 domain (~60 residues) binds proline-containing sequences capable of forming a polyproline type II helix.

Some NRTKs lack SH2 and SH3 domains but possess subfamily-specific domains used for protein-protein interactions. For example, members of the Jak family contain specific domains that target them to the cytoplasmic portion cytokine receptors. The NRTK Fak possesses two domains that mediate protein-protein interactions: an integrin-binding domain and a focal adhesion-binding domain. The NRTK Abl contains a nuclear localization signal and is found in both the nucleus and the cytoplasm. In addition to SH2 and SH3 domains, Abl possesses and F actin-binding domain and a DNA-binding domain.

Another modular domain, present in the Btk/Tec subfamily of NRTKs and in many other signaling proteins, is the plecktrin homology (PH) domain. PH domains bind to phosphatidylinositol (PtdIns) lipids that have been phosphorylated at particular positions on the head group (Lemmon and Ferguson, 1998). Concentrations of specific PtdIns lipids in the cell membrane, such as PtdIns-3,4-P3, increase as a consequence of PI-3K activation. Proteins can be recruited to activated signaling

complexes at the membrane through PH domain interactions with phosphorylated PtdIns lipids.

Regulation of Receptor Tyrosine Kinases

Tyrosine Autophosphorylation

Activation of RTKs typically requires two processes: enhancement of intrinsic catalytic activity and creation of binding sites to recruit downstream signaling proteins. For the majority of RTKs, both of these processes are accomplished by autophosphorylation on tyrosine residues, a consequence of ligand-mediated oligomerization. In general, autophosphorylation of tyrosines in the activation loop within the kinase domain results in stimulation of kinase activity, and autophosphorylation of tyrosine in the juxtamembranes, kinase insert, and carboxy-terminal regions generates docking sites for modular domains that recognize phosphotyrosine in specific sequence contexts. The two well-established phosphotyrosine-binding modules present within signaling proteins are the SH2 domain and the phosphotyrosine-binding (PTB) domain (Kuriyan and Cowburn, 1997).

All RTKs thus far identified contain between one and three tyrosines in the kinase activation loop, which comprises subdomains VII and VIII of the protein kinase catalytic core. Phosphorylation of these tyrosines has been shown to be critical for stimulation of catalytic activity and biological function for a number of RTKs, including the insulin receptor, FGF receptor, VEGF receptor, PDGF receptor, Met (hepatocyte growth factor receptor), and TrkA. The major exception to catalytic enhancement via activation loop autophosphorylation is the EGF receptor.

In principle, receptor autophosphorylation could occur in *cis* (within a receptor) or in *trans* (between receptors). In the first case, ligand-induced dimerization would cause a conformational change in the receptor that would facilitate *cis*-autophosphorylation. In the second case, no conformational change need occur upon dimerization; a simple proximity effect would provide sufficient opportunity for *trans*-autophosphorylation to occur. Based on structural studies of the insulin receptor kinase domain, steric considerations indicate that activation loop tyrosines in PTKs can only be phosphorylated in *trans*. Other autophosphorylation sites (e.g. in the juxtamembrane region or carboxy-terminal tail) could potentially be autophosphorylated in *cis*.

Dimerization

Tyrosine phosphorylation is the essential modification that occurs during RTK activation. Ligand-induced oligomerization of RTKs is the mechanism by which tyrosine autophosphorylation is triggered (Ullrich and Schlessinger, 1990; Heldin, 1995). Ligand binding to the extracellular portion of RTKs mediates the noncovalent oligomerization of monomeric receptors or induces a structural rearrangement in heterotetrameric receptors (e.g. the insulin receptor), facilitating tyrosine autophosphorylation in the cytoplasmic domains.

Whether receptor dimerization is sufficient for signal transmission or whether higher-order oligomerization is required has not been fully resolved; it likely depends on the particular ligand-RTK system. For receptors that bind dimeric ligands, such as the PDGF receptor, a receptor dimer is likely to be a competent signaling unit. However, not all dimeric configulations of a receptor are capable of signaling. The introduction of cross-linking cysteine residues into the transmembrane helix of ErbB2, an EGF receptor family member, indicates that ErbB2 activation is dependent on the relative orientation of the two receptors in the dimer (Burke and Stem, 1998). For Eph receptors, biochemical studies show that although a dimeric ephrin is sufficient for receptor autophosphorylation, a tetramer ephrin is necessary to elicit the full range of biological responses in cells (Stein *et al.*, 1998). In most cases, RTK dimerization is probably sufficient for transducing the biological signal.

Ligand binding stabilizes a dimeric configulation of the extraceelular domains of RTKs, but the spatial relationship between the tyrosine kinase-containing cytoplasmic domains within the dimer is not well understood. The cytoplasmic domains may associate only transiently, acting as enzyme and substrate for the other, or they may interact stably to form symmetric (or asymmetric) dimer before and/or after autophosphorylation. For those RTKs whose kinase activity is stimulated via activation loop phosphorylation, the transient association model appears to be consistent with the available biochemical data. In this model, all sites could conceivably by autophosphorylated within the dimer (i.e. higher-order receptor interactions would not be required).

If the two cytoplasmic domains in the ligand-mediated dimer from a stable complex before autophosphorylation, steric constraints would preclude trans-autophosphorylation of a subset of sites (those nearest the kinase domains), in which case higher-order receptor association would be necessary to complete

autophosphorylation. For the EGF receptor, which does not undergo activation loop autophosphorylation, biochemical evidence suggests that a cytoplasmic domain dimer required for catalytic enhancement (Mohammadi *et al.*, 1993; Sherrill, 1997a); autophosphorylated but monomeric EGF receptors are not activated. Interestingly, all of the identified autophosphorylation sites in the EGF receptor are in the long carboxy-terminal tail of the receptor. It is conceivable (from steric considerations) that all of these sites could be autophosphorylated by the cytoplasmic domain dimer, although evidence exists for autophosphorylation occuring between pairs of EGF receptor dimers (Sherrill, 1997b). A further level of positive regulation may come from Src phosphorylation of Tyr-845 in the activation loop (Sato *et al.*, 1995; Biscardi *et al.*,1999).

Regulation of Nonreceptor Tyrosine Kinases

The most common theme in NRTK regulation, as in RTK regulation, is tyrosine phosphorylation. With few exceptions, phosphorylation of tyrosines in the activation loop of NRTKs leads to an increase in enzymatic activity. Activation loop phosphorylation occurs via *trans*-autophosphorylation or phosphorylation by a different NRTK. Phosphorylation of tyrosines outside of the activation loop can negatively regulate kinase activity. PTPs restore NRTKs to their basal state of activity or, in some cases, positively regulate NRTK activity (Tonks and Neel, 1996).

Src Tyrosine Kinase

Src and its family members contain a myristoylated amino terminus, a stretch of positively-charged residues that interact with phospholipid head groups, a short region with low sequence homology, an SH3 domain, an SH2 domain, a tyrosine kinase domain, and a short caboxy-terminal tail. Src possesses two important regulatory tyrosine phosphorylation sites. Phosphorylation of Tyr-527 in the carboxy-terminal tail of Src by the NRTK Csk represses kinase activity (Nada *et al.*, 1991). The importance of this phosphorylation site is underscored by v-Src, an oncogenic variant of Src that is a product of the Rous sarcoma virus. Owing to a carboxy-terminal truncation, v-Src lacks the negative regulartory site Tyr-527 and is constitutively active, leading to uncontrolled growth of infected cells (Cooper, 1986). Moreover, substitution of this tyrosine with phenylalanine in c-Src results in activation (Kmiecik and Shalloway, 1987). A second regulatory phosphorylation site

in Src is Tyr-416, an autophosphorylation site in the activation loop. Maximal stimulation of kinase activity occurs when Tyr-416 is phosphorylated , and a Tyr-416 → Phe mutation can suppress the transforming ability of the activating Tyr-527 → Phe mutation (Kmiecik and Shalloway, 1987).

Both the SH2 and SH3 domains have been implicated in the negative regulation of Src activity (Superti-Furga and Courtneidge, 1995); mutations in the SH2 and SH3 domains that disrupt binding of phosphotyrosine and proline-rich sequences, respectively, activate Src. The mechanisms by which the SH2 and SH3 domains repress Src kinase activity have been elucidated through X-ray crystallographic studies.

Epidermal Growth Factor Receptor (EGFR)

Already in the mid-1970s, experiments were performed that allowed the identification of an EGF-binding, plasma membrane located protein, the EGF receptor. Subsequent biochemical studies revealed an enormous variety of receptor properties.

EGFR Architecture

The EGF receptor is a transmembrane glycoprotein of approximately 170,000 D. A part of the receptor is highly homologous to the chicken v-erb B protein. The external domain of the EGF receptor contains the amino terminus of the molecule, 622 amino acid residues and 12 sites where N-linked glycosylation might occur. Furthermore, the external domain contains 2 cysteine-rich domains. The EGF binding domain of the receptor has been identified to reside between the cystein-rich domains. The EGF receptor contains one hydrophobic domain of 23 amino acid residues which spans the membrane.

The internal domain of the receptor is composed of 542 amino acid residues. It contains a region of approximately 300 amino acid residues that is homologous to the catalytic domain of the protein kinase encoded by the src gene family of oncogenes. Thus the EGF receptor belongs to the class of tyrosine kinase receptors. The C-terminal domain is believed to have a number of regulartory functions. The first element in this domain is the CAIN, necessary for receptor internalization and increased cytosolic Ca^{2+}. This regulartory domain contains also a number of tyrosine residues that can be phosphorylated by the receptor itself. The autophosphorylation tyrosines have been implicated in binding receptor substrates by their SH2 domains (Songyang *et al.*, 1993).

EGFR Activation

The prevalent model of EGFR activation, shared by most RTKs, proposes that the binding of ligand to the extracellular domain induces dimerization. The juxtapostion of intracellular kinase domains promotes the phosphorylation of multiple C-terminal tyrosine residues *in trans*, including Tyr-1068, 1086, 1148 and 1173. Receptor phosphorylation does not increase its kinase activity but, in a fashion comparable with dissociated G-protein subunits, its function is to generate docking sites for downstream signaling molecules. The receptors for platelet-derived growth

factor (PDGF), colony-stimulating factor 1 (CSF-1) and nerve growth factor are RTKs in which individual autophosphorylation sites are strictly required for association with specific signaling molecules. In contrast, no individual EGFR autophosphorylation site appears to be essential for the recognition and association of at least four signaling proteins (Soler *et al.*, 1994). Unregulated RTK activity and mutation of autophosphorylation sites can lead to aberrant signal transduction, as for example occurs in cellular transformation by various *v-erbB* oncogenes (Kato *et al.*, 1987).

There are two general mechanisms by which signaling molecules are regulated by interacting with RTKs: firstly, enzymes may undergo a change in activity when they interact with the receptor. This usually arises through a conformational change in the enzyme or by phosphorylation on specific tyrosine residues by the receptor. Secondly, cytosolic enzymes can be physically juxtaposed with their membrane – bound substrates, upstream activator, or downstream effectors as a result of translocating to the plasma membrane. It is the specific set of intracellular molecules that binds to an activated receptor that will mediate which signal transduction pathways will be stimulated. Cross-talk between these pathways may augment or desensitise their respective signals, presumably in order to finely tune cellular responses.

To date, at least 30 proteins have been found to interact either directly or indirectly with the EGFR. Whether all of these molecules bind a receptor dimer at once is not clear, but steric consideration suggest this is unlikely. One solution lies in the recent identification of the docking proteins Gab1 (Holgado-Madruga *et al.*, 1996) and p62 (Richard *et al.*, 1995). Docking proteins bring several proteins together as a consequence of their multiplicity of homology domain binding sites. Gab1 can bind the EGFR, GRB2, PLCγ, PI3K and PTP-1D, but not Nck (Holgado-Madruga *et al.*, 1996), while p62 can bind PLCγ and GRB2 (Richard *et al.*, 1995). Although Gab1 appears to be a substrate for the EGFR tyrosine kinase, it is not yet clear whether Gab1 remains bound to the receptor via any of their common binding partners, or whether Gab1 complexes are detached from receptor complexes. Adapter proteins perform a similar function to docking proteins but they use multiple homology domains to link activated receptors to downstream enzymatic effectors.

Interaction of the EGF Receptor With the Cytoskeleton

It is tempting to suggest that the actin microfilament system acts as a matrix for growth factor induces signal transduction. This would imply that the components involved in these signal transduction cascades are in one way of another associated with the microfilament system. Evidence accumulated that growth factor receptors, amongst them the EGF receptor, were associated with the cytoskeleton. Thus it was shown by biochemical and ultrastructural methods that 20-25% of the EGF receptor population of A431 cells was insoluble to Triton X-100 (Roy *et al.*, 1989) and thus by definition associated with the cytoskeleton. In contrast, a mutated EGF receptor in which the cytoplasmic domain of the receptor was deleted, did not bind to the cytoskeleton (van Belzen et al., 1990). Further characterization of the cytoskeleton-bound EGF receptors in A431 cells revealed that these receptors belonged to the high affinity class (Wiegant et al., 1986). Considering that the high affinity responses are primarily responsible for EGF-induced signal transduction (Defize *et al.*, 1989; Bellot *et al.*, 1990), these observations suggest that EGF receptor-cytoskeleton interaction is important for the cellular response towards EGF.

Subsequently the association between the EGF receptor and the cytoskeleton was further analyzed in detail. Selective extraction of A431 cells into the three major cytoskeletal fractions, i.e. the microtubules, the microfilaments and the intermediate filaments, and EGF binding studies on these fractions revealed that the EGF receptors were associated with the actin microfilament system (van Bergen en Henegouwen *et al.*, 1992). These results were confirmed by immunofluorescence studies, which demonstrated a clear co-localization between the EGF receptor and the actin microfilaments, but not between the EGF receptor on the one hand and the microtubules, the intermediate filaments or stress fiber actin on the other hand (van Bergen en Henegouwen *et al.*, 1992). Because a wide variety of actin binding proteins are known to present substrates of the EGF receptor, it can not be concluded whether the receptor binds directly or indirectly to actin. Therefore, both the EGF receptor and actin were purified to homogeniety and subsequent co-sedimentation assays demonstrated unequivocally that the receptor itself is an actin binding protein, with no other proteins involved (den Hartigh *et al.*, 1992). Of particular interest was the finding that the amino acid sequence of the EGF receptor domain involving amino

acid residues 986-999 appeared to have homology with the actin binding domain of profilin of *Acanthamoeba* (Vandekerckhove *et al.*, 1989).

In order to establish the possibility that this domain of the receptor indeed represents the actin binding domain, a synthetic peptide was prepared which was identical to the EGF receptor domain 984-996. The peptide was radiolabeled and binding studies indeed demonstrated the ability of the peptide to bind specifically to F-actin *in vitro*. In addition, the peptide was able to compete with purified EGF receptor for binding to F-actin, demonstrating that this receptor domain represents the only actin binding domain of the receptor. In contrast, a peptide identical to receptor sequence 1001-1013 did not bind to actin, nor did it compete with the receptor for binding to actin (den Hartigh *et al.*, 1992).

In addition to the EGF receptor, various components involved in EGF-induced signal transduction also appeared to be associated with the cytoskeleton. Thus it was shown that cytoskeleton isolated from A431 cells contained high activities of PI kinase, PIP kinase, PLC and DG kinase (Payrastre *et al.*, 1991). Like the EGF receptor, these proteins were found to be associated with the actin microfilament system. Interestingly, the cytoskeleton associated kinase activities were significantly increased upon treatment of intact cells with EGF (Payrastre *et al.*, 1991). The EGF induced activation and translocation of PLCγ 1 to the cytoskeleton in rat hepatocytes was demonstrated (Yang *et al.*, 1994).

These findings indeed suggest that the actin microfilament system might function as a matrix to align the signal transduction components. In this respect it is of interest to mention that the EGF-induced actin polymerization is related to membrane ruffling, and that the early signal transduction events occur predominantly in these newly formed membrane ruffles. Thus an extensive study on the localization of F-actin, EGF receptor, PLCγ 1 and tyrosine phosphorylated proteins in A431 cells treated with EGF for 2 and 5 minutes respectively demonstrated that immediately after the formation of the membrane ruffles following addition of EGF to the cells, a strong co-localization was observed between F-actin on the one hand and EGF receptors, PLCγ 1 and tyrosine phosphorylated proteins on the other. This co-localization was most apparent 2 minutes after addition of EGF. In particular the appearance of tyrosine phosphorylated proteins after addition of EGF was confirmed to the membrane ruffles, and only after longer incubation periods in the presence of

EGF these tyrosine phosphorylated proteins appeared throughout the cells. These findings indicated that the membrane ruffles constitute the signal transduction organelles of the cells following addition of EGF, and actin play an essential role in the establishment of these membrane ruffles. Actin is phosphorylated on serine residues upon treatment of the cells with EGF (van Delft *et al.*, 1995). This phosphorylation may play a role in the generation of optimal conditions for signal transduction.

Phosphatidylinositol-3 Kinase (PI3K)

Membrane phospholipids including phosphatidylinositol are key substances mediating the control of cell division. It was supposed for many years that phosphatidylinositol (PtdIns) and its phosphorylated derivatives (PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3) took part in the transduction of mitogen signals only through their hydrolysis by phospholipase C to well known cell division mediators such as diacylglycerol and inositol phosphates. During the recent years these suggestions have been significantly changed, first of all due to the discovery and broad investigation of phosphatidylinositol-3 kinase (PI3K), the enzyme phosphorylating PtdIns in the 3-OH position of the inositol ring.

Initially PI3K was the subject of interest because of its known ability to form complexes with some viral oncoproteins such as v-src and v-ros and also because of involvement of intracellular PI3K in the viral transformation process. Later, in 1997, the possibility of malignant transformation of cells as the result of transfection with DNA containing a fragment of a viral or cellular PI3K gene was shown. Parallel investigations of biochemical properties of PI3K led to rather unexpected results. The two-subunit (regulatory p85 and catalytic p110) molecule of PI3K appears to possess both lipid kinase and protein kinase activity. Activation of the dimeric p85/p110 PI3K molecule occurs through phosphorylation of a tyrosine residue by either receptor (platelet, insulin-like, or epidermal growth factor receptors) or non-receptor (p60-src) tyrosine kinases. Experiments with the use of some specific inhibitors of PI3K and/or cell transfection with different PI3K gene variants reveal PI3K being a mediator in the control of at least two very important cell functions, namely, cell division (as a necessary component of the signaling pathway initiated by growth factors) and apoptosis (the progress of which is inhibited by PI3K).

Recently, clear progress in the investigation of the mechanism of PI3K action has revealed the main mediators of its action. For instance, the event cascade leading to the delay of apoptosis is initiated by complex formation between PtdIns-phosphate products of the PI3K-catalyzed reaction and protein kinase B (PKB, also named Akt or Akt/PKB). The latter enzyme plays an important role in the regulation of the activity of many genes controlling, directly or indirectly, the apoptotic process. PI3K-mediated transduction of mitogen signal is realized in another way. In spite of the

traditional view of PtdIns and its derivatives as the main components of mitogen signal, the role of PI3K in the regulation of cell division appears not to be restricted to synthesis of these compounds. Not so long ago it was shown that PI3K may directly control the activities of individual components of the RAS/RAF/ERK-mitogenic pathway by complex formation with some signal proteins; the enzyme acts in this case as a serine--threonine protein kinase.

Among the other important PI3K functions involved directly or indirectly in mitogen signal transduction, the involvement of PI3K in receptor down-regulation (endocytosis and degradation of activated growth factor receptors), in control of lysosomal enzyme synthesis, and in reorganization of actin cytoskeleton during the course of malignant transformation process and/or mitogen stimulation of cells should also be emphasized.

In general, PI3K is now considered as one of the most important regulatory proteins, being involved in a number of diverse signaling pathways and controlling the main functions of the cell. PI3K activation in malignant cells after exposure to radiation or other stress and also the above-mentioned anti-apoptotic effect of PI3K indicate the important role of this enzyme in the control of both malignant cell resistance to damaging agents and the sensitivity of malignant tumors to chemo- or radiotherapy.

PI3K: General Properties (Krasilnikov, 2000)

Phosphatidylinositol-3 kinase is a heterodimer of two subunits, catalytic and regulatory, with molecular weights of 110 kD (p110) and 85 kD (p85), respectively Cloning experiments with the use of PI3K cDNA have revealed at least five isoforms of each subunit. The regulatory p85 subunit consists of several domains including the SH3 domain, two proline rich fragments, and two SH2 domains separated by the iSH2 (inter SH2) sequence. The iSH2 domain provides the interaction between the p85 and p110 subunits, and the two SH2 domains are responsible for binding of the p85/p110 heterodimer with receptor tyrosine kinases. It is supposed that due to the ability of the regulatory p85 subunit to interact with both the catalytic p110 subunit and receptor tyrosine kinases directed membrane targeting of p110 occurs, initiating complex formation between the enzyme and its phospholipid substrate.

The catalytic p110 subunit of PI3K is homologous to protein kinases and possesses both serine--threonine protein kinase and phosphoinositide kinase activities. Phosphorylation of PtdIns and phosphoinositides PtdIns(4)P and PtdIns(4,5)P_2 occurs in the D_3-position of the inositol ring leading to formation of PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, respectively. Three classes of the PI3K protein superfamily are now known. All of these possess the protein kinase activity, the difference is preferentially in the substrate specificity of the phosphoinositide kinase site. Thus, the first class includes p85/p110 heterodimers reacting with all phosphoinositides, PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2. These are now often referred to as phosphoinositide-3 kinases; the term reflects their substrate specificity more correctly than the traditional name, phosphatidylinositol-3 kinases. The second class involves enzymes phosphorylating preferably PtdIns and PtdIns(4)P. Finally, the third class includes PI3K that possesses additionally a specific protein transfer function and has a structural and functional resemblance with the yeast analog of PI3K, vps34p (vacuolar protein sorting). Unlike the enzymes from the first and the second classes, this one uses only PtdIns as a substrate.

Two main processes lead to PI3K activation: p85/p110 heterodimer assembly and interaction of the heterodimer with activator proteins. As mentioned above, the binding of the catalytic and regulatory subunits occurs via the iSH2 domain of the latter. The p85/p110 heterodimer assembly does not result itself in marked enzyme activation. Moreover, some investigators reported the activity of the catalytic subunit being decreased when the p85/p110 complex is formed *in vitro*. An additional interaction with specific activator proteins is required for the subsequent activation of the heterodimer. The main activator proteins have tyrosine-phosphorylated amino acid sequences including both some receptor (receptors of platelet, epidermal, or insulin-like growth factors), and non-receptor (p60-src) tyrosine kinases. The binding of phosphotyrosine sites of activator proteins with SH2 domains of the PI3K regulatory subunit causes a conformational change of the heterodimer leading to enzyme activation.

There are, however, other mechanisms of PI3K activation. An extra activation of the heterodimer may occur while direct interaction between the PI3K catalytic subunit and one of several cellular proteins takes place. A good example is the

complex formation between p21-ras and p110 resulting in activation of PI3K. The diversity of ways for PI3K activation to occur and also the multi-substrate specificity and double-enzymatic activity (lipid kinase and protein kinase) of the enzyme likely determine its key role in the control of cell growth and survival.

PI3K Signaling Pathway (Krasilnikov, 2000)

Investigations of recent years have shown the involvement of PI3K in the control of cell division being realized through at least two possible mechanisms: the first includes phosphoinositide production (PI3K lipid kinase activity), the second includes direct interaction of PI3K with some cellular signal proteins, when the protein kinase activity of PI3K may play a critical role.

Traditional opinions concerning the role of phosphoinositides in cell growth control were based on their role as substrates of phospholipase C, the enzyme which is activated by receptor tyrosine kinases during cell division. When accumulated in the cells due to phospholipase C action, the hydrolysis products of phosphoinositides (diacylglycerol and inositol phosphates) activate protein kinase C, thus stimulating one of the most important signaling pathways of the cell. However, as was shown later, the phosphoinositides may have an independent significance in mitogen signal transduction, because of their ability for direct interaction with some signal proteins. The role of phosphoinositides in activation of protein kinase B (PKB), which is involved preferentially in the control of cell apoptosis, will be considered below. As for the protein mediators of cell division, protein kinase C (PKC) should be distinguished first of all. The PKC activation appears to occur not only via binding with diacylglycerol formed by hydrolysis of phospholipids, but also via the interaction with PI3K lipid products. Great progress in the study of the control mechanisms of PI3K was achieved following the discovery of a new family of serine--threonine protein kinases, phosphoinositide-dependent kinases (PDK). These are activated by the lipid products of PI3K, 3OH-phosphoinositides (hence the name of the family), and responsible for the phosphorylation and activation of a number of signaling protein kinases, including both PKB and PKC. Thus, two steps of PI3K-dependent activation of protein kinase C may be distinguished: PKC interaction with diacylglycerol, the phosphoinositide hydrolysis product, and phosphorylation of PKC by PDK family enzymes. Also, the data obtained from the studies on binding of

phosphoinositides with SH2-containing proteins should be taken into account. These indicate one of the PI3K products, PtdIns(3,4,5)P_3, interacts with SH2-domains of proteins competing with phosphotyrosine peptides. The same effect may exist for an additional pathway of activation of SH2-containing signaling proteins which is independent from receptor tyrosine kinases.

The subject of particular interest is the involving of PI3K in receptor «down-stream» processes, including endocytosis and degradation of activated growth factor receptors. As known, binding of ligand with growth factor receptor and activation of its phosphotyrosine kinase domain is followed by internalization of the receptor into intracellular vesicles and its consequent degradation in lysosomes. The whole process and, in particular, the activated receptor transfer into lysosomes appear to be under PI3K control. For instance, the studies on the down-stream handling of platelet growth factor receptor have revealed that some mutations in the phosphotyrosine site of the receptor molecule responsible for PI3K binding may cause an almost total blockage of the receptor transfer into lysosomes. The same effect is caused by wortmannin, a specific PI3K inhibitor. However, PI3K possesses an ability to direct control of lysosomal enzyme activity by stimulation of the transfer of *de novo* synthesized hydrolases into lysosomes.

The ability of PI3K to direct binding with some cellular proteins and also the fact that the enzyme possesses not only lipid kinase, but also protein kinase activity opened new opportunities for studying its role in intracellular signaling pathways. The p85/p110 heterodimer *in vivo* forms complexes with a broad spectrum of cellular molecules including tyrosine kinases, Grb2, p21-ras, rac, Cdc42, tubulin, etc. The subject of principal significance for understanding the role of PI3K in the control of RAS/RAF/ERK signaling pathway is complex formation between the catalytic subunit of PI3K and p21-ras. PI3K binds only with the GTP-form of ras resulting in PI3K activation observed both *in vitro* and *in vivo*. The same PI3K activation effect occurs when the heterodimer p85/p110 binds some other G-proteins, for instance, rac or Cdc42. And, on the other hand, complex formation between PI3K and p21-ras is accompanied by an increased amount of activated (GTP-bound) form of ras. Moreover, the presence of PI3K appears to be necessary both for stimulation of the RAS/RAF/ERK pathway and for induced transformation of cells, and, in addition, in

some cases PI3K activity inhibition may cause total blockage of transformation. It should be mentioned that the mutual control between PI3K and p21-ras is rather complex and does not corresponded to a linear model of mitogen signal transfer. PI3K is supposed to activate p21-ras (possibly via membrane targeting of SOS-proteins) and is activated simultaneously via its binding with the GTP-form of ras or other G-proteins.

However, the significance of PI3K for the RAS/RAF/ERK signaling pathway is not limited by its influence on p21-ras. Recently, the important role of serine--threonine protein kinase activity of PI3K in the control of cellular MAP-kinases was demonstrated. Experiments using different classes of PI3K whose lipid and protein kinase activity components differ revealed that only the protein kinase activity of PI3K causes the activation of cellular MAP-kinases. The level of the synthesis of phosphoinositides (lipid products of PI3K) did not influence markedly the MAP-kinase activities. Thus, the general scheme of PI3K-dependent control of cellular mitogen-transduction signaling pathways consists of several stages, the main being: PI3K activation via the binding of p85/p110 heterodimer with tyrosine-phosphorylated proteins and/or small G-proteins (p21-ras, rac, Cdc42); the synthesis of 3-OH phosphoinositides which are the sources of both diacylglycerol and inositol phosphates and activators of some protein kinases (PKB, PKC, PDK) they can directly interact with; serine--threonine phosphorylation of secondary PI3K messengers and the activation of MAP-kinases. Also, the scheme of PI3K involvement in cellular metabolism should be supplemented with PI3K-dependent control of stress-activated signaling pathways.

PI3K in the Control of Apoptosis (Krasilnikov, 2000)

Among other cellular protein targets of PI3K, a particular role belongs to these which are involved in the cell response to stress. Moreover, although in the control of cell division PI3K plays a rather secondary role, its role in the control of cell survival and resistance to stress is a key one. This conclusion was made on the basis of the discovery and investigation of a PI3K/PKB-dependent signaling pathway.

The mitogenic activity of growth factors and cytokines does not always correlate with their ability to prevent cell death. The platelet and insulin-like growth

factors are good examples of compounds possessing anti-apoptotic activity. However, the fibroblast growth factor or the epidermal growth factor possessing high mitogenic activity have a negligible influence on cell survival. A partial explanation of these facts was obtained from comparative studies on signaling pathways activated by growth factors in target cells. A stimulation of the traditional RAS/RAF/ERK-pathway usually does not result in significant anti-apoptotic effect. The ability to prevent apoptosis was detected in serine--threonine protein kinase B (PKB), which is activated by some growth factors. Studies on PKB activation pathways have shown that PI3K is a mediator of an activator signal for PKB.

PI3K is now considered as one of the main intracellular factors responsible for the transmission of anti-apoptotic signal and controlling the survival of cells. For instance, overexpression of PI3K in cells is accompanied by a strongly marked anti-apoptotic effect and causes a significant increase in cell survival under the influence of radiation. On the contrary, PI3K specific inhibitors cause increased apoptosis and decreased cell survival. The data obtained from many experiments indicate the PKB activation by complex formation between this enzyme and lipid products of PI3K is a key event in the realization of the anti-apoptotic effect of PI3K. There are two main stages of PKB activation: the binding of PH (pleckstrin homology)-domain of PKB with PtdIns(3)P and/or PtdIns(3,4)P_2, the main products of lipid kinase reaction catalyzed by PI3K, and the phosphorylation in Thr-308 position by PDK-1 kinase (phosphoinositide-dependent kinase-1). PI3K-dependent activation of PKB occurs independently from the influence of PI3K on the RAS/RAF/ERK pathway: in the first case, the binding of PKB with lipid products of PI3K is sufficient, but in the second one, as mentioned above, the involvement of the protein kinase component of PI3K in the effect of PI3K on MAP kinases is strongly necessary.

What is the further route of the signal from PKB and what is the nature of signaling pathways leading from PKB and controlling the survival of cells? Several mechanisms independently activated via PKB which can lead to the block of apoptosis are known at present. First, it should be mentioned that no basic proteins belonging to the Bcl family of the most widely distributed negative apoptosis regulators are known among the direct targets of PKB. The only exception known is Bad, which being phosphorylated by PKB is prevented from its binding to Bcl-2.

Proteases of the caspase family are known to be PKB mediators, which are activated during apoptosis. PKB inhibits their activities; this property may serve as a basis of its anti-apoptotic effect. Another possible effector of PKB is p70 S6 kinase, which possesses a distinct anti-apoptotic property and, being phosphorylated directly by PKB kinase, displays increased activity. Finally, data obtained from recent investigations have revealed an important role of GSK-3 (glycogen synthetase kinase-3) in the induction of programmed cell death. The direct phosphorylation of GSK-3 by PKB kinase leading to a drastic decrease in GSK-3 activity is one of its regulatory mechanisms.

Undoubtedly, the scheme of signal transduction pathways initiated by PI3K and PKB presented here is rather incomplete. Knowledge on the nature of secondary messengers involved in signal promotion from PI3K and PKB is expanding from day to day; new data on the role of these substances in the control of cell response to stress are appearing. For instance, new data were obtained on the involvement of integrin-associated protein kinases (ILK, integrin-linked kinase) in realization of the anti-apoptotic effect of PI3K. The problem on the role of stress-activated kinases (JNK family) in the control of apoptosis and signal transduction from PI3K and PKB is under comprehensive study.

PI3K and Cell Aging (Krasilnikov, 2000)

The ability of PI3K to control key functions of the cell such as proliferation or apoptosis became the stimulus for studying the role of PI3K in the control of cell aging, another key function of the cell. The cell aging phenomenon is known to develop after the cell achieves the Hayflick limit, i.e., after the cell has passed through a definite number of divisions to the resting stage. The passage of cells to resting stage ahead of time and initiation of cell aging is appreciated by some researchers as one of the programs of defense resembling programmed cell death (apoptosis) and is activated under the influence of damaging agents. The fact that some substances such as ceramide involved in mitogen signal transduction and possessing apoptotic activity may (unlike traditional mitogens) effectively influence on the rate of cell aging is indirect evidence for that assumption.

Now several experimental proofs have been obtained that indicate that PI3K is involved in the control of aging. First of all, experiments with *Caenorhabditis elegans* reveal a homology between *Age1*,one of the genes of aging, and the gene encoding the PI3K catalytic subunit in mammals and demonstrate the involvement of *Age1* in the control of development of *C. elegans*. Convincing evidence for the involvement of PI3K in the control of cell aging have been obtained from the experiments on normal fibroblasts *in vitro*. Comparative analysis of the effects of PI3K inhibitor LY2940002 and MEK-1 (kinase phosphorylating ERK1/ERK2) inhibitor PD58029 on fibroblasts has shown that both substances inhibit cell proliferation. However, only in the first case (when the PI3K inhibitor acts) cell growth retardation was accompanied by a complex of specific phenotypic alterations which are normally typical for aging fibroblasts: galactosidase activation, overexpression of collagenase gene, and decreased expression of *EPC-1* gene (early population doubling level cDNA 1) which is a specific marker of proliferating fibroblasts. This suggests that under PI3K-dependent control of cell aging the components of the anti-apoptotic signaling pathway controlled by PI3K and independent of the RAS/RAF/ERK cascade have the most important significance. Apparently, it is the activation of the anti-apoptotic pathway and especially PKB that mainly determines the involvement of PI3K in the control of cell aging.

PI3K in Malignant Transformation of Cells (Krasilnikov, 2000)

As mentioned above, initial interest in PI3K was due mostly to the involvement of PI3K in malignant transformation of cells. Some viral oncoproteins such as src, abl, T-antigen form complexes with PI3K, and the presence of PI3K in a cell is required for realization of their transforming potential. Experiments with mutant forms of those proteins have shown that the loss of their ability to form complexes with PI3K results in a dramatic decrease in the transforming activity of the oncoproteins. As a result of complex formation between oncoproteins and PI3K, the enzyme becomes activated and the level of PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 increases; also, the transforming efficiency decreases in accordance with the decrease in intracellular concentration of phosphoinositides.

The question on the independent oncogenic activity of PI3K remained without answer for a long time, but in 1997 the viral analog of a gene encoding the PI3K

catalytic subunit p110 was found in the genome of ASV 16 (avian sarcoma virus 16). Studies on the transforming potential of the gene encoding the catalytic subunit p110 named *p3k* have shown that both viral and cellular variants of this gene cause morphologic transformation in chicken embryo fibroblasts *in vitro*. What components of the PI3K-induced signaling pathway take a direct part in malignant transformation of cells? Now, with no doubt, these are the lipid products of PI3K and protein kinases they activate, such as the above-mentioned protein kinase B (PKB) encoded by the Akt protooncogene. The transformation of cells by the *p3k* gene is shown to be accompanied by a drastic increase in both the PKB activity and the rate of phosphorylation of endogenous substrates. An important role in the process of PI3K-dependent transformation belongs to the stimulation of the RAS/RAF/ERK-kinase cascade and the increase in AP-1 transcription factor activity. The complex changes in cellular signaling pathways, namely, the appearance of constantly generated PI3K-dependent mitogen signal and the activation of some protooncogenes (*src*, *ras*, *rac*, etc.), and also PKB-dependent pathway stimulation, which leads to partial blockage of apoptosis and increased survival of cells, are possibly the basis for the transforming effect of PI3K.

Studies on the role of PI3K in the formation of the actin cytoskeleton deserve separate attention. It is well known that both the induction of cell proliferation and the malignant transformation of cells are accompanied by reorganization of the actin cytoskeleton. Data from recent studies indicate that one of the key elements in the control of this process is PI3K. It has been shown that PI3K activation appears to be an essential condition for reorganization of actin filaments in a cell. Oncoprotein rac, which belongs to the small G-protein group and is involved in signal transduction via the stress-activated kinases of JNK family, is considered as a possible effector of PI3K. The other possible pathway for PI3K to control actin assembly is based on the lipid kinase activity of PI3K. The main role in this pathway belongs to one of the PI3K substrates, PtdIns(4,5)P_2, which interacts with actin binding proteins, thus stimulating the actin polymerization process. Intracellular activation of PtdIns(4,5)P_2 metabolizing enzymes (phospholipase C and PI3K), in particular, under the influence of mitogenic/oncogenic factors, leads to a decreased amount of PtdIns(4,5)P_2 bound with actin and, as a result, to actin depolymerization.

The discovered oncogenic activity of PI3K is evidence for an important role of the enzyme in carcinogenesis and tumor growth. In fact, some data indicate changes in phosphoinositide level during malignant tumor progression. Our study on PI3K expression in breast cancer tumors has revealed in 79% of cases a significant activation of PI3K compared to adjacent normal tissue. It was recently shown that the effect of some tumor growth suppressor genes are activated via PI3K inhibition. One of these is a product of suppressor gene product PTEN/MMCA1, a phosphatase which dephosphorylates PtdIns(3,4,5)P_3. Specific inhibitors of PI3K (wortmannin, LY 294002) cause a significant retardation of cell growth in culture and prevent the cells from being malignantly transformed *in vitro*. However, the problem concerning the anti-tumor activity of PI3K antagonists or inhibitors and, on the whole, their usage in cancer therapy is far from completely resolved. Further investigations are required.

In general, the progress achieved in recent years in studying PI3K allows us to place this enzyme among the main signaling proteins of the cell. The diversity of ways for PI3K activation and also its unique biochemical properties (multisubstrate specificity and both lipid and protein kinase activity) determine its critical role in the control of key functions in the cell: growth and survival, aging, malignant transformation. The main PI3K effectors are mitogen-transducing signaling proteins (protein kinase C, phosphoinositide-dependent kinases, small G-proteins, MAP-kinases) which are activated either via interaction with lipid products of PI3K or through PI3K-dependent phosphorylation of proteins. The anti-apoptotic effect of PI3K is realized through the activation of proteins from another regulatory pathway, the protein kinase B (PKB) and PKB-dependent enzymes (GSK-3, ILK).

Nevertheless, the mechanism of some PI3K effects, such as PI3K-dependent control of malignant transformation, remain unclear. PI3K possesses a direct oncogenic activity and also potentiates the effects of other oncogenes activating and increasing the transforming activity of many of the known oncoproteins (ras, rac, Akt, src). A number of cell damaging factors cause a drastic increase in PI3K activity. That activation of PI3K leads to delay of apoptosis in cells with damaged DNA structure and also causes an extra activation of cellular oncoproteins and stimulates uncontrolled cell growth and, hence, may have perhaps great significance in carcinogenesis.

The problem of malignant transformation is closely related to another PI3K function, the control of cell aging. A decrease in PI3K activity causes an increase in aging rate in normal cells, but the mechanism of PI3K-dependent control of cell aging and the role of individual effectors of PI3K are still unknown. It may be that, as in the case of PI3K-induced malignant transformation, the activation of mitogen-dependent proteins and (as appears to be the most important) the anti-apoptotic signal constantly generated by PI3K play an important role in the control of aging. However, studies in this field are just beginning, and it is unclear whether the PI3K activity changes during cell aging and the overexpression of PI3K leads to significant delay in the aging process. Undoubtedly, in the near future studies will answer these and other questions on the mechanism of the effect of PI3K in normal and malignant cells.

It should be noted in conclusion that PI3K, when being identified as one of the key signaling proteins, lets us tie together many events which occur in cells under the action of mitogenic or oncogenic factors and also stress, and, at first glance, seem to be independent. Every year new data appear concerning the description of novel PI3K effectors or revealing a correlation between known cell proteins and PI3K-dependent signaling pathways. We are on the way to understanding the basic principles of coordinated control of biochemical signaling pathways and their significance for fundamental cell properties: growth, aging, transformation.

Ras Signaling Pathway and MAP Kinase (Karp, 1999)

A key component of many RTK reaction cascades in the protein **Ras**. The *Ras* gene was originally discovered as a viral oncogene, that is, a gene carried by certain tumor viruses that enables the virus to transform cells to a malignant state. Subsequent research showed that, like other oncogenes, *ras* was also present as part of the normal genome of animals, including humans. During the early 1980s, several human tumor cells were found to contain a mutant version of *ras*. Subsequent studies have found that an altered version of the *ras* gene is found in about 30 percent of all human tumors. Given its importance in the development of human cancers, the product of the *ras* gene, a protein called Ras, became a focus of research.

Ras is small G protein residing at the inner surface of the plasma membrane, and is a member of several signaling pathway. Unlike heterotrimeric G proteins, Ras consists of a single small (21kDa) subunit. Like other G proteins, Ras cycles between an inactive GDP-bound form and an active GTP-bound form. In its active form, Ras stimulates effectors that lie downstream in the signaling pathway. Ras has a very weak GTPase activity and, left by itself, would remain in the active, GTP-bound state for 30 minutes or so. In the cell, Ras activity is regulated by a number of GTPase-activating proteins (GAPs) that stimulate the Ras GTPase about 10^5-fold and return the protein to the inactive state. Ras-GAPs have a unique way of stimulating the enzyme activity of their Ras target: they provide an amino acid residue (an arginine) that becomes a critical part of the active sites of the Ras GTPase. Mutation in the *ras* gene that lead to tumor formation prevent the protein from hydrolyzing the bound GTP back to the GDP form, even in the presence of the GAP. As a result, the mutant version of Ras remains in the "on" position, sending a continual message downstream along the signaling pathway, keeping the cell in the proliferative mode. Disease involving abnormal cell proliferation also result from alterations in Ras-GAPs. Mutations in one of the Ras-GAP genes (*NF1*) cause neurofibromatosis 1, a disease in which patients develop large numbers of benign tumors (neurofibromas) along the sheaths that line the nerve trunks.

In its best-studied role, Ras is a key component of a signaling pathway that leads all the way from the outer surface of the plasma membrane to the DNA of the nucleus. The pathway is activated when a growth factors, such as EGF or PDGF, binds to the extracellular domain of its receptor. The step in the Ras signaling

pathway that follow binding of a growth factor to an RTK are depicted in next figure. Briefly, the phosphotyrosines that are generated in the cytoplasmic domain of the RTK by autophosphorylation act as binding sites for a specific SH2 protein called Grb2. Grb2 is not a protein with catalytic activity, but one that functions solely as an adaptor molecule that links other proteins into a complex. The structure of Grb2 can be seen to consist of three distinct domains, each of which binds to an other protein. One domain of the Grb2 molecule binds to the phosphorylated RTK at the inner surface of the membrane, while another domain binds to a protein called Sos.

Sos is a guanine nucleotide exchange factor for Ras, that is, a Ras-GEF. GEFs activate G proteins by stimulating the exchange of GDP with GTP. In the unstimulated cell, Ras remains bound to GDP. When a ligand binds to the RTK and recruits the Grb2-Sos to the inner surface of the membrane, the Sos protein binds to Ras, causing it to lose its GDP, which is replaced by GTP, thus activating Ras. The primary, and possibly sole, function of Ras-GTP is to recruit another protein, called Raf, to the plasma membrane. Once it is localized at the plasma membrane, Raf becomes activated as a protein kinase that initiates an orderly chain of phosphorylation reactions called the **"MAP kinase cascade"**. The MAP kinase cascade is similar to the cascade of reactions triggered by cAMP during glucose mobilization, but even more complex. Once activated, the last protein kinase in the cascade (MAPK) translocates to the nucleus where it phosphorylates and activates specific transcription factors, such as Elk-1. Elk-1 binds to the promoter regions of a number of genes, including *c-fos* and *c-jun*. The products of these genes, Fos and Jun, interact to form a heterodimeric transcription factor called AP-1 that activates genes involved in cell proliferation.

The same basic pathway from an RTK through Ras to the activation transcription factors is found in all eukaryotes investigated, from yeast through flies and nematodes to mammals. Evolution has adapted the pathway to meet many different ends. In yeast, for example, the MAP kinase cascade is required for maintenance of cell shape and to respond to mating pheromones; in fruit flies, the pathway is utilized during the differentiation of the photoreceptors in the compound eye; and in flowering plant, the pathway transmits signals that initiate a defense against pathogens. Mammals have a number of different isoforms for each of the proteins that make up the MAP kinase cascade. Pathways consisting of different isoforms can transmit different types.

VITAE

Flight Officer Teerayut Suramana

Personal Data

Date of Birth May 15, 1966

Place of Birth Bangkok, Thailand

Education

1980-1985 Bordindeja (Sin Sinhaseni) High School, Bangkok, Thailand

1986-1990 Bachelor of Science in Pharmacy
Chulalongkorn University, Bangkok, Thailand

1998-1999 Master of Science in Toxicology
Faculty of Medicine, Chulalongkorn University

1999-2003 Doctor of Philosophy in Biopharmaceutical Sciences
Faculty of Pharmaceutical Sciences, Chulalongkorn University

Experiences

1990-1991 Hospital Pharmacist
Wing 71 Hospital Suratthani, Royal Thai Air Force

1991-1993 Production Pharmacist
Suphong Pharmaceutical Company, Bangkok

1993-1995 Product Research and Development (R&D) Pharmacist
Suphong Pharmaceutical Company, Bangkok

1995-1998 Medical Representative (Up-Country)
Takeda (Thailand), Bangkok

1993-now Community Pharmacist
Teerayut Pharmacy, Bangkok and Nonthaburi

2003-now Lecturer in Pharmacology and Toxicology Unit,
Department of Medical Sciences, Faculty of Sciences,
and Health Science Research Institute, Research Institute,
Rangsit University, Pathumthani