Cell Cycle- and Apoptosis-regulatory Protein-1 Is Involved in Apoptosis Signaling by Epidermal Growth Factor Receptor*

Arun K. Rishi1,5‡§, Liyue Zhang1,5, Yingjie Yu1,5, Yan Jiang1,5, Jyoti Nautiyal5, Anil Wali4†§, Joseph A. Fontana1,5, Edi Levi5, and Adhip P. N. Majumdar1,5†§

From the 1Veterans Affairs Medical Center and the Departments of 5Internal Medicine and 4Surgery and the 4Karanos Cancer Institute, Wayne State University, Detroit, Michigan 48201

CARP-1, a novel apoptosis inducer, regulates apoptosis signaling by diverse agents, including adriamycin and growth factors. Epidermal growth factor receptor (EGFR)-related protein (ERRP), a p38 inhibitor, inhibits EGFR and stimulates apoptosis. Treatments of cells with ERRP or Iressa (an EGFR tyrosine kinase inhibitor) results in elevated CARP-1 levels, whereas antisense-depletion depletion of CARP-1 causes inhibition of apoptosis by ERRP. CARP-1 is a tyrosine-phosphorylated protein, and ERRP treatments cause elevated tyrosine phosphorylation of CARP-1. CARP-1 contains multiple, nonoverlapping apoptosis-inducing subdomains; one such subdomain is present within amino acids 1–198. Wild-type or CARP-1-(1–198) proteins that have substitution of tyrosine 192 to phenylalanine abrogate apoptosis within amino acids 1–198. Wild-type or CARP-1-(1–198) proteins that promote apoptosis-inducing subdomains; one such subdomain is present within amino acids 1–198. Wild-type or CARP-1-(1–198) proteins that have substitution of tyrosine 192 to phenylalanine abrogate apoptosis by ERRP. In addition, apoptosis mediated by wild type or CARP-1-(1–198), and not CARP-1-(1–198)Y192F, results in activation of caspase-9 and increased phosphorylation of p38 MAPK. However, the expression of dominant-negative forms of p38 MAPK activators MKK3 or MKK6 proteins inhibits apoptosis induced by both the full-length and truncated (amino acids 1–198) proteins. Together, data demonstrate that tyrosine 192 of CARP-1 is a target of apoptosis signaling, and CARP-1, in turn, promotes apoptosis by activating p38 MAPK and caspase-9.

Programmed cell death (apoptosis) is essential for the development and maintenance of cellular homeostasis. The pathways regulating apoptosis serve as important targets for many anti-cancer agents currently utilized for treatment of diverse malignancies. Recently, we reported identification of a novel perinuclear protein CARP-1, also known as CCAR1 (1). CARP-1 regulates apoptosis signaling by chemotherapeutic agent adriamycin and by a novel retinoid [3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437), [3-(1-adamantyl)-4-Hydroxyphenyl]-2-naphthalene carboxylic acid belongs to a novel class of apoptogenic adamantyl retinoids that induce growth arrest and stimulate apoptosis in a wide variety of malignant cell types, including breast and prostate cancer and leukemia, by an retinoid acid receptor/retinoid X receptor-independent mechanism (2–4). Expression of CARP-1, on the other hand, induces apoptosis while causing elevated levels of CDK1 p21WAF1/CIP1 and inhibiting the expression of a number of cell cycle-regulatory proteins such as c-Myc, cyclin B, and topoisomerase IIa (1). Whereas it is evident that CARP-1 is an important apoptosis signal transducer, it is unclear how CARP-1-dependent apoptosis is accomplished. Understanding the apoptosis-inducing pathways utilized by CARP-1 will define mechanism(s) of action of agents such as adriamycin.

Members of the EGFR family of receptor tyrosine kinases, which includes EGFR, ErbB-2/HER-2, ErbB-3/HER-3, and ErbB-4/HER-4, collectively referred to as EGFRs, serve as critical mediators of the cellular communication network regulating complex biological processes such as growth, differentiation, motility, or death (5, 6). Since deregulated signaling by EGFRs is frequently noted in a variety of human cancers (7–9), interference with growth factor receptor activation and/or with intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of novel and selective anti-cancer therapies (10–12). Small molecule inhibitors of EGFR, such as gefitinib (Iressa) and erlotinib (Tarceva; OSI-774), monoclonal antibodies to EGFR (cetuximab/IMC-C225/Erbux), and HER-2 (trastuzumab/Herceptin) are being utilized as anti-cancer therapeutics.

ERRP, a recently isolated pan-EGFR inhibitor, is a 53–55-kDa protein that possesses substantial homology to the extracellular ligand-binding domain of EGFR and its family members (13). ERRP is a secretory protein that forms an inactive heterodimer with EGFR, causing inhibition of EGFR-dependent signaling events (14). ERRP inhibits proliferation and induces apoptosis as well as attenuates ligand-induced activation of EGFR and HER-2 in cancer cells that express varying levels of EGFR and other member(s) of its family, specifically HER-2 (14–16). However, the intracellular signaling pathways involved in ERRP-dependent inhibition of cell growth and induction of apoptosis remain to be delineated.

In light of the fact that CARP-1 regulates cell growth and that growth factors regulate CARP-1, we speculated that CARP-1 is a key intracellular transducer of growth factor receptor-dependent apoptosis signaling. To test our hypothesis, we investigated apoptosis-inducing pathways that are activated following inhibition of EGFRs. Herein we report that treatments of cells with ERRP that results in inhibition of constitutive activity of EGFR enhances expression of CARP-1 and apoptosis and that tyrosine phosphorylation of CARP-1 is essential for its regulatory function. Our studies also reveal that apoptosis induction by CARP-1 involves elevated phosphorylation of mitogen-activated protein kinase p38 and activation of proapoptotic caspase-9.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, Ham’s F-12 medium, fetal bovine serum, and a Lipofectamine-based transfection kit

2 The abbreviations used are: EGFR, epidermal growth factor receptor; RNAi, RNA interference; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; ER, endoplasmic reticulum; eIF-2a, eukaryotic initiation factor 2a; WT, wild type.
were purchased from Invitrogen. Anti-EGFR (clone LA22) and anti-phospho-EGFR (Y1173, clone 9H2) antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-SAPK-4 (p38α) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphoysine and anti-phosphoserine antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA), whereas phospho-p38 (Thr180/Tyr182) 28B10 monoclonal antibody and antibodies for procaspase-3, caspase-9, Myc epitope tag, p38(α/β), and eIF-2α (phospho- and nonphospho-) were purchased from Cell Signaling (Beverly, MA). The caspase-9 colorimetric assay kit was purchased from BioVision Research Products (Mountain View, CA). Phosphoehichrom- 

were purchased from BD Biosciences.

**Recombinant Plasmid Constructs**—The construction of plasmid clone 6.1 expressing Myc–His-tagged wild-type CARP-1 has been described before (1). The plasmids pRC/RSV-FLAG-MKK3(Ala) and pcDNA3-FLAG-MKK6(Ala) encoding dominant-negative MKK3 and MKK6 proteins, respectively, were a kind gift from Dr. Ajay Rana (Texas A&M University, College Station, TX) and have been described before (17). The plasmid pRC/RSV-FLAG-MKK3(Ala) encodes a FLAG-tagged MKK3 mutant protein where threonine 193 of the wild type MKK3 has been replaced with alanine, whereas plasmid pcDNA3-FLAG-MKK6(Ala) encodes a FLAG-tagged MKK6 mutant protein where lysine 82 of the wild type MKK6 has been replaced with alanine (17). The ERRP-Myc–His-tagged clone 1 plasmid (14) was digested with a combination of HindIII and PmeI restriction enzymes, followed by purification of a cDNA fragment encoding Myc–His-tagged ERRP and its subsequent ligation into vector plasmid pLNCX2 (BD Biosciences/Clontech) to generate plasmid pLNCX2-ERRP-Myc–His clone 1.

Various recombinant plasmids for expression of mutant CARP-1 proteins (see Table 1) were generated by a combination of standard PCR amplification and cloning methodologies (18). CARP-1 cDNA fragments derived from PCR amplification and/or digestion with specific restriction enzyme(s) were subcloned into the vector plasmids pcDNA3/Myc-HisA (Invitrogen) or pLNCX2 (BD Biosciences/Clontech) to generate constructs that encode CARP-1 proteins having Myc and His tags positioned at the carboxyl termini (see Table 1). A construct encoding the CARP-1 (1–198) protein having tyrosine at position 192 substituted with phenylalanine was generated by subcloning a 0.6-kb PCR-amplified CARP-1 cDNA fragment. This PCR amplification utilized CARP-1 sense oligonucleotide AS-6.1 (1) and an antisense oligonucleotide AS-6.31 (5'-ACCCTCTCCCAGGAT-ACA-3') and 1901–1919 (5'-GCCGCCACGTGTCGAGAAG-3') were directionally subcloned as double-stranded fragments at the unique BamHI and EcoRI sites of the vector plasmid pSIREN-RetroQ (BD Biosciences/Clontech). The plasmids along with the empty vector were transfected into PT-67 cells, and stable, puromycin-resistant sublines were obtained for production of CARP-1 RNAi retroviruses. The retroviral titers were determined as before (1). For RNAi-mediated CARP-1 knockdown, HBC cells were transduced with retroviral supernatants for 5–7 days, and levels of CARP-1 were determined by Western immunoblotting. In addition, RNAi-transduced cells were either untreated or treated with purified ERRP for an additional 48 h, followed by determination of apoptosis as below.

**Immunoprecipitation, Western Blot, Apoptosis, and Caspase-9 Assays**—Logarithmically growing cells were either untransfected or transfected with various plasmids for 24 h, followed by their lysis to prepare protein extracts. In certain instances, cells were treated with immunoadfinity-purified ERRP or commercially available EGFR inhibitor Iressa. For immunoprecipitation, aliquots of cell lysates containing 1.0 mg of proteins were incubated with the indicated antibodies and Sepharose G at 4 °C for 3 h. Immunoprecipitates or the protein extracts were electrophoresed on 9–12% SDS-polyacrylamide gels, followed by their transfer to nitrocellulose membranes. The filters were independently probed with the indicated antibodies per the manufacturer’s guidelines and following previously published protocols (14–16). For enrichment of phosphorylated proteins, the cells were first transfected with plasmids encoding wild-type or mutant CARP-1 proteins, followed by treatments of cells with immunoadfinity-purified ERRP for various times. Equal amounts (1 mg) of cell lysates were then applied to phosphoprotein enrichment columns; the complexes were washed and eluted following the manufacturer’s suggested guidelines. The enriched phosphoproteins along with the protein lysate from transfected cells were electrophoresed on SDS-PAGE, followed by transfer of proteins to the nitrocellulose membranes. Western immunoblots analyses of these complexes were carried out by first probing the membranes with anti-Myc tag antibodies followed by reprobing with anti-phosphotyrosine antibodies. In addition, cell lysates were prepared from untreated as well as treated cells to determine apoptosis by utilizing a cell death detection enzyme-linked immunosorbent assay kit (Roche Diagnostics). The net
CARP-1 Regulates Apoptosis by EGFR

ERRP Inhibits EGFR and Induces CARP-1 Expression and Apoptosis, whereas Loss of CARP-1 Inhibits Apoptosis by ERRP—Whether and to what extent ERRP inhibition of EGFRs causes apoptosis and the role of CARP-1 in regulating this event were first investigated. We utilized HBC cells that were either transduced with retroviruses expressing ERRP or treated with immunoaffinity-purified recombinant ERRP. PT-67 cells were transected with pLNCX2-ERRP-Myc-His clone 1 or pLNCX2 vector plasmids followed by the selection of multiple, independent neomycin-resistant sublines. Expression of ERRP in 10 independent sublines was analyzed by Western immunoblot using anti-Myc tag antibodies (not shown). PT-67 ERRP clone 7 and 10 sublines were found to express the highest levels of ERRP and were chosen for further experiments. The retroviral titers were determined as detailed before (1). Each PT-67 ERRP subline produced retroviruses with a titer of ~0.5 × 10^5 colony-forming units/ml, whereas pLNCX2 vector subline 1 displayed a titer of ~10^4 colony-forming units/ml.

ERRP causes growth inhibition of a variety of cancer cells, and cell growth inhibition by ERRP involves, in part, induction of apoptosis (15, 16). Whether retroviral-mediated expression of ERRP causes apoptosis in HBC cells was investigated next. HBC cells were cultured in the absence or presence of retroviruses encoding vector or ERRP for 48 h, and cell lysates were utilized to determine apoptosis. Retroviral transduction of ERRP caused ~5-fold higher apoptosis when compared with apoptosis noted in untreated or vector-transduced cells (Fig. 1A). In addition, HBC cells were either untreated or treated with 5 μg/ml of purified ERRP protein, and flow cytometric analyses were carried out at the end of the treatment periods to determine the number of live cells or cells undergoing apoptosis. Approximately 4- and 8-fold increased apoptosis was noted in cells treated with ERRP for 12 and 24 h, respectively, when compared with their untreated counterparts (not shown).

Since ERRP is a pan-ErbB inhibitor, we further examined whether retroviral transduction of ERRP results in attenuation of constitutive activity of EGFR. HBC cells were cultured in the absence or presence of retroviruses encoding vector or ERRP for various times, followed by determination of activity and expression of EGFR by Western immunoblotting. As shown in Fig. 1A, transduction of HBC cells with ERRP results in reduced EGFR activity at as early as 6 h of treatment, whereas reduction of the constitutive EGFR levels was noted after 48 h of transduction with ERRP. Transduction with retroviruses expressing vector over a similar period did not affect either the activity or expression of EGFR. ERRP-dependent inhibition of EGFR activity was further ascertained by treating HBC cells with 5 μg/ml immunoaffinity-purified ERRP for various times followed by determination of constitutive activity and expression of EGFR. Similar to our observations in Fig. 1A, the data revealed time-dependent attenuation of EGFR activity in response to purified ERRP (Fig. 1C). Collectively, our data suggest that retroviral transduction of ERRP results in inhibition of EGFR and induction of apoptosis. These data corroborate our previous observations demon-
showing attenuation of EGFR and stimulation of apoptosis of cancer cells by recombinant ERRP (14, 15).

Whether apoptosis induction by ERRP involves CARP-1 was investigated by transducing cells with ERRP or by treating cells with purified ERRP for various times. Western blot analysis revealed elevated levels of CARP-1 in cells that were either transduced with ERRP or treated with purified ERRP for the indicated times essentially as detailed in Fig. 1C. Expression of CARP-1 and Actin proteins was determined essentially as in A. Columns in the histogram represent means of CARP-1 levels in three independent Western blots; bars, S.E. C, cells were treated with noted doses of Iressa for the indicated times. Expression of CARP-1 and actin proteins was determined essentially as in A.

The role of CARP-1 in regulating ERRP-dependent apoptosis was then examined as below. We first utilized multiple MDA-MB-468 HBC sublines that stably express CARP-1 antisense. They were transduced with retroviruses expressing vector or ERRP, and cell lysates were analyzed for apoptosis. Although treatments with ERRP retroviruses resulted in a modest but statistically significant increase in apoptosis of subline 9 cells when compared with their vector-transduced counterparts, overall ERRP-dependent apoptosis was significantly reduced in sublines 9 and 10 when compared with apoptosis noted in ERRP-transduced sublines 1 and 4 (Fig. 3A). Thus, apoptosis in the presence of ERRP was greatly inhibited in cells that had reduced levels of CARP-1 (sublines 9 and 10) when compared with those with normal levels of CARP-1 (sublines 1 and 4). Moreover, the above HBC sublines expressing reduced CARP-1 also displayed lower apoptosis in response to affinity-purified ERRP when compared with those that expressed normal levels of CARP-1 (not shown).

To further validate role of CARP-1 in apoptosis, PT-67 cells were transfected with vector or CARP-1 RNAi plasmids followed by selection of multiple, independent puromycin-resistant sublines. One subline each encoding the vector, CARP-1 RNAi-1 or CARP-1 RNAi-2, was chosen, and viral titers were determined as before (1). Each of the RNAi sublines yielded a titer of \( \times 10^4 \) colony-forming units/ml. Western blot analysis of HBC cells revealed a 50% and 20% reduction in CARP-1 expression in cells that were transduced with CARP-1 RNAi-1 and RNAi-2, respectively, when compared with its levels in untransduced cells (Fig. 3B). In an analogous study, HBC cells were either untransduced or transduced with vector or CARP-1 RNAi viruses and subsequently cultured in the absence (control) or presence of ERRP, followed by determination of apoptosis. That RNAi-dependent depletion of CARP-1 results in inhibition of apoptosis by ERRP was revealed when CARP-1 RNAi-1-transduced cells showed reduced apoptosis in the presence of ERRP when compared with the ERRP-dependent apoptosis...
CARP-1 Regulates Apoptosis by EGFR

FIGURE 3. Depletion of CARP-1 inhibits apoptosis by ERRP. A, cells expressing normal (indicated as 1 and 4) or 50% reduced CARP-1 (indicated as 9 and 10) were transduced with 5 ml of vector (Vector) or ERRP (ERRP 810) retroviruses for 48 h, and apoptosis was determined as in Fig. 1A, 8. B, HBC cells were untransduced (Control) or transduced with 5 ml of CARP-1 RNAi retroviruses as described under “Experimental Procedures.” Cell lysates were analyzed by Western immunoblotting for expression of CARP-1 and actin proteins as in Fig. 2A. A representative Western blot is shown. CARP-1 expression relative to its levels in untransduced cells, noted as Control (WT), is presented as a histogram. Columns represent mean values derived from two independent Western blots; bars, S.E. RNAi-1 and -2 denote retroviral stocks encoding two CARP-1 RNAi sequences described under “Experimental Procedures.” C, HBC cells that were either untransduced (WT) or transduced with the indicated RNAi viruses, followed by their treatments without (Control) or with 5 μg/ml ERRP for 48 h essentially as in Fig. 1C. The cell lysates were analyzed for apoptosis as in A. Columns in histograms in A and C represent data from three independent experiments; bars, S.E. Data in A were analyzed using a two-tailed Student’s t test, taking p < 0.05 as the level of significance. *, p < 0.01, compared with the corresponding vector-transduced controls.

noted in untransduced or vector-transduced cells (Fig. 3C). Taken together, the results demonstrate that depletion of CARP-1 results in inhibition of apoptosis by ERRP and that CARP-1 is involved in ERRP-dependent apoptosis.

CARP-1 Is a Phosphoprotein, and ERRP Induces Tyrosine Phosphorylation of CARP-1—Signaling pathways for cell growth and apoptosis frequently involve post-translational modifications, such as phosphorylation/dephosphorylation of the mediatory proteins. We have previously noted that CARP-1 is a perinuclear apoptosis inducer that interacts with 14-3-3-3 (1). Available literature suggests that members of the 14-3-3 family of proteins bind with their target proteins and peptides in a phosphorylation-dependent as well as -independent manner (19, 20). Further, it was recently found that CARP-1 possesses a unique motif for serine phosphorylation following tandem mass spectrometric and microsequencing analyses of cellular phosphoproteins (21). Whether this unique serine phosphorylation motif of CARP-1 is involved in its binding with 14-3-3 or regulates apoptosis by CARP-1 remains to be determined. Members of the 14-3-3 family of proteins often sequester proapoptotic molecules, such as Bad and Bax, and thus interfere with apoptosis signaling. This prompted us to speculate that CARP-1-phosphorylation at serine(s), threonine(s), and/or tyrosine(s), the residues that are commonly targeted during phosphorylation-dependent signaling, may be involved in regulating apoptosis by CARP-1. To test this possibility, we first determined whether phosphorylation of CARP-1 also occurs at tyrosine residues. COS-7 monkey kidney cells were transfected with plasmid expressing Myc- His-tagged wild-type CARP-1 (1). The CARP-1 protein was immunoprecipitated with anti-Myc tag or anti-stress-activated protein kinase 4 (SAPK-4; p38) antibodies, and the immunoprecipitates were subjected to Western blot analysis with anti-phosphotyrosine antibody. The data revealed the presence of tyrosine-phosphorylated CARP-1 in immunoprecipitates, derived from anti-Myc tag (lane 2) but not from anti-SAPK-4 (lane 1) antibodies (Fig. 4A). In an analogous experiment, MDA-MB-468 HBC cells were transfected with plasmid expressing Myc- His-tagged wild-type CARP-1 (1) or 50% reduced CARP-1 (indicated as Fig. 4B) or ERRP (lane 2), and apoptosis was determined as in Fig. 1A, 8. HBC cells were untransduced (Control) or transduced with retroviruses encoding ERRP. The CARP-1 protein was immunoprecipitated with anti-Myc tag or anti-SAPK-4 antibodies, followed by Western blot analysis with anti-phosphotyrosine antibody as above. Tyrosine-phosphorylated CARP-1 was noted in the immunoprecipitates that were derived from anti-CARP-1 antibodies but not from anti-SAPK-4 antibodies (not shown).

Whether apoptosis signaling by ERRP induce tyrosine phosphorylation of CARP-1 was investigated next. The HBC subline stably expressing Myc- His-tagged wild-type CARP-1 (subline Z-4; see below) was either untransduced or transduced with retroviruses encoding ERRP. The CARP-1 protein was immunoprecipitated with anti-Myc tag or anti-SAPK-4 antibodies, followed by Western blot analysis with anti-phosphotyrosine antibody. The data revealed that tyrosine phosphorylation of CARP-1 was elevated following treatments with ERRP in a time-dependent manner (Fig. 4B). Thus, apoptosis signaling induced by ERRP cause increased tyrosine phosphorylation of CARP-1.

CARP-1 Tyrosine at Position 192 Is a Target of Apoptosis Signaling—To define mechanisms of apoptosis signaling by CARP-1, we proceeded to map apoptosis-inducing subdomain(s) of CARP-1. To this end, we first subcloned CARP-1 CDNA fragments to generate recombinant constructs for expression of Myc- His-tagged CARP-1 mutant proteins as described under “Experimental Procedures.” The recombinant plasmids and the mutant CARP-1 peptides encoded by them are summarized in Table 1. Since CARP-1 protein contains putative DNA (bivalent) binding as well as cold-shock RNA binding motifs (1), we first transduced HBC cells with retroviruses that encode vector, wild-type CARP-1, CARP-1 proteins lacking either DNA (bivalent) binding, cold-shock RNA binding motif, or both of the motifs. Transduction of various CARP-1 proteins resulted in elevated apoptosis when compa-
red with their vector-transduced or untransduced counterparts (not shown), suggesting that CARP-1-mediated apoptosis in HBC cells does not require cold-shock RNA binding or the DNA (bhelical) binding motifs. Whether these domains play a role in CARP-1 binding with RNA and/or DNA in a manner dependent on or independent of specific cis-sequences remains to be determined.

Since CARP-1 is a tyrosine phosphoprotein and ERRP treatments elevate tyrosine phosphorylation of CARP-1 (Fig. 4), we speculated that apoptosis signaling pathways target specific tyrosine residue(s). Support for this speculation is derived, in part, from Prosite data base analysis (22) of the CARP-1 protein, where the presence of multiple putative motifs for tyrosine kinase phosphorylation was indicated. Two such putative motifs were present within CARP-1-(1–198) protein around tyrosines at positions 42 and 192. The CARP-1 sequence RVLEVATY (positions 185–192) displayed a randomized probability of 4.083 × 10⁻⁴ with the consensus (R/K)X₂(X/D/E)X₂,Y for tyrosine phosphorylation. Moreover, alignment of amino-terminal sequences of CARP-1 proteins of human, mouse, dog, chimpanzee, Gallus, Xenopus, and honey bee (Apis mellifera) revealed conservation of the putative domain around tyrosine 192 (Fig. 5A). On the basis of this information, we generated a construct for expression of CARP-1-(1–198) protein that harbors substitution of tyrosine 192 to phenylalanine. Transduction of CARP-1-(1–198) protein resulted in 2–3-fold elevated apoptosis, compared with the vector-transduced HBC and COS-7 cells (Fig. 5B). Expression of the CARP-1-(1–198Y¹⁹²F) protein, however, failed to stimulate apoptosis (Fig. 5B).

CARP-1 protein harbors apoptosis-promoting subdomains outside of the 1–198 region was revealed when MDA-MB-468 HBC, PC-3 human prostate cancer, or HCT-116 human colon cancer cells were transduced with retroviruses encoding wild-type CARP-1 or CARP-1 mutants for a period of 48 h, followed by determination of apoptosis. Transduction of wild-type CARP-1 or its mutants, with the exception of CARP-1-(1–198Y¹⁹²F), caused apoptosis induction (Table 1).

In addition, HBC cells were transduced with retroviruses expressing vector, CARP-1-(1–198), or CARP-1-(1–198Y¹⁹²F) proteins. In some samples, cells were additionally transduced with ERRP retroviruses after 24 h of their transduction with CARP-1 proteins, and incubation continued for further 24 h followed by apoptosis determination. Expression of ERRP, CARP-1-(1–198), or a combination of both caused 2–3-fold increase in apoptosis, compared with the cells that were either untransduced or transduced with vector-encoding viruses (Fig. 6A). In contrast, expression of CARP-1-(1–198Y¹⁹²F) failed to cause elevated apoptosis (Fig. 6A). Moreover, apoptosis in ERRP-transduced cells was inhibited in the cells that expressed CARP-1-(1–198Y¹⁹²F) protein (Fig. 6A). The data in Fig. 6A suggest that CARP-1 tyrosine 192 is a target of apoptosis signaling pathways.

Whether apoptosis signaling induced by ERRP involves tyrosine and/or serine phosphorylation of CARP-1-(1–198) and the extent to which such signals specifically target tyrosine 192 was studied by conducting the following experiments. COS-7 or HCT-116 cells were first transfected with construct encoding Myc-His-tagged CARP-1-(1–198). After 24 h, the Myc-His-tagged CARP-1 protein was immunoprecipitated by anti-phosphotyrosine, anti-phosphoserine, or anti-SAPK-4 antibodies followed by Western immunoblotting with anti-Myc tag antibodies. The tyrosine-phosphorylated CARP-1-(1–198) protein was noted in immunoprecipitates derived from anti-phosphotyrosine (lanes 2 and 5) but not from anti-SAPK-4 (lane 1) or anti-phosphoserine (lanes 3 and 6) antibodies (Fig. 6B). The data indicate that CARP-1-(1–198)

---

**TABLE 1**

List of CARP-1 constructs and CARP-1 proteins derived from them

| pcDNA3/Myc-His vector | pLNCX2 vector | CARP-1 proteins (amino acids) | Expected molecular mass | Apoptosis induction |
|-----------------------|---------------|------------------------------|-------------------------|---------------------|
| 6.1       | 16.3          | 1–150                        | 130                     | Yes                 |
| 8.6       | 17.1          | 1–636(de)1668–1150           | 125                     | Yes                 |
| 10.2      | 18.1          | 1–150(de)198–1150            | 120                     | Yes                 |
| 12.1      | 19.1          | 1–636(de)198–1150            | 115                     | Yes                 |
| 24.1      | 31.1          | 1–898                        | 95                      | Yes                 |
| 25.1      | 32.1          | 1–654                        | 75                      | Yes                 |
| 26.1      | 33.3          | 1–545                        | 51                      | Yes                 |
| 27.1      | 34.1          | 1–198                        | 25                      | Yes                 |
| 54.1      | 63.1          | 197–454                      | 31                      | Yes                 |
| 53.4      | 62.1          | 452–654                      | 28                      | Yes                 |
| 55.3      | 64.3          | 603–898                      | 35                      | Yes                 |
| 50.3      | 59.2          | 896–1150                     | 36                      | Yes                 |
| 40.1      | 42.1          | 1–198Y¹⁹²F                   | 25                      | No                  |
|           | 60.1          | 1–1150Y¹⁹²F                  | 130                     | No                  |

---

**FIGURE 4.** CARP-1 is a tyrosine phosphoprotein, and ERRP causes elevated tyrosine phosphorylation of CARP-1. A, cells were first transfected with the indicated plasmid, and 24 h after transfection, cell lysates were prepared. Lanes 1 and 2, co-immunoprecipitation using noted antibodies and 1000 µg of protein lysate was carried out prior to Western blotting. Lane 3, 100 µg of protein lysate was analyzed along with immunoprecipitates on 10% SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody, B, the indicated HBC subline was either untransduced or transduced with 5 ml of ERRP (noted as ERRP(q97)) retroviruses for the indicated times. Co-immunoprecipitation and Western immunoblotting was performed as in A. The presence of phosphorylated CARP-1 is indicated on the left in both panels, whereas the approximate location of molecular weight markers is indicated on right of B.
protein is a tyrosine-phosphorylated, but not serine-phosphorylated, protein. That apoptosis signaling by ERRP involves tyrosine phosphorylation of CARP-1-(1–198) and targets tyrosine 192 was then investigated. COS-7 cells were transfected with plasmid encoding CARP-1-(1–198) or CARP-1-(1–198Y192F) protein. Twenty-four hours after transfection, the cells were either untreated or treated with purified ERRP for additional 24 h. Protein lysates were derived followed by immunoprecipitation with phosphoprotein enrichment beads. The immunocomplexes were analyzed by Western blotting in conjunction with anti-Myc tag antibodies, followed by blotting of membranes with anti-phosphotyrosine antibodies as in Fig. 4B. ERRP treatment caused increased total as well as tyrosine phosphorylation of CARP-1-(1–198) but not that of CARP-1-(1–198Y192F) protein (Fig. 6C). Interestingly, CARP-1-(1–198Y192F) protein displayed elevated overall phosphorylation when compared with CARP-1-(1–198) protein following enrichment with phosphobeads. Whether CARP-1-(1–198Y192F) protein is additionally phosphorylated at specific threonine residues, and the extent to which substitution of tyrosine 192 to phenylalanine promotes this phenomenon remains to be clarified. Nevertheless, the data in Figs. 4 and 6 strongly suggest that ERRP causes elevated tyrosine phosphorylation of the wild-type and CARP-1-(1–198) proteins, and tyrosine 192 is a target of phosphorylation in the presence of ERRP.

The extent to which substitution of tyrosine 192 in full-length CARP-1 protein regulates ERRP-dependent apoptosis was investigated. In the first instance, HBC cells were either untransduced or transduced with vector,
ERRP, Myc-His-CARP-1 (WT), Myc-His-CARP-1-(1–1150Y192F), or a combination of these retroviruses, followed by determination of apoptosis. Similar to our observations in Fig. 6A, transduction of cells with ERRP or Myc-His-CARP-1 (WT) or in combination resulted in 2–3-fold elevated apoptosis, compared with untransduced or vector-transduced cells (Fig. 7A). Transduction of cells with Myc-His-CARP-1-(1–1150Y192F) or in combination with ERRP failed to induce apoptosis (Fig. 7A). The data suggest that substitution of tyrosine 192 to phenylalanine in full-length CARP-1 interferes with apoptosis induction by ERRP. Involvement of the motif surrounding CARP-1 tyrosine 192 in regulating apoptosis by ERRP was further investigated by utilizing stable HBC sublines that express wild-type CARP-1 or CARP-1 mutant having in-frame deletion of amino acids 151–197. Expression of Myc-His-tagged wild-type or mutant CARP-1 in multiple sublines was determined by Western immunoblotting in conjunction with anti-Myc tag antibodies. All of the sublines revealed overexpression of Myc-His-tagged wild-type or mutant CARP-1 when compared with the untransfected cells (Fig. 7B). Although, in the wild-type untransfected HBC cells, the Myc tag antibodies displayed a weak cross-reactivity to a protein that is of similar size to Myc-His-CARP-1, our subsequent immunocytochemical analyses in conjunction with anti-His tag antibodies revealed specific, punctate, perinuclear staining for CARP-1 proteins in wild-type CARP-1 when compared with the apoptosis noted in their vector-transduced counterparts (Fig. 7C). HBC sublines that overexpress CARP-1 (WT) or CARP-1-(1–150(del)198–1150) failed to show significant increase in ERRP-dependent apoptosis over their vector-transduced counterparts (Fig. 7C). Together, data in Fig. 7 underscore involvement of CARP-1 tyrosine 192 in apoptosis signaling by ERRP.

### Apoptosis Signaling by CARP-1 or ERRP Involves Activation of p38 MAPK

— Members of the p38 family of MAPKs regulate signaling by a variety of environmental stresses, including ionizing radiation, heat shock, osmotic shock, and oxidative stress (23, 24). Four p38 isoforms (p38α also known as SAPK2a, p38β2 (SAPK2b), p38γ (SAPK3), and p38δ (SAPK4)) are known to activate common as well as distinct substrates in response to various stress stimuli (23, 25). Activation of p38α induces apoptosis, whereas p38β2 activation promotes cell survival (26). We investigated whether apoptosis induction by CARP-1 or ERRP involves activation of p38 MAPK. HBC cells were transduced with ERRP or CARP-1 proteins, and the cell lysates were analyzed by Western blotting for the presence of activated (phospho-p38) as well as total p38 activities (Fig. 8A). As expected, expression of CARP-1-(1–198) protein caused apoptosis similar to that noted for wild type CARP-1, whereas CARP-1-(1–198Y192F) failed to cause apoptosis (Fig. 5), we next examined whether expression of CARP-1-(1–198) protein also caused activation of p38 MAPK. HBC cells were transduced with retroviruses encoding vector plasmid, CARP-1-(1–198), or CARP-1-(1–198Y192F), followed by analysis of activated p38 and its levels as in Fig. 8A. Since CARP-1-(1–198) protein caused apoptosis similar to that noted for wild type CARP-1, whereas CARP-1-(1–198Y192F) failed to cause apoptosis (Fig. 5), we next examined whether expression of CARP-1-(1–198) protein also caused activation of p38 MAPK. HBC cells were transduced with retroviruses encoding vector plasmid, CARP-1-(1–198), or CARP-1-(1–198Y192F), followed by analysis of activated p38 and its levels as in Fig. 8A. Expression of CARP-1-(1–198Y192F), on the other hand, failed to activate p38 (Fig. 8B). Levels of endogenous p38 did not change in untransduced cells; however, expression of CARP-1-(1–198Y192F) resulted in increased levels of activated p38 (Fig. 8C).
or CARP-1-transduced HBC cells (Fig. 8B). Thus, expression of ERRP or CARP-1 causes activation of p38 MAPK, and apoptosis signaling pathways that target tyrosine 192 of CARP-1 regulate p38 activation.

Activation of p38 isoforms is regulated by specific upstream MAPK kinases (MAPKKs or MKKs). To date, two MKKs (MKK6 and MKK3) are known to activate p38 MAPKs. MKK6 is a common activator of p38α, p38β2, p38γ, and p38δ, whereas MKK3 activates only p38α, p38γ, and p38δ (23). Whether CARP-1-dependent p38 activation is critical for induction of apoptosis was investigated by utilizing plasmids encoding dominant-negative MKK3 (pRc/RSV-Flag-MKK3(Ala)) or MKK6 (pcDNA3-Flag-MKK6(Ala)) proteins in conjunction with retroviruses encoding vector plasmid, wild-type CARP-1, or CARP-1-(1–198) proteins. HBC cells were either untransfected or transfected with plasmids expressing MKK3(Ala) or MKK6(Ala) proteins for 12 h, followed by their transduction with retroviruses expressing vector plasmid, wild-type CARP-1, or CARP-1-(1–198) proteins for an additional 48 h. Apoptosis levels were analyzed essentially as in Fig. 1. Expression of wild-type or CARP-1-(1–198) proteins caused ~6-fold higher apoptosis than that noted in cells that were transduced with vector-encoding retroviruses (Fig. 8C). Expression of dominant negative MKK3 or MKK6 proteins, on the other hand, inhibited apoptosis induced by wild type CARP-1 as well as CARP-1-(1–198) proteins (Fig. 8C). Furthermore, Western blot analyses of protein lysates derived from HBC cells transfected with either MKK6(Ala) or MKK3(Ala) prior to their transduction with wild type CARP-1 or CARP-1-(1–198) proteins failed to show activated p38 (not shown). Together, data in Fig. 8 demonstrate that apoptosis induction in the presence of ERRP or CARP-1 involves activation of p38 MAPKs.

Apoptosis Induction by CARP-1 Involves Activation of Caspase-9—Initiation of apoptosis often involves activation of the ICE family of proteases/caspases. We have previously noted that apoptosis induction following retroviral transduction of CARP-1-Myc-His fusion protein resulted in reduced levels of procaspase-3 (1). Since CARP-1 possesses multiple, nonoverlapping apoptosis-promoting subdomains (Table 1), we investigated whether apoptosis induction by CARP-1 and its mutants involved activation of caspases. Expression of wild-type CARP-1 as well as CARP-1-(1–198) proteins (Fig. 8C). Furthermore, Western blot analyses of protein lysates derived from HBC cells transfected with either MKK6(Ala) or MKK3(Ala) prior to their transduction with wild type CARP-1 or CARP-1-(1–198) proteins failed to show activated p38 (not shown). Together, data in Fig. 8 demonstrate that apoptosis induction in the presence of ERRP or CARP-1 involves activation of p38 MAPKs.

FIGURE 8. CARP-1-mediated apoptosis involves activation of p38 MAPK. HBC cells were either untreated (Control) or treated with 5 ml of the noted retroviral supernatants for the indicated times. A and B, protein lysates (100 μg/lane) were analyzed by Western blotting using anti-phospho-p38 antibodies. The membranes were then probed with anti-p38 as well as anti-actin antibodies. The presence of these proteins is noted on the left side of the respective panels. C, cells were transduced with the indicated retroviruses for 48 h. In certain cases, the cells were transfected with plasmids expressing dominant-negative MKK3 (MKK3 (Ala)) or MKK6 (MKK6 (Ala)) proteins for 24 h prior to their treatments with retroviruses. The cell lysates were utilized for determination of apoptosis as in Fig. 1C. Columns in the histogram represent means of three independent experiments; bars, S.E.

FIGURE 9. CARP-1-dependent apoptosis involves activation of caspase-9. HBC cells were either untreated (Control) or transduced with 5 ml of noted retroviral supernatants for 48 h. Activation of caspase-9 in respective cytosolic protein lysates (100 μg/sample) was determined as described under “Experimental Procedures.” Columns in the histogram represent means of three independent experiments; bars, S.E. WT Cells, wild-type HBC cells; Vector, pLNCX2 vector-encoding viruses.
CARP-1 Regulates Apoptosis by EGFR

ERRP

EGFRs

Y_P

CARP-1

Caspase-9

Activation

p-P38 MAPK

Apoptosis

FIGURE 10. Schematic of CARP-1-dependent apoptosis signaling. ↓, inhibition.

(1–198Y192F) proteins (not shown). Together, these observations suggest that CARP-1-dependent apoptosis involves activation of caspase-9.

DISCUSSION

Members of the EGFR family of receptor tyrosine kinases mediate a variety of pathways and cellular responses, including proliferation, differentiation, and apoptosis (27). EGFRs play critical roles in the development and progression of many malignancies and are generally considered indicators of poor prognosis (8). Transforming growth factor-α/epidermal growth factor and their class of peptide growth factors are known to bind EGFRs and stimulate cell growth and proliferation (6, 30). Inhibition of activation of EGFRs and interference with growth factor-activated signal transduction pathways is, therefore, a promising strategy for development of novel and selective targeted therapies (see Ref. 11 and Refs. therein). Several of the currently utilized therapies that target the EGFR family of receptors are thought to induce cell growth inhibition that involves, in part, induction of apoptosis.

CARP-1/CCAR1 is a recently identified perinuclear protein that mediates apoptosis signaling by diverse agents, including anthracycline toxin Adriamycin. CARP-1 interactions with cytoplasmic signal transducers such as 14-3-3 protein coupled with CARP-1-mediated down-regulation of several cell cycle-regulatory proteins, including c-Myc and cyclin B1, play an important role in CARP-1-dependent inhibition of cellular growth (1). In the current investigation, we examined the pathways that regulate CARP-1 to better define molecular determinants of CARP-1-dependent cell growth-inhibitory and apoptosis-promoting signaling. In light of the fact that growth factor-dependent signaling pathways regulate CARP-1 (1), we speculated that signaling events following activation of cell surface growth factor receptors target CARP-1 protein, and cell growth-inhibitory effects elicited by inhibition of EGFRs involve CARP-1. To this end, we utilized a recently identified pan-ErbB inhibitory protein, ERRP (13, 16), to study whether and to what extent negative regulation of EGFR by ERRP and attenuation of subsequent intracellular signaling events involved CARP-1. ERRP binds EGFR and forms inactive heterodimers and causes inhibition of transforming growth factor-α-dependent activation of EGFR and downstream mediators of this signaling, thus regulating cellular growth and proliferation processes (14). The findings presented in this report, summarized in Fig. 10, show for the first time that CARP-1 is involved in regulating growth-inhibitory signaling resulting from attenuation of EGFRs. In addition to corroborating our previous observations where ERRP treatments inhibited EGFR activity and expression, together with our current observation that ERRP increases CARP-1 expression, these findings strongly suggest that CARP-1 may regulate EGFR-dependent intracellular signaling events that control diverse processes, such as cell growth and proliferation. That CARP-1 is an important regulator of EGFR signaling processes is further supported by the fact that antisense or RNAi-mediated depletion of CARP-1 results in inhibition of apoptosis in the presence of ERRP.

Intracellular signaling cascades often involve the formation of multimolecular signaling complexes at particular spatial positions within the cell. These assemblies are formed through protein-protein interactions of the member proteins that utilize modular domains on one molecule to short sequence motifs on another. The 14–3–3 family of proteins are such an emerging family of proteins and protein domains that bind and regulate proteins involved in intracellular signaling (such as Raf, phosphatidylinositol 3-kinase, and MEKK), cell cycle (Cdc25 and CDK25), apoptosis (BAD and ASK-1), and transcriptional regulation (p53, FKHRL, and TAZ). CARP-1-dependent apoptosis signaling involves CARP-1 interactions with 14–3–3/Stratifin, whereas overexpression of 14–3–3/Stratifin inhibits apoptosis by CARP-1 (1). Since CARP-1 possesses a novel serine phosphorylation motif (21) and in light of the fact that the 14–3–3 family of proteins often bind to serine/threonine-phosphorylated residues in their target proteins (19, 20), it remains to be determined whether CARP-1 interactions with 14–3–3/Stratifin are dependent on prior specific serine/threonine phosphorylation of CARP-1 and the extent to which CARP-1 interactions with 14–3–3/Stratifin regulate apoptosis signaling by ERRP.

Mediators and/or effectors of apoptosis signaling often have specific protein interaction domains, such as the death domain, death effector domain, caspase recruitment domain, and Bcl-2 homology domains (31). In the absence of a known apoptosis-regulatory protein interaction domain in CARP-1, we utilized deletional mutagenesis to first determine whether CARP-1 possesses distinct apoptosis-inducing region(s). This approach revealed the presence of multiple, novel apoptosis-inducing subdomains in CARP-1, since several nonoverlapping CARP-1 peptides possessed apoptosis-inducing properties, as summarized in Table 1. The precise definitions of these subdomains, including the one within the CARP-1–(1–198) protein remain to be elucidated. Nevertheless, our current mutagenesis data revealed that apoptosis signaling targets tyrosine 192 of CARP-1. Involvement of specific tyrosine phosphorylation of CARP-1 during apoptosis signaling is further supported by the facts that CARP-1 is a tyrosine-phosphorylated protein and that apoptosis signaling following treatment of cells with ERRP caused increased phosphorylation of specific tyrosine residue of CARP-1. Indeed, functional properties of several mediators of growth, transformation and apoptosis signaling, including EGFRs, are regulated by phosphorylation at specific tyrosine residues, whereas aberrant activation of receptor and/or nonreceptor tyrosine kinases is frequently noted in dysregulation of growth factor signaling networks (32). CARP-1 harbors multiple, apoptosis-inducing subdomains and also regulates apoptosis by adriamycin (1). However, it remains to be determined whether apoptosis signaling by adriamycin targets distinct or overlapping subdomain(s) of CARP-1 in a manner dependent on or independent of involvement of phosphorylation of specific tyrosine, threonine, and/or serine residues within such domain(s).

A variety of extrinsic and intrinsic signals lead to activation of specific caspases to trigger apoptosis. Extrinsic signaling involves stimulation of death receptors and is frequently initiated by activation of caspase-8 and -10. Intrinsic signaling for apoptosis, on the other hand, is often due to the DNA damage, injury to mitochondria, or endoplasmic reticulum (ER) stress. The DNA damage and growth factor withdrawal-induced apo-
CARP-1 Regulates Apoptosis by EGFR

Apoptosis signaling activates caspase-9, whereas ER stress-induced apoptosis requires activation of caspase-12 (33). ER stress is induced by factors including accumulation of unfolded protein aggregates (unfolded protein response) or by excessive protein traffic, and unfolded protein response, in turn, activates a set of ER-located sensors, such as PERK kinase-dependent phosphorylation of eIF-2α, resulting in attenuation of protein synthesis (28). Whereas apoptosis induction following overexpression of wild-type CARP-1 or its nonoverlapping mutants involved activation of caspase-9, the apoptosis signaling did not involve ER stress-dependent pathways, since neither activation of caspase-12 nor increased phosphorylation of eIF-2α was noted (not shown).

Apoptosis-inducing intracellular signaling utilizes distinct transducers that include members of Jun N-terminal kinase (JNK) and p38 MAPK family of proteins (see Ref. 17 and references therein). Although EGFR and ErbB-2 receptors are usually associated with proliferation, overexpression of these receptors or aberrant stimulation by ligands also activates apoptosis in a manner dependent on p38 MAPK (29). The data in this report show ERRP inhibition of EGFRs, resulting in CARP-1 induction that, in turn, leads to activation of p38 MAPKs. This observation is consistent with what was noted for p38 MAPKs in regulating apoptosis signaling by EGFRs. That p38 MAPK is downstream of CARP-1 during apoptosis signaling by growth factor receptors is supported by the observations in Fig. 8, where abrogation of p38 activation results in inhibition of CARP-1-dependent apoptosis. In conclusion, we report that negative regulation of EGFRs causes inhibition of cell proliferation and induction of apoptosis. A novel apoptosis-inducing protein CARP-1/CCAR1, in turn, regulates these events in a manner dependent on phosphorylation of a specific tyrosine residue of CARP-1 and downstream activation of p38 MAPK and caspase-3 and -9.

REFERENCES

1. Rishi, A. K., Zhang, L., Boyanapalli, M., Wali, A., Mohammad, R. M., Yu, Y., Fontana, J. A., Hatfield, J. S., Dawson, M. I., Majumdar, A. P. N., and Reichert, U. (2003) J. Biol. Chem. 278, 33422–33435
2. Shao, Z. M., Dawson, M. I., Li, X. S., Rishi, A. K., Sheikh, M. S., Han, Q. X., Ordenez, J. V., Shroot, B., and Fontana, J. A. (1995) Oncogene 11, 493–504
3. Hu, C. K. A., Rishi, A. K., Li, X. S., Gerald, T. M., Dawson, M. I., Schiffer, C., Reichert, U., Shroot, B., Poirier, G. C., and Fontana, J. A. (1997) Blood 89, 4470–4479
4. Liang, J. Y., Fontana, J. A., Rao, J. N., Ordenez, J. V., Dawson, M. I., Shroot, B., Wilber, J. F., and Feng, P. (1999) Prostate 38, 228–236
5. Hackel, P. O., Zwick, E., Prenzel, N., and Ullrich, A. (1999) Curr. Opin. Cell Biol. 11, 184–189
6. Glenney, J. R. (1992) Biochim. Biophys. Acta 1134, 113–127
7. Porter, A. C., and Vaillancourt, R. R. (1998) Oncogene 15, 1343–1352
8. Rajkumar, T., Gullick, W. J. (1994) Breast Cancer Res. Treat. 29, 3–9
9. Lohrisch, C., and Piccart, M. (2001) Semin. Oncol. 28, Suppl. 18, 3–11
10. Kriscina, M. H., and Yarden, Y. (2000) J. Cell. Biochem. Suppl. 34, 52–60
11. Raie, J. M., and Lipmann, M. E. (2004) Breast Cancer Res. Treat. 83, 99–107
12. Ritter, C. A., and Arteaga, C. L. (2003) Semin. Oncol. 30, Suppl. 1, 3–11
13. Yu, Y., Rishi, A. K., Turner, J. J., Liu, D., Black, E., Moshier, J. A., and Majumdar, A. P. N. (2001) Am. J. Physiol. 280, C1083–C1089
14. Marciniak, D. J., Moragoda, L., Mohammad, R., Yu, Y., Nagothu, K. K., Aboutanemel, A., Sarkar, F. H., Adsay, V. N., Rishi, A. K., and Majumdar, A. P. N. (2003) Gastroenterology 124, 1337–1347
15. Marciniak, D. J., Rishi, A. K., Sarkar, F. H., and Majumdar, A. P. (2004) Mol. Cancer Ther. 3, 1615–1621
16. Xu, H., Yu, Y., Marciniak, D. J., Rishi, A. K., Sarkar, F. H., Kucuk, O., and Majumdar, A. P. N. (2005) Mol. Cancer Ther. 4, 435–442
17. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) Mol. Cell. Biol. 16, 1247–1255
18. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. G., and Struhl, K. (eds) (1990) Current Protocols in Molecular Biology, Vol. 2, John Wiley and Sons, Inc., Hoboken, NJ
19. Tzivion, G., Shen, Y. H., and Zou, J. J. (2001) Oncogene 20, 6331–6338
20. Rittinger, K., Rudman, J., Xu, J., Volinia, S., Cantley, L. C., Smerdon, S. J., Gamblin, S. J., and Yaffe, M. B. (1999) Mol. Cell. 4, 153–166
21. Beausoleil, S. A., Jedrychowski, M., Schwartz, D. R., Elias, J. E., Villen, J., Li, J., Cohn, M. A., Cantley, L. C., and Gygi, S. P. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 12130–12135
22. Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. (2000) Trends Biochem. Sci. 25, 147–150
23. Enslen, H., Brancho, D. M., and Davis, R. J. (2000) EMBO J. 19, 1301–1311
24. Kyriakis, J. M., and Avruch, J. (2001) Trends Biochem. Sci. 26, 807–886
25. Goedert, M., Cuenda, A., Craxton, M., Jakes, R., and Cohen, P. (1997) EMBO J. 16, 3563–3571
26. Nemoto, S., Xiang, J., Huang, S., and Lin, A. (1998) J. Biol. Chem. 273, 16415–16420
27. Casalini, P., Iorio, M. V., Galmozzi, E., and Menard, S. (2004) J. Cell. Physiol. 200, 343–350
28. Kaufman, R. J. (1999) Genes Dev. 13, 1211–1233
29. Tikhomirov, O., and Carpenter, G. (2004) J. Biol. Chem. 279, 12988–12996
30. Tzivion, G., Shen, Y. H., and Zou, J. J. (2001) Oncogene 20, 6331–6338
31. Reed, J. C., Doctor, K. S., and Godzik, A. (2004) Sci. STKE 2004, 9–29
32. Noble, M. E. M., Endicott, J. A., and Johnson, L. N. (2004) Science 303, 1800–1805
33. Degterev, A., Royce, M., and Yuan, J. (2003) Oncogene 22, 8543–8567