Oncogenic Raf-1 Induces the Expression of Non-histone Chromosomal Architectural Protein HMG1-C via a p44/p42 Mitogen-activated Protein Kinase-dependent Pathway in Salivary Epithelial Cells*

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The enzyme activity of mitogen-activated protein kinase (MAP kinase) increases in response to agents acting on a variety of cell surface receptors, including receptors linked to heterotrimeric G proteins. In this report, we demonstrated that Raf-1 protein kinase activity in the mouse parotid glands was induced by chronic isoproterenol administration in whole animals. To investigate the molecular nature underlying cellular responses to Raf-1 activation, we have stably transfected rat salivary epithelial Pa-4 cells with human Raf-1-estrogen receptor fusion gene (ΔRaf-1:ER) and used mRNA differential display in search of messages induced by ΔRaf-1:ER activation. Through this approach, the gene encoding non-histone chromosomal protein HMG1-C was identified as one of the target genes activated by oncogenic Raf-1 kinase. Activation of Raf-1 kinase resulted in a delayed and sustained increase of HMG1-C expression in the Pa-4 cells. The induction of HMG1-C mRNA level is sensitive to both the protein synthesis inhibitor cycloheximide and transcription inhibitor actinomycin D. The role of the extracellular signal-related kinase (ERK) signaling pathway in the HMG1-C induction was highlighted by the result that the MAP kinase (MEK) inhibitor, PD 98059, blocked ΔRaf-1:ER- and 12-O-tetradecanoylphorbol-13-acetate-stimulated HMG1-C induction. Altogether, these findings support the notion that the Raf/MEK/ERK signaling module, at least in part, regulates transcriptional activation of the chromosomal architectural protein HMG1-C.

The Ras/Raf/MEK1/MAP kinase signaling pathway mediates diverse responses to extracellular stimuli and initiates cellular proliferation and oncogenic transformation in mammalian cells (1–5). Results from biochemical and genetic studies have emphasized that members of the Raf family of serine/threonine kinases (Raf-1, A-Raf, and B-Raf) are the pivotal components of intracellular signal transduction pathways that relay signals from a plethora of activated cell surface receptors to a variety of intracellular effectors (4, 5). Once activated, the membrane-associated Raf-1 phosphorylates and activates a set of dual specificity protein kinases MEK1 and MEK2 (6–8), which in turn phosphorylate and activate the ubiquitous p42/p44 MAP kinases (ERK2/ERK1 for extracellular signal-regulated kinase) (9, 10). Activation of ERK1/2 induces pleiotropic effects ranging from phosphorylation of membrane-bound proteins, such as cystalysosomal phospholipase A2, to the activation of transcription factors, such as Elk-1 (11–14).

Although ERK1 and ERK2 are likely to be the essential mediators of Raf-1 activation, there are situations in which Raf-1 exerts biological effects in the absence of detectable ERK1/2 activation (15–18). Recent observations that Raf-1 activates ede25 protein phosphatase in HeLa cells (19) and p70 S6 kinase in Chinese hamster ovary cells (20) support the idea that there are branch points in the Raf/MEK/ERK signaling cascade. Moreover, it is also demonstrated that Raf-1 activates JNK (c-Jun terminal kinase) in 3T3 cells through an autocrine manner (21). Hence, it is possible that these different pathways are not mutually exclusive and could be activated in a cell type-specific manner to mediate intracellular signaling events.

The pathway linking G protein-coupled receptors to Raf-1 activation is still poorly understood. Recent reports indicate that the Raf-1 signaling pathway can be activated by a number of receptors linked to Gαi, Gαq, or Gαs. For example, triggering of α2-adrenergic receptor (22), m1 muscarinic receptor (23), and α1- and β-adrenergic receptors (24, 25) can all potently stimulate ERK activities. Moreover, the activation of MAP kinase appears not to be exclusively linked to cell proliferation. Accumulating evidence has suggested that persistent activation of the MAP kinase might lead to cell differentiation or hypertrophy in a cell type-specific manner (5). For instance, the sustained ERK activation following stimulation by nerve growth factor is believed to be necessary and sufficient for PC12 cell differentiation and neurite outgrowth (26). Moreover, recent evidence using antisense RNA against ERKs suggests that ERK activation results in a hypertrophy of the cardiac myocytes (27).

Using the β-adrenergic model of salivary hyperplasia/hypertrophy, we and others have shown previously that stimulation with β-agonist isoproterenol results in a transient induction of immediate-early gene (c-fos and c-jun) expression, followed by a delayed induction of tissue-specific proline-rich protein gene expression and the enlargement of mouse and rat salivary glands (28). An understanding of molecular processes that contribute to salivary adaptive responses may provide new insights into cell signaling and mechanisms controlling long-term...
cellular responses to extracellular stimuli. In this study, we showed that chronic exposure of mice to the β-agonist isoproterenol effectively activates Raf-1 protein kinase activity in the parotid glands. To correlate salivary-specific gene expression with Raf-1 activation, we have attempted to identify the target gene(s) regulated by the activated Raf-1 kinase pathway in the salivary Pa-4 cells. To this end we present evidence that persistent activation of Raf-1 kinase using the regulatable and oncogenic form of Raf-1, ΔRaf-1:ER (29), induces the expression of non-histone chromosomal protein HMGI-C.

HMGI-C protein belongs to the family of HMGI(Y). The HMGI(Y) family of proteins is barely detectable in the differentiated adult tissues. Developmental studies revealed that the mouse HMGI-C gene is expressed predominantly during the embryogenesis prior to 15.5 days post-coitum (30). The exact biological function of HMGI-C is less well defined. There is evidence that suggests that the disruption of the HMGI-C gene causes growth retardation in pygmy mice (31). HMGI-C protein has been inferred to play a role in cell proliferation, based on the observation that HMGI-C is abundant in the fully transformed cells (32, 33). Moreover, dysregulation of HMGI-C expression has also been assumed to be one of the causative factors involved in the development of various human tumors such as lipoma, uterine leiomyoma, and pleomorphic salivary adenoma (34, 35). Using antisense technology to block HMGI-C protein synthesis, the retrovirally induced neoplastic transformation of rat thyroid cells is suppressed (33). Therefore, it is suggested that one of the specific changes brought about during the transformation process is the constitutive elevation of HMGI-C expression, and the induction of HMGI-C expression is directly correlated with cell transformation. However, the specific signaling pathway and molecular mechanism activating HMGI-C expression are still elusive. The results described here go further in characterizing the mechanism governing HMGI-C gene activation. By analyzing the expression pattern of HMGI-C steady state mRNA in cells treated with inhibitors of transcription or protein synthesis, in the presence of Raf-1 activator, we propose that the HMGI-C gene is a secondary response gene to Raf-1 activation. We also present evidence that there is a direct link between the activation of Raf/MEK/ERK signaling module and the induction of transformation-associated chromosomal architectural protein HMGI-C expression. To our knowledge, this is the first report that delineates the signaling pathway that activates HMGI-C expression. Our finding may provide a key element to the understanding of the mechanisms underlying salivary adaptive responses and cell transformation in general.

**MATERIALS AND METHODS**

**Cell Culture and Stable Transfection**

The normal rat parotid acinar cell line Pa-4, also known as parotid C5 cells (36), was plated on Primaria culture dishes (Falcon) in Dulbecco’s modified Eagle’s/F12 (1:1) medium supplemented with 2.5% fetal bovine serum, insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (1.1 μM), and glutamate (5 mM) and were maintained in a humidified atmosphere containing 5% CO2 and 95% air at 35°C. All culture media contained 60 mM D-glucose, 2 mM l-glutamine, 10 mM NaHCO3, 137 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.25 mM KH2PO4, 0.8 mM Na2HPO4, 1 mM Na2EDTA, 10 mM HEPES, 10 mM NaF, 1 mM aspartate, 1 mM glutamate, 100 mM theophylline, 1 μM sodium butyrate, 1 mM sodium vanadate, 1 mM EDTA, 1 mM MgCl2, 50 mM NaCl, 100 mM NaH2PO4, 5% dialyzed fetal bovine serum and 0.8% dextran (Sigma). The Pa-4 cells were maintained in this medium at 35°C in 10% CO2.

**Regulation of Raf-1 Secondary Response Gene Expression**

Isoproterenol was administered as described previously (36); each mouse received a daily intraperitoneal injection of 2 mg of dl-isoproterenol HCl in 0.5 ml of 140 mM NaCl for the periods indicated. Mice were sacrificed at 30 min after the last injection, and the parotid glands were removed surgically. After being rinsed with cold phosphate-buffered saline, the isolated glands (0.1–0.2 g) were homogenized immediately in 1–2 ml of glycerol buffer (GLB (29)) containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin A, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium PPi, 100 mM β-glycerophosphate at 4°C. The homogenates were incubated at 4°C for 10 min, and the insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4°C. The protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad Laboratories).

The cell extracts from Pa-4 and Pa-4 ΔRaf-1:ER cells were prepared in the same manner.

**mRNA Differential Display**

Total RNA was isolated from untreated Pa-4 cells and estradiol-treated Pa-4 ΔRaf-1:ER cells using the TRIzol reagent (Life Technologies, Inc.). The isolated RNA was then treated with RNase-free DNase (Life Technologies, Inc.) according to the manufacturer’s instructions to remove any DNA contaminant. Differential display PCR reaction was carried out using RNAimage™ 1 and 2 kits (GenHunter), and a total of 48 different primer pairs were used with [35S]ATP included in the reaction mixture to label the PCR products. The radiolabeled cDNA fragments were resolved and sequenced or analyzed by autoradiography. The differentially expressed bands were identified and excised from the gels. The cDNA fragment was eluted from the gel by boiling the gel slice in 10 μl Tris-HCl (pH 7.5), 1 mM EDTA (TE) solution. After ethanol precipitation, the cDNAs were amplified by a second round of PCR using corresponding primer pairs. PCR samples were then electrophoresed on a 2% agarose gel. The DNA bands were recovered from the gel after electrophoresis and visualized by autoradiography. Northern blot analyses. Northern blots confirm the authenticity of the differences in mRNA expression levels between parental Pa-4 and Pa-4 ΔRaf-1:ER cells as demonstrated by the differential display. After confirmation, PCR products were subsequently cloned into pCR-TRAP vector (GenHunter) and subjected to DNA sequencing analyses using the Sequenase version 2.0 kit (Amersham). For further sequencing analyses, cDNA clones were isolated by screening a rat parotid cDNA library, synthesized by us, using the 32P-labeled fragments obtained from mRNA differential display as probes. Comparison of DNA sequence homology with the GenBank™ and the EMBL data bases was performed using the BLAST program (37).

**Western Analysis**

Equal amounts of protein from mouse parotid-soluble extracts or cell-soluble lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electroblotted onto Immobilon-P membrane (Millipore), and the membranes were incubated with anti-Raf-1 antibody (C-12, Santa Cruz) or anti-ELK-2a antibody (kindly provided by Dr. Roger Duncan, USC). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) and the enhanced chemiluminescence (ECL) detection system (Amersham) were used to detect bound antibodies.

**Kinase Assays**

Raf-1 Immune Complex Kinase Assay—Raf-1 kinase activity was analyzed by the immune complex kinase assay as modified from Samuels et al. (29). Briefly, the immunoprecipitation of endogenous p74Raf1 or ΔRaf-1:ER was carried out by incubating 1 μg of soluble tissue extract protein or 100 μg of soluble cell lysate protein in 1 ml of GLB with 1 μg of either anti-Raf-1 antibody (C-12, Santa Cruz) or anti-ER antibody (HC-20, Santa Cruz) for 1 h at 4°C. The immune complexes were precipitated by centrifugation, and the pellets were washed three times with 1 ml of GLB each after addition of 20 μl of protein A-agarose beads (Santa Cruz) to the reaction mixtures and incubation at 4°C with rotation for 2 h. The in vitro kinase activities of p74Raf1 and ΔRaf-1:ER were assessed by incubating the immune complexes in a 30-μl reaction mixture containing 25 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM MnCl2, 1 mM dithiothreitol, 1 μM ATP, and 20 μCi of [γ-32P]ATP with 100 ng of recombinant MEKI (Santa Cruz) as substrate at 30°C for 30 min. The reaction was stopped by adding SDS-loading buffer and boiled for 5 min. The protein A-agarose beads were collected by centrifugation, and the
supernatant was resolved through a 12% SDS-PAGE, followed by electrophoresis. The membrane was exposed to x-ray film to visualize the level of MEK phosphorylation.

**Assay for MEK1**—Briefly, 100 μg of cell lysate protein was incubated with 1 μg of anti-MEK1 (C-18, Santa Cruz) antisera for 1 h at 4 °C. The immune complexes were collected by centrifugation as described in the assay for Raf-1. The in vitro MEK kinase assay was carried out by incubating the immune complexes in a 30-μl reaction mixture containing 40 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM dithiothreitol, 40 mM ATP, 2 μCi of [γ-³²P]ATP, and 600 ng of recombinant p42 MAPK protein (Santa Cruz) as substrate at 30 °C for 30 min. The reaction mixture was resolved by SDS-PAGE, and the proteins were transferred to membranes and exposed to x-ray film for autoradiography.

**ERK Immune Complex Kinase Assay**—Briefly, 100 μg of cell lysate protein was incubated with 2 μg of goat anti-ERK2 IgG (C-14, Santa Cruz) for 1 h at 4 °C. The immune complexes were collected by the addition of 20 μl of protein G plus-agarose beads (Santa Cruz) to the reaction mixture and incubating with rotation for 2 h at 4 °C, and the pellet was washed three times with 1 ml of cold GLB each, and one more time with buffer containing 25 mM Tris (pH 7.5), 137 mM NaCl, 40 mM MgCl₂, 10% glycerol (v/v). The in vitro kinase activities of ERK1/2 were determined by incubating immune complexes in a 30-μl reaction mixture containing 40 mM HEPES (pH 7.4), 40 mM MgCl₂, 200 μM ATP, 1 μCi of [γ-³²P]ATP, and 10 μg of myelin basic protein (MBP) as substrate at 30 °C for 30 min. The reaction was stopped by adding SDS-loading buffer and boiled for 5 min. The protein G plus-agarose beads were spun down, and the supernatant was electrophoresed through a 16.5% SDS-PAGE followed by electroblotting. The membrane was exposed to x-ray film to determine the ERK1/2 activities by visualizing MBP phosphorylation.

**Northern Blot Analysis**

Total cellular RNA (20 μg) from Pa-4 and estradiol-treated Pa-4 Raf-1:ER cells was electrophoresed through a 1.5% agarose gel in the presence of 2.2 mM formaldehyde, transferred to nylon membranes, and UV-crosslinked. The prehybridization and hybridization steps were carried out using QuickHyb solution from Stratagene according to manufacturer’s instructions. Each differentially expressed cDNA fragment was UV-crosslinked. The prehybridization and hybridization steps were resolved by SDS-PAGE, and the proteins were transferred to membranes and exposed to x-ray film for autoradiography.

**Measurement of Cloning Efficiency in Agarose**

Pa-4 and Pa-4 Raf-1:ER cells were plated at 2,000 cells per 35-mm culture dish in 1 ml of 0.35% (w/v) low-melting-temperature agarose solution diluted with medium in the absence or presence of 1 μM estradiol. Different concentrations of PD 98059 were also added together with estradiol to the Pa-4 Raf-1:ER cells. The 1-ml overlay media was changed every 3 days. After 28 days, the cells were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 0.2 mg/ml. The stained plates were photographed, and the number of colonies greater than 0.4 mm in diameter was counted and analyzed.

**RESULTS**

**Isoproterenol-induced Raf-1 Activation in the Mouse Parotid Glands**—Chronic isoproterenol administration results in the enlargement of salivary glands and the induction of salivary-specific gene expression in rodents (28). It has been reported that the β-agonist isoproterenol induces MAP kinase activation through G<sub>i</sub>-coupled receptors in CHO cells (25). Thus, we first examined whether chronic isoproterenol treatment stimulates Raf-1 kinase activity in the parotid glands of mice parallel with the known phenomenon of isoproterenol-mediated salivary adaptive responses. As shown in Fig. 1A, daily injections of β-agonist effectively induced mouse parotid Raf-1 activity as measured by its activity on rMEK1 in a time-dependent manner, reaching a maximum at the eighth daily injection. This gradual increase in Raf-1 activities in the mouse parotid glands correlated well with the kinetics of salivary enlargement mediated by chronic isoproterenol stimulation (28). Moreover, the observation of this delayed Raf-1 activation via β-adrenergic receptor is also consistent with the notion that daily isoproterenol treatment leads to a down-regulation of the intracellular cAMP level (38). We postulate that this delayed Raf-1 activation is, in part, mediated by a derepression mechanism, since cAMP is known to markedly decrease Raf-1 kinase activity (39). To rule out the possibility that the observed Raf-1 activation was caused by an increase in the amount of endogenous Raf-1 protein in the parotid extracts, immunoblot analysis of Raf-1 protein from these extracts was carried out. As shown in Fig. 1B, the amount of Raf-1 protein remains at approximately the same level following chronic isoproterenol injection regimes. Therefore, Raf-1 activity is elevated rather than its protein level. The amount of translation factor eIF-2α in the extracts was also determined by Western analysis as an internal control (Fig. 1B) and shown to be constant over the 8 days of study. The result suggests that the delayed Raf-1 activation by β-adrenergic receptor agonist may participate in the events leading to isoproterenol-induced salivary hyperplasia and hypertrophy. However, the exact mechanism remains to be elucidated.

**Characterization of the Stable Cell Line Pa-4 ΔRaf-1:ER**—To investigate the primary biochemical events following persistent Raf-1 activation in the salivary cells, a chimeric construct containing the catalytic domain of human Raf-1 fused to the hormone binding domain of human estrogen receptor (ΔRaf-1: ER) (29) was used to establish stably transfected cells. The amino-terminal half of the Raf-1 protein contains two conserved regulatory domains CR1 and CR2. The catalytic domain is located in the carboxyl-terminal part of the protein. Deletion or mutation of the regulatory domains of the Raf-1 can generate constitutively active kinase. Fusion of the catalytic domain of the Raf-1 (ΔRaf-1) and the hormone binding domain of the estrogen receptor (ER) generates a β-estadiol-regulated kinase. The catalytic activity of ΔRaf-1:ER protein remains very low in the absence of estradiol and is induced dramatically within minutes after estradiol addition (29). This conditional construct therefore provides a useful tool for studying the role of Raf-1 in mediating gene expression and cellular responses. It has been shown previously in a variety of cell types that the activation of ΔRaf-1:ER fusion protein and its downstream MEK and ERK activities, by the addition of estradiol to the growth medium, is independent of other signaling pathways.
immunoprecipitated ERK2 as described under “Materials and Methods.” The radioactivities of 32P-labeled MBP bands are indicated. The data were resolved by SDS-PAGE, blotted onto a nylon membrane, and exposed to x-ray film for autoradiography. The 32P-labeled ERK2 bands are indicated. Stimulation of MEK activity, using recombinant ERK2 as a substrate, as described in “Materials and Methods.” The reaction mixture was assayed for MEK activity, with 1 μM estradiol (Est) or vehicle (Ctl) overnight. Cell lysates were prepared as described under “Materials and Methods.” Equal amounts (20 μg) of cellular protein were loaded in each lane and resolved by SDS-PAGE. Endogenous Raf-1 and ectopic ΔRaf-1:ER proteins were probed with anti-Raf-1 antibody and anti-hER antibody as described in Fig. 1B. B, induction of ΔRaf-1:ER activity (first and second panel). Parental Pa-4 and Pa-4Raf-1:ER cells were treated with 1 μM estradiol (Est) or vehicle (Ctl) for 1 h. The ectopically expressed ΔRaf-1:ER proteins were either immunoprecipitated with HC-20 (Santa Cruz), an antibody against the ligand binding domain of human estrogen receptor (first panel) or with anti-Raf-1 antibody (second panel). Raf-1 activity was assayed as described in Fig. 1A. The 32P-labeled recombinant MEK1 bands are indicated. Stimulation of MEK activity (third panel). Cells were treated as described in the legend to the first panel. One hundred micrograms of cell extracts were assayed for MEK activity, using recombinant ERK2 as a substrate, as described in “Materials and Methods.” The reaction mixture was resolved by SDS-PAGE, blotted onto a nylon membrane, and exposed to x-ray film for autoradiography. The 32P-labeled ERK2 bands are indicated. Stimulation of ERK activity (fourth panel). Cells were grown as described in the legend to the first panel. Samples were assayed with immunoprecipitated ERK2 as described under “Materials and Methods.” The radioactivities of 32P-labeled MBP bands are indicated. The data shown are the representative of six independent experiments.

A stable clone of Pa-4 cells expressing ΔRaf-1:ER with a high stimulated ΔRaf-1:ER activity was isolated and hereafter referred to as Pa-4Raf-1:ER. Western blot analyses of Pa-4Raf-1:ER cell extracts with anti-Raf-1 antibody demonstrated the expression of ΔRaf-1:ER fusion protein exclusively, while anti-Raf-1 antibody detected both endogenous p74Raf-1 and ectopic ΔRaf-1:ER in these Pa-4Raf-1:ER cells (Fig. 2A). Thus, anti-Raf-1 antibody was used in the immune complex kinase assays to evaluate the induction of ΔRaf-1:ER activities. A result comparable with that obtained with anti-Raf-1 antibody on the basal and induced ΔRaf-1:ER activities was observed with anti-Raf-1 antibody, except that a lesser degree of rMEK1 phosphorylation was demonstrated when anti-Raf-1 antibody was used (Fig. 2B, first versus second panel). This was probably caused by a lower efficiency in immunoprecipitation of the ΔRaf-1:ER fusion protein by anti-Raf-1 antibody. As expected, the basal ΔRaf-1:ER activity in Pa-4Raf-1:ER cells were greatly reduced when 2.5% charcoal-stripped serum was used to constitute the growth medium; however, the remaining residual activity was still high enough to elevate the basal level of MEK1 and ERK2 activities when compared with those in the Pa-4 cells (Fig. 2B). This is probably due to an incomplete inactivation of the ΔRaf-1:ER fusion protein. Addition of estradiol to the parental Pa-4 cells had no discernible effect on Raf-1 activity and its downstream MEK1 and ERK2 activities (Fig. 2B). In contrast, 1 μM estradiol led to an activation of ΔRaf-1:ER activity, as demonstrated by an increase in phosphorylation of its substrate, rMEK1. The activation of ΔRaf-1:ER resulted in a subsequent activation of MEK1 and ERK2 as shown by the increases in phosphorylation of their substrates, ERK2 and MBP, respectively (Fig. 2B, third and fourth panels). This specific activation of ΔRaf-1:ER can last for more than 16 h (data not shown) in the presence of estradiol, as reported in other cell types (29).

Identification of ΔRaf-1:ER-regulated Genes in Pa-4 Cells—To identify differentially expressed genes as a consequence of cellular responses to Raf-1 activation, we have attempted to identify genes regulated by ΔRaf-1:ER activation in Pa-4 epithelial cells using the technique of mRNA differential display. In total, 48 artificial primer pairs were used, and the mRNA expression patterns of parental Pa-4 cells were compared with Pa-4Raf-1:ER cells incubated with 1 μM estradiol for 16 h to induce ΔRaf-1:ER activities. Message levels with apparent differences, which were reproducibly observed between the basal and estradiol-treated Pa-4Raf-1:ER cells were pursued. To confirm the gene expression patterns observed in the mRNA differential display analysis, cDNA fragments were recovered from gels and reamplified by PCR using the corresponding primer pairs. The reamplified fragments were used as hybridization probes for Northern analyses, as well as cloned into pCR-TRAP vector for sequence analyses. The estimated sizes of the transcripts and the results of homology search against GenBankTM and EMBL data bases of six differentially expressed genes are summarized in Table I. Among them, a 112-base pair insert of clone 2C3 shared 83% homology with the 3′-untranslated region of mouse HMGIC (42), a transformation-associated non-histone chromosomal protein. Since rat HMGIC cDNA has not been characterized yet, we screened a Lambda Zap cDNA library prepared from mRNA of estradiol-treated Pa-4Raf-1:ER cells, with cDNA fragment 2C3 as a probe. The longest cDNA clone rHMGIC-C9 is approximately 3.3 kilobases in size, of which 321 nucleotides encode a single reading frame of 107 amino acids preceded by 412 nucleotides in 5′-untranslated region (GenBankTM accession number U89695). Alignment of HMGIC-C-derived amino acid sequences from different species indicated that the predicted rat HMGIC-C amino acid sequence is highly homologous to that of the human (43) and mouse (42) HMGIC-C with 8- and 5-amino acid differences, respectively (Fig. 3).

Regulation of HMGIC Message Levels by Raf-1 via an ERK-dependent Pathway—After identifying HMGIC-C as one of the Raf-1-regulated genes, we wished to ascertain whether the HMGIC-C gene is a primary or secondary response gene to the Raf-1 signaling pathway activation. Pa-4Raf-1:ER cells were treated with 1 μM estradiol to activate ΔRaf-1:ER kinase activities, for 0.5, 2, 4, 6, 12, 24, 48, and 72 h, and Northern blot analyses were performed. As seen in Fig. 4, HMGIC-C cDNA probe hybridized with a single band around 4.2 kilobases, from the total RNA isolated from Pa-4Raf-1:ER cells, which is consistent with the published sizes of mouse and human...
acids shared by all HMGI, HMGY, and HMGI-C proteins are also present the three DNA binding domains. The conserved C-terminal calculated relative to the level measured in cells treated with vehicle mean and analyzed by Instantimager 228. The fold induction shown is the maximize the alignment. The domains I, II, and III (black boxes)

The Pa-4

were treated with vehicle alone, 20 μM, or 100 μM PD 98059 for 60 min prior to 6 h of estradiol stimulation. This selective inhibitor allowed us to demonstrate that Raf-1-mediated HMGI-C mRNA induction is blocked when ERK activation by MEK is inhibited (Fig. 5A). The result confirms that oncogenic Raf-1 induces HMGI-C expression via an ERK-dependent pathway. In addition, when Pa-4Raf-1:ER cells were exposed to either 20 μM or 100 μM PD 98059, in the absence of estradiol treatment, the basal HMGI-C mRNA expression was reduced (Fig. 5A, lanes 3 and 5 versus lane 1) to a level comparable with that in the parental Pa-4 cells (data not shown). This inhibition of basal HMGI-C expression may be caused by a decrease in the elevated ERK2 activities in the untreated Pa-4Raf-1:ER cells (Fig. 2B, lane 3 versus lane 1).

It has been suggested that protein kinase C is capable of mediating ERK activation by phosphorylating Raf-1 directly (47). To determine the role of protein kinase C and ERK in inducing HMGI-C expression, we treated parental Pa-4 cells with TPA and measured HMGI-C expression by Northern analysis. As shown in Fig. 5B, treatment of cells with phorbol ester mimics the effect of ΔRaf-1:ER stimulation and resulted in an unequivocal increase in HMGI-C mRNA level in the parental cells. Moreover, PD 98059 treatment nearly abolished HMGI-C induction by TPA (Fig. 5C). Thus, the activation of the Raf/MEK/ERK signaling cascade is necessary and, perhaps, sufficient for elevating HMGI-C steady state mRNA level.

Mechanism of Activation of HMGI-C Expression by ΔRaf-1:ER—We next investigated the mechanism by which activation of ΔRaf-1:ER leads to an increase in the expression of HMGI-C mRNA. To determine whether concurrent protein synthesis is required for the ERK pathway to induce HMGI-C expression, we performed experiments using cycloheximide, an inhibitor of protein synthesis. As reported, at 10 μg/ml cycloheximide, the incorporation of 3H-amino acids into the acid-insoluble fraction was more than 99% inhibited (48). In our study, treatment with cycloheximide alone for 4 h had no obvious effect on the steady-state HMGI-C mRNA level in the Pa-4ΔRaf-1:ER cells, as compared with that of β-actin (Fig. 6, lanes 1 and 3). Apparently, there was no superinduction, known to modulate immediate-early c-fos gene expression, of HMGI-C expression after protein synthesis was inhibited. Moreover, this cycloheximide treatment clearly abolished the induction of HMGI-C mRNA expression by ΔRaf-1:ER (Fig. 6, lanes 2 and 4), suggesting that ongoing protein synthesis is necessary for HMGI-C gene activation.

To further understand the nature of Raf-1-regulated HMGI-C message induction, the stability of HMGI-C mRNA in the Pa-4ΔRaf-1:ER cells was studied in the presence of actino-

**TABLE I**

| Fragment name | Primer for RT<sup>a</sup> | Primers for PCR | Size of PCR products (bp) | mRNA size (kb) | mRNA level | Corresponding gene |
|---------------|--------------------------|-----------------|---------------------------|---------------|------------|-------------------|
| 2A1           | T11A                    | H-AP2, T11A     | 191                       | 3.0           | Down       | Human MAL-b protein |
| 2C2           | T11C                    | H-AP2, T11C     | 217                       | 1.3           | Up         | Mouse platelet factor 4 |
| 2C3           | T11C                    | H-AP2, T11C     | 109                       | 3.6           | Up         | Mouse HMGI-C |
| 10G1          | T11G                    | H-AP10, T11G    | 272                       | 2.6           | Up         | No significant similarity |
| B4            | T11G                    | H-AP3, T11G     | 125                       | 4.0           | Down       | Rat epithelial sodium channel α subunit |
| D11           | T11G                    | H-AP4, T11G     | 200                       | 1.5           | Down       | Mouse keratin D |

<sup>a</sup> RT, reverse transcription reactions.

**Fig. 3. Comparison of the derived amino acid sequence of rat HMGI-C with that of human and mouse.** The derived amino acids of rat HMGI-C (this work) are aligned with that of human (43) and mouse (42). Dashed lines indicate identity whereas dots are introduced to maximize the alignment. The domains I, II, and III (black boxes) represent the three DNA binding domains. The conserved C-terminal acidic tails shared by all HMGI, HMGY, and HMGI-C proteins are also boxed.

**Fig. 4. Kinetics of HMGI-C mRNA induction in Pa-4ΔRaf-1:ER cells.** The Pa-4ΔRaf-1:ER cells were treated with 1 μM estradiol for indicated times. Twenty micrograms of total RNA from cells at various times after estradiol addition were fractionated, transferred to membrane, and hybridized with 32P-labeled probes of HMGI-C and β-actin as indicated. The levels of hybridized HMGI-C messages were detected and analyzed by Instantimager 228. The fold induction shown is the mean ± S.E. from at least three independent experiments and was calculated relative to the level measured in cells treated with vehicle only. Blot shown is one representative.
mycin D to block new transcription. It had been shown that actinomycin D completely inhibited the incorporation of [3H]uridine into acid-soluble fraction at 5 μg/ml (48). Total RNA was prepared from Pa-4 ΔRaf-1:ER cells that have been treated with 1 μM estradiol to activate Raf-1 pathway or vehicle alone, as a control, for 6 h in the presence or absence of 5 μg/ml actinomycin D. Equal amounts of RNA were used in the Northern analysis. As shown in Fig. 7, the induction of HMGI-C by activated ΔRaf-1:ER was completely blocked by co-incubation with actinomycin D. This suggests that the stability of the HMGI-C message is not enhanced by Raf-1 activation. Moreover, the message levels of HMGI-C decreased faster than that of β-actin in the presence of actinomycin D, indicating a shorter half-life than β-actin. These results imply that the induction of HMGI-C mRNA expression following ΔRaf-1:ER activation, at least in part, was caused by transcriptional activation of the HMGI-C gene promoter.

MEK/ERK-dependent Transformation of Pa-4 ΔRaf-1:ER Cells Induced by ΔRaf-1:ER Activation—Anchorage-independent growth is one of the hallmarks of cell transformation. To analyze the role of the Raf/MEK/ERK pathway in cell transformation, the Pa-4 ΔRaf-1:ER cells were further tested in vitro for their ability to form colonies in soft agarose. Compared with the parental Pa-4 cells, the estradiol-treated Pa-4 ΔRaf-1:ER cells have an approximately 15-fold increase in soft agarose colony-forming efficiency (Fig. 8, A and B). Estradiol alone did not induce foci formation or morphological changes in untransformed Pa-4 cells. In the absence of estradiol, the basal ΔRaf-1:ER activity in Pa-4 ΔRaf-1:ER cells failed to result in the outgrowth of foci of transformed cells. Moreover, addition of 5 μM or 20 μM PD 98059 led to a concentration-dependent decrease of the colony-forming efficiency in soft agarose induced by ΔRaf-1:ER activation (Fig. 8D). There was no gross growth inhibition of Pa-4 ΔRaf-1:ER cells by PD 98059 at 20 μM when
the cells were cultured in the regular dishes for 96 h (data not shown). Taken together, our results raise the possibility that there is a correlation between the HMGI-C induction and cell transformation by the activation of oncogenic Raf-1.

DISCUSSION

A central goal in the field of molecular biology and signal transduction biology is to elucidate the mechanism through which short-lived signals, emanating from the surface of the cell, can be conveyed internally to effect long-term adaptive changes in cellular behavior, such as proliferation, differentiation, and oncogenic transformation. In the present study we set out to investigate the signaling pathway linking β-adrenergic receptor stimulation to the activation of Raf-1 protein kinase in whole animals. We found that daily isoproterenol administration of the β-agonist isoproterenol induced a delayed activation of Raf-1 activities in the mouse parotid glands. It is of interest to note that the kinetics of Raf-1 activation correlates with the time course of the enlargement of mouse parotid glands as well as the time course for the induction of salivary-specific proline-rich protein gene expression following daily isoproterenol injection (28). Thus, our observation of a time-dependent Raf-1 kinase activation by isoproterenol treatment suggests that Raf-1 may play a critical role in promoting long-term adaptive changes in rodent salivary glands. Most of these changes in salivary glands are believed to be brought about by modulating gene expression at the level of transcription or post-transcriptional modification (49, 50). For this reason, the mechanism by which altered gene expression occurs in response to Raf-1 activation in salivary cells is an important topic. The availability of estradiol-regulated forms of Raf-1 kinase provides a unique opportunity for us to express the conditional form of Raf-1 kinase in salivary epithelial Pa-4 cells to investigate biochemical mechanism underlying long-term cellular adaptive response to Raf-1 activation. We report here that the HMGI-C gene is one of the target genes that respond to Raf-1 activation.

The result from the actinomycin D experiment implies that the increase in steady-state HMGI-C mRNA levels is a result of transcriptional activation rather than enhanced mRNA stability. In addition, HMGI-C gene expression responds to Raf-1 activation after a lag of more than 2 h and requires ongoing protein synthesis. Taken together, a mechanism different from that of the primary response or early response genes is likely to be involved in regulating HMGI-C gene expression. Many genes are classified as the immediate-early Raf-1 response genes based on the original definition of a transcriptional response to Raf-1 activation that is both insensitive to protein synthesis inhibitors and transient. Three of the best known examples among these immediate-early Raf-1 response genes are the c-fos (51), egr-1 (52), and HB-EGF (21, 53). However, very little work has been done to identify and characterize the secondary response genes to Raf-1 activation. Analysis of the Raf-1 response element(s) in HMGI-C promoter/enhancer should provide new insights into the important factors involved in this adaptive response to Raf-1 activation.

To demonstrate that HMGI-C induction by Raf-1 activation is mediated via an ERK-dependent pathway, we have shown that TPA, known as a protein kinase C activator, is capable of modulating HMGI-C expression (Fig. 5B). It has been reported that protein kinase C stimulates ERK activation through a direct phosphorylation of Raf-1 (47). Although both TPA and ΔRaf-1:ER activate HMGI-C gene expression, they have different kinetics in terms of elevating steady state HMGI-C mRNA levels. The difference might reflect either the duration of activation, TPA induction being transient possibly due to protein kinase C down-regulation, or the possibility that ΔRaf-1:ER activates an additional pathway that modulates HMGI-C expression. In an independent line of investigation to assess the putative role of ERK in Raf-1-induced HMGI-C expression, we used PD 98059, a cell-permeable inhibitor of the ERK upstream kinase (MEK) (44, 45). This compound has been shown to block MEK-mediated tyrosine phosphorylation, while not affecting other tyrosine kinases (e.g. Src) or serine/threonine kinases (e.g. protein kinase A, protein kinase C, or Raf) (45). The Pa-4ΔRaf-1:ER cells were treated with PD 98059, a dose-dependent inhibition of ΔRaf-1:ER- and TPA-stimulated HMGI-C expression was observed (Fig. 5, A and C). However, it is still conceivable that the MEK downstream kinase(s) other than ERK1/2 might be involved or that TPA may also activate other signaling pathway to modulate HMGI-C expression. Taken together, our results strongly indicate that the Raf/MEK/ERK signaling cascade is involved in activating HMGI-C expression.

The molecular mechanisms underlying the activation of immediate-early gene expression by the Raf/MEK/ERK module have been studied extensively (21, 51, 52). For example, it has been demonstrated that Raf-1 through MEK activates ERK, which in turn efficiently phosphorylates several sites in the carboxyl-terminal of ternary complex factor/Elk-1. Phosphoryl-
ation elevates the transcriptional activator potential of ternary complex factor/Elk-1, which is required to form a complex with serum response factor at the serum response element for the induction of \(c\text{-}fos\) expression (54). However, the Raf/MEK/ERK cascade-mediated \(c\text{-}fos\) induction is transient and superinduced by cycloheximide treatment, which is different from the mode of regulation observed for \(HMGI\text{-}C\) induction. Therefore, it is possible that a different pathway, which is also activated by ERK phosphorylation, is responsible for activating the expression of the Raf-1 secondary response gene \(HMGI\text{-}C\). Moreover, the murine \(HMGI\text{-}C\) gene has been cloned, and several putative transcription factor binding sites for activation transcription factor (ATF), Sp1, Ets, and E\(_2\)F have been identified in the first 247 nucleotides of the 5' flanking region (30). While the rat \(HMGI\text{-}C\) 5'-proximal flanking region is highly homologous to that of the mouse \(HMGI\text{-}C\) gene, the presence of these transcription factor binding sites in the 5'-upstream region cannot fully explain the delayed induction of \(HMGI\text{-}C\) expression. This conclusion is based on the reasoning that the above-mentioned transcription factors are either constitutively expressed or are immediate targets of various signal transduction pathways and de novo protein synthesis is indispensable for \(HMGI\text{-}C\) gene activation. We are presently engaged in the identification and characterization of cis- and trans-factors that lead to the transcriptional activation of the \(HMGI\text{-}C\) promoter.

What is the physiological and pathological importance of the \(HMGI\text{-}C\) induction? \(HMGI\text{-}C\) protein is a member of the high mobility group (HMG) proteins, which comprise a significant fraction of non-histone proteins of eukaryotic chromatin. HMG proteins were originally discovered as abundant heterogeneous fraction of non-histone proteins of eukaryotic chromatin. HMG proteins (also referred to as “A-T” hooks) and a highly acidic C domain, which has been hypothesized as the location of multiple casein kinase II phosphorylation sites (59). Recently, results from many experiments (60–64) have shed light on the putative role(s) of the HMG(Y) family of proteins and their possible involvement in the transcriptional regulation of nuclear gene expression. Particularly, HMG1 and HMGY proteins have no intrinsic transcriptional activation activity; rather, these two gene products have been demonstrated to transactivate a number of promoters by facilitating the activities of other transcription factors such as NF-\(k\)B, ATF2, Tst-1/Oct-6, and Sp1 (60–64). Through protein-protein and protein-DNA interactions, these proteins have been shown to have the ability to change DNA conformation and are therefore named the “architectural protein” (65). However, the role of \(HMGI\text{-}C\) in terms of transcriptional regulation is not as well characterized as HMG(Y). By analogy to HMG1 and HMGY, we propose that HMG1 proteins by bringing distant transcription factors already bound to the promoter/enhancer region into close proximity and results in higher levels of transcription of the target genes because the HMG1-C protein shares about 50% amino acid sequence identities with that of HMG1 and HMGY (43). The target gene or interacting protein partners for HMG1-C remains to be determined. It has been reported that HMG1-C is involved in the development of a variety of tumors, including the pleomorphic salivary adenoma (34). The Pa-4 Raf-1:ER cells with activated Raf-1:ER also show the phenotype of transformed cells, as demonstrated by a soft agarose assay.

In conclusion, we have shown that the activation of the Raf/MEK/ERK module is involved in the sustained activation of \(HMGI\text{-}C\) expression by an oncogenic and regulatable form of Raf-1 kinase introduced into the salivary Pa-4 cells. The results from the experiments using protein synthesis and transcription inhibitors suggest that the mechanism underlying \(HMGI\text{-}C\) induction is dependent on new protein synthesis, which in turn activates \(HMGI\text{-}C\) transcription. The time course of induction studies has demonstrated that in response to Raf-1 activation, the mRNA levels increase after a lag of 2 h. Therefore, new protein(s) responsible for elevating \(HMGI\text{-}C\) expression should present in the cells at approximately 2 h after Raf-1 activation. The nature of this regulatory protein(s) and its cognate target sequence in the \(HMGI\text{-}C\) promoter/enhancer region remain to be determined. Further investigation to identify the putative regulatory proteins and \(HMGI\text{-}C\) target gene(s) will ultimately assist in delineating the mechanism(s) that underlies long-term adaptive survivable responses and cell transformation in general by Raf-1 activation.

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**Regulation of Raf-1 Secondary Response Gene Expression**

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