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PROTEIN PHOSPHORYLATION IN THE NERVOUS SYSTEM

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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The City University of New York

Abstract

PROTEIN PHOSPHORYLATION IN THE NERVOUS SYSTEM

by

HUIAI YANG

Advisers: Professor Yigal H. Ehrlich and Professor Probal Banerjee

Ecto-protein kinases are enzymes which are responsible for protein phosphorylation occurring in the extracellular matrix. Extracellular phosphorylation has been found to be associated with the growth and transformation of parasites, and its involvement in the formation of bone tissues has been discovered. Using cultured telencephalons from seven-day chicken embryos, we have investigated the role of extracellular phosphorylation in nervous system.

Extracellular phosphorylation assays performed on attached cells have revealed several proteins as substrates of ecto-protein kinases. When antibodies generated against protein kinase C isozymes were added to the assay buffer, the PKC δ antibody selectively inhibited the phosphorylation of two proteins with apparent molecular weights of 12 and 13 kilodaltons. This suggests that there is a PKC δ-like ecto-protein kinase located on the surface of cultured

chicken telencephalons, and that there is a novel signal transduction pathway mediated by extracellular phosphorylation in the nervous system.

Similar to the regulation of extracellular phosphorylation by ecto-protein kinases, important cellular events such as mitosis and transformation are regulated by specific intracellular protein kinases. Raf-1 is such a kinase which is also a key player in MAP kinase-mediated mitosis and transformation. Additionally, its involvement in apoptosis has been recently observed.

Since Raf-1 phosphorylation plays a key role in regulating the MAP kinase pathway, we investigated the effect of 8-OH-DPAT, a serotonin 1A receptor agonist, on Raf-1 phosphorylation in a hippocampal hybrid neuroblastoma cell line (HN2-5). We also investigated the interactions of phosphorylated Raf-1 with some proteins of the apoptotic pathway. Our results showed that though increased Raf-1 phosphorylation was not detected by 1-D gel, further resolution with the 2-D gel did reveal elevated Raf-1 phosphorylation in cells treated with 8-OH-DPAT. Immunoprecipitation studies showed that the phosphorylated Raf-1 molecule is co-immunoprecipitated with antibodies against caspase 8 and Bad, but not with an antibody against Bcl-2. Caspase 8 belongs to a family of cystein proteases, which are activated by apoptotic signals. Bad and Bcl-2 belong to the Bcl-2 family of proto-oncogenes, which regulate mitochondrial functions and apoptosis. The formation of Raf-1- caspase 8 and Raf-1-Bad complexes suggest that Raf-1 might be also involved in regulating the apoptotic pathway.

This book is dedicated to

my parents

Frances

Binbin

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ABBREVIATIONS

Bad	Bcl-xL/Bcl-2-associated death promoter
Erk1/Erk2	extracellular signal-regulated kinases
FLICE	caspase 8
MAPK (MAP kinase)	mitogenesis-activating protein kinase
MEK	MAP kinase kinase, the kinase that phosphorylates MAP kinas
MEKK	MEK kinase, the kinase that phosphorylates MEK
PI3-K	phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase
PKB (Akt)	protein kinase B
PKC	protein kinase C
SAPK	stress-activated protein kinase

CHAPTER ONE

EXTRACELLULAR PHOSPHORYLATION IN CULTURED CELLS FROM CHICKEN TELENCEPHALONS

1-1. Introduction

Protein phosphorylation is a major regulatory mechanism of cellular homeostasis. It is a chemical reaction in which a phosphate group is transferred from a nucleotide triphosphate to a protein. This process is normally an intracellular event; however, it has been found that protein phosphorylation can also occur extracellularly and such phosphorylation is not an artifact of leakage of kinases into the extracellular fluids. Thus, extracellular protein phosphorylation might be a novel signal transduction pathway that cells use to regulate certain important functions. In order to understand the role of extracellular protein phosphorylation in the nervous system, we proposed to test the hypothesis that extracellular protein phosphorylation is found in the nervous system and is catalyzed by a membrane-bound enzyme with the catalytic domain facing outwards, namely, an ecto-protein kinase.

Specific Aims:

The goal of this thesis was to investigate the role of extracellular protein phosphorylation in the nervous system. We hypothesized that extracellular protein phosphorylation can be found in the nervous system and the enzyme catalyzing the reaction can be characterized. We proposed to test the hypothesis with the following questions.

The first task was to establish a condition where extracellular phosphorylation can occur measurably. To address this, we used cultured chicken telencephalons from seven-day embryos, and the phosphorylation assays were performed on attached cells. Radioactive Pi that is a small molecule and can penetrate cell membrane was used to monitor intracellular phosphorylation, and radioactive ATP that cannot penetrate the cell membrane was used to test extracellular phosphorylation. Labeled proteins were identified by autoradiography.

The second task was to characterize the protein kinases catalyzing the extracellular phosphorylations of identified protein substrates. To achieve this, we used known protein kinase substrates and known antibodies generated against a specific protein kinase to evaluate the effect of these exogenously added reagents on extracellular phosphorylation.

Background and significance:

Protein phosphorylation is involved in the regulation of cell growth and motility (Nishizuka, 1984; Hashimoto et al., 1989; Cantley et al., 1991; Gulbins et al., 1993). In the nervous system, protein phosphorylation has been associated with neuronal development, differentiation, plasticity, and aging (Sorkin et al., 1984; Akers et al., 1986; Reymann et al., 1988; Colley et al., 1990; Bading et al.,1991; Routtenberg, 1991; Buxbaum et al., 1993; Hollenbeck, 1993; Waxham et al., 1993; Manser et al., 1994; Rocchi et al., 1995; Worley et al., 1996).

Protein kinases are enzymes that catalyze protein phosphorylation, in which the γ-phosphate group of adenosine triphosphate (ATP) is transferred to a protein substrate. The amino residues receiving the phosphate group are serine, threonine, and tyrosine; other amino residues functioning as phosphorelay centers have been found in prokaryotes and yeasts (Appleby et al., 1996). The number of protein kinases identified so far has increased dramatically in recent years. The majority of protein kinases are found inside the cell, where they participate in an array of signal transduction pathways (Hunter, 1987; Hanks et al., 1988; Kemp et al., 1990; Dvir et al., 1993; Segil et al., 1991; Wang et al. 1993; Goldberg et al., 1996).

The focus of my study is on protein phosphorylation that occurs extracellularly and on protein kinases responsible for this type of phosphorylation. ATP can be secreted into extracellular matrix (Bishop et al., 1959; Ågren et al., 1969;Gordon, 1986). Exogenously added ATP has an effect

on cellular osmolarity, synthesis, and programmed cell death (Hempling et al., 1969; Stewart et al., 1969; Rozengurt et al., 1977;Kitagawa, 1980; Hosoi et al., 1989; Kuroki et al., 1989; Zheng et al., 1991).

In the early fifties, a substance in dry powders of spinal roots was found to be able to induce capillary dilation, and that substance was later proved to be ATP. Ever since then the possibility of ATP functioning as a neurotransmitter has been pursued vigorously (Holton et al., 1953,1954), and ATP has met several criteria set for any molecule qualifying as a neurotransmitter (Whittaker, 1968b, 1972). Firstly, ATP is found at a high concentration in the nerve endings, and synaptic release of ATP in different regions of the brain has been demonstrated (Nyman et al., 1963; Potter et al., 1980). Secondly, ATP has been shown to be a constituent of cholinergic synaptic vesicles and is associated with the release of acetylcholine from synapses (Dowdall et al., 1974; Silinsky, 1975; Richardson, et al., 1987; Unsworth et al., 1990). Thirdly, depolarization can induce the ATP release from synapses, thus resulting in a fast excitatory synaptic potential (White, 1977; White et al., 1980; Wieraszko et al., 1989a,b; Silinsky et al., 1992; Edwards et al., 1993; Galligan et al., 1994).

In addition to being a neurotransmitter, ATP can function as a neuromodulator as well. Its effects include stimulation of norepinephrine uptake, enhancement of DNA synthesis, induction of mitogenesis and cell differentiation, stimulation of synthesis or release of cytokines and neurotrophic factors, induction of habituation, and modulation of intrasynaptosomal free Mg^{++} concentration (Stone, 1981; Kuroda, 1983; Ehrlich et al., 1988; Hardwick et al.,

1989; Wang et al., 1990; Neary et al., 1991,1996; Cheever et al., 1994; Ciccarelli et al., 1994; Garcia-Martin et al., 1995; Jamieson et al., 1996).

A possible mechanism by which extracellular ATP exerts its effects is by binding to membrane-bound proteins. Membrane, the barrier separates intracellular and extracellular environments, plays a very important role in the physiology of cells (DePierre et al., 1973; DePierre et al., 1974a,b; Whittaker, 1966; Whittaker, 1968a). In earlier studies it was found that membrane fractions obtained from different tissue preparations can be phosphorylated and their phosphorylations regulate certain functions; some of these phosphorylations are cAMP-dependent (Shlatz et al., 1971; Weller et al., 1971; Guthrow et al., 1972; Roses et al., 1973; Chang et al., 1974; Ehrlich et al., 1974;Lemay et al., 1974; Carpenter et al., 1979; Mastro, 1979). Such phosphorylation regulates the activity of membrane-bound proteins like ion channels and receptors which transduce transmembrane signals into cells (Nestler et al., 1983; Whittemore et al., 1984; Sibley et al., 1987; Nishibe et al., 1990; Keller et al., 1992; Nellen et al., 1994; Wrana et al., 1994; Sculptoreanu et al., 1993; Smith et al., 1996).

Receptors that bind to ATP or its derivatives are called purinoceptors. ATP receptor can activate ion channels either directly or through G proteins. In either case, the channels involved are Na^{+}, K^{+}, or Ca^{++} channels which depolarize neurons (Bean, 1992; Illes et al., 1993). Activation of adenylate cyclase, phospholipase C, Ca^{++} influx, and mobilization of intracellular Ca^{++} have also been reported (Eriksson et al., 1989; Gonzalez et al., 1989; Van Der Merwe et al., 1989; Dubyak et al., 1988, 1990a,b, 1991; Mahoney et al., 1992; Mastuoko

et al., 1995).

ATP can be broken down to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine by ATPases found in the extracellular space, or by a group of enzymes, which are membrane-bound and have their catalytic subunit facing outwards, namely, ecto-ATPase, -ADPase, or -AMPase (Ågren et al., 1971a,b; Ågren et al., 1973; DePierre et al., 1974a,b). Both ATP and its degradation products can bind to specific receptors and exert various effects (Burnstock, 1978; Kuroda, 1983; Silinsky, 1984; Karnovsky, 1986; Lin et al., 1991; Bonitati et al., 1993; Dubyak et al., 1993; Kitakaze et al., 1994; Clifford et al., 1997).

In addition to receptor-mediated signal transduction, ATP is hypothesized to be directly utilized by either membrane-bound ecto-protein kinases, or extracellularly located soluble exo-protein kinases. Extracellular phosphorylation catalyzed by these types of protein kinases offers a possible new avenue for initiating signal transduction.

Ecto-protein kinase activity has been reported in tumor cells (Ågren et al., 1970; Ronquist et al., 1970; Ågren et al., 1971a,b; Kübler et al., 1982b), fibroblasts, myoblasts, osteoblasts (Mastro et al., 1976; Chiang et al., 1979; Chen et al., 1991a,b; Zhu et al., 1997), spermatozoa (Majumder, 1978; Haldar et al., 1986), fat cells (Kang et al., 1979), hepatocytes (Sommarin et al., 1981), and aortic endothelial cells (Pirrotton et al., 1992). Immune and nervous systems also contain cells displaying ecto-protein kinase activity (Ågren et al., 1974; Schlaeger et al., 1976; Remold-ODonnell, 1978; Emes et al., 1982; Amano et al.,

1984; Kang et al., 1984; Ehrlich et al., 1986; Dusenbery et al., 1988; Zhang et al., 1988; Myers et al., 1990; Naik et al., 1991; Volonté et al., 1994).

Some of these ecto-protein kinases have been characterized to be cAMP dependent or sensitive to ganglioside (Kang et al., 1978; Majumder, 1978; Sommarin et al., 1981; Tsuji et al., 1988; Kübler et al., 1989). Ecto-protein tyrosine kinase activity and autophosphorylation of an alkaline phosphodiesterase/nucleotide pyrophosphatase (PC-1) were also reported (Lasher et al., 1988; Skubitz et al., 1988; 1995; Belli et al., 1995). Both lipid- and protein-anchorage have been proposed to explain the attachment of ecto-protein kinases to the plasma membrane (Jordan et al., 1992; Paas et al., 1995) Investigation on the ecto-protein kinase activity in my study was performed on cultured cells obtained from the telencephalons of 7-day chicken embryos. Development of GABAergic, cholinergic, and serotonergic neurons derived from chicken embryos has been elucidated (Mangoura et al., 1988a,b; Okado et al., 1992). Catecholamine, indoleamine and free amino acid levels have been recorded throughout embryogenesis (Hevor et al., 1988; Huether et al., 1991). Since the developmental stages of chicken embryo *in vivo* and *in vitro* have been well documented, it provides a good model system (Hamburger et al., 1951; Thampy et al., 1983; Vernadakis et al., 1986; Mangoura et al., 1988a; Tokioka et al., 1993; Suda et al., 1994).

A set of criteria has been established to detect ecto-protein kinase activity (Kinzel et al., 1986; Hogan et al., 1995). One criterion is that the phosphorylation assay has to be carried out with attached cells. Another is that exogenously

added and membrane impermeable reagents can either stimulate or inhibit extracellular protein phosphorylation. **Figure 1.1** shows protein bands identified to be the substrates of ecto-protein kinases, which have the apparent molecular weight of 116 kDa (116k), 105 kDa (105K), 67 kDa (67K), 53 kDa (53K), 17 kDa (17K), 13 kDa (13K), and 12 kDa (12K). Radioactive ATP and Pi are used to label extracellular protein phosphorylation and intracellular protein phosphorylation respectively. Addition of non-radioactive Pi should have effect only on the intracellular phosphorylation without affecting extracellular phosphorylation. Apyrase, an enzyme inactivates ATP and does not penetrate cell membrane, should only affect extracellular phosphorylation (Molnar et al., 1961). Based on these criteria, 53K and 67K proteins were proved to be phosphorylated both intracellularly and extracellularly; and the rest of the proteins shown in **Figure 1.1** are phosphorylated only extracellularly.

The purpose of my study was to characterize the ecto-protein kinases which phosphorylate the above identified protein bands. By adding different reagents to the phosphorylation assays, I was able to identify different ecto- protein kinases involved in extracellular phosphorylation in cultured cells from chicken telencephalons.

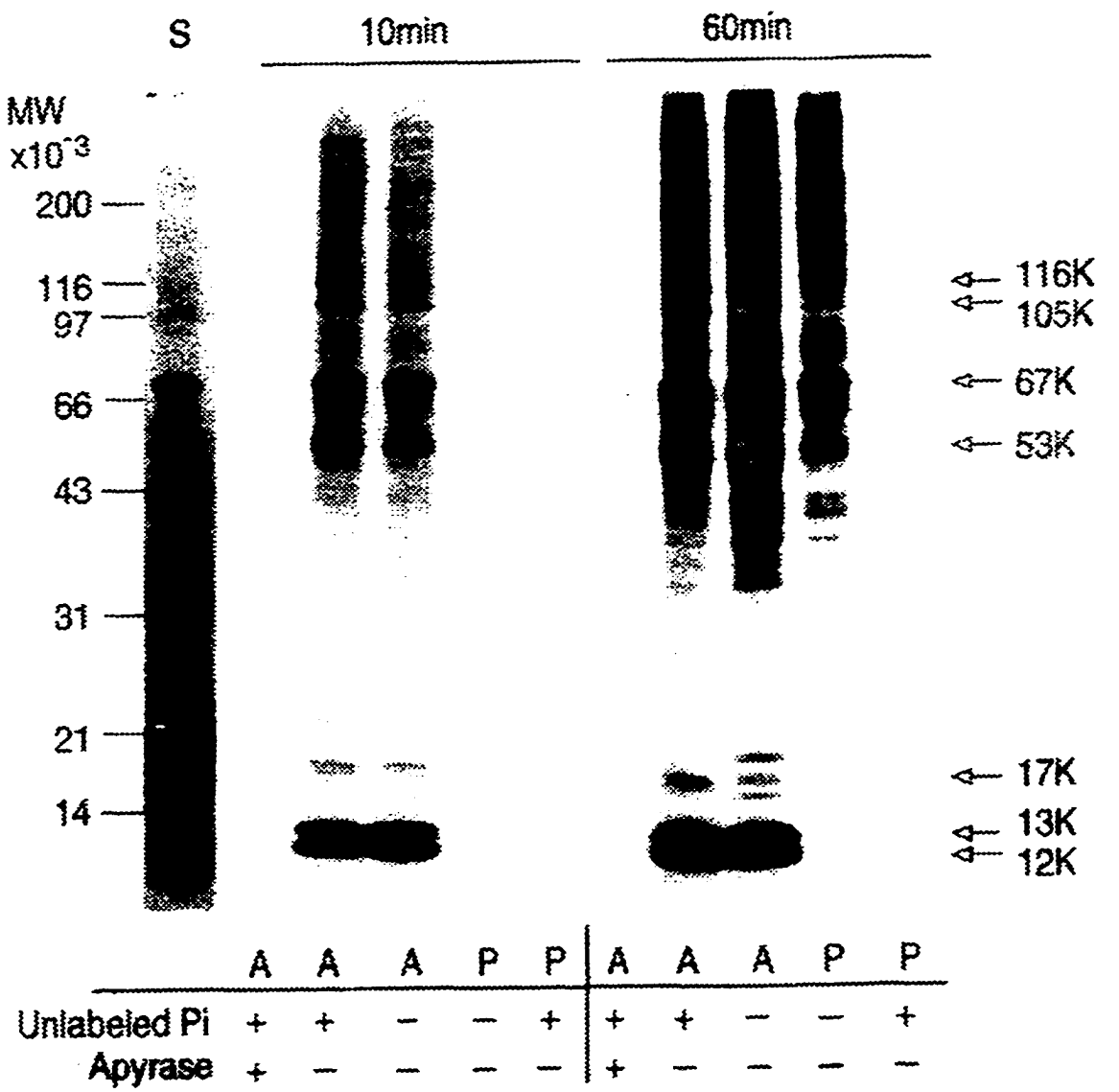

Figure 1.1. Identification of endogenous substrates of ecto-protein kinases. **Left:** Coomassie Brilliant Blue staining of the proteins. **Right:** the autoradiograph of proteins labeled for 10 minutes or 60 minutes with 15 μCi per well of 0.1μM [γ-^{32}P]ATP(lanes marked **A**) or 15 μCi per well of ^{32}P(lanes marked **P**). The reactions were carried out with (+) or without (-) addition of 1mM Na_2HPO_4 and 5units/ml of apyrase (Sigma). Molecular weights are shown on the left. The arrows on the right indicate the position of the protein substrates (Hogan et al., 1996).

1-2. Results.

Phosphorylation of 12K and 13K proteins is inhibited by exogenously added myelin basic protein, but not by α-dephosphorylated casein.

Known protein kinase substrates were added exogenously to identify the properties of ecto-protein kinases. Two substrates were used: one is α-dephosphorylated casein, the substrate of casein kinase, and the other is myelin basic protein, the substrate of protein kinase C (PKC) (Kübler, et al., 1982; Kishimoto et al., 1985). When casein and myelin basic protein (MBP) were applied to the cells, both substrates can be phosphorylated. However, only MBP inhibits the phosphorylations of 12K and 13K as shown in **Figure 1.2**, suggesting that there are at least two different ecto-protein kinases, and that the ecto-protein kinase that phosphorylates 12K and 13K proteins may be related to PKC.

12K and 13K proteins are phosphorylated by an ecto-PKC δ-like protein kinase.

In the PKC regulatory domain, there is an amino acid sequence (19-36) that can serve as a pseudosubstrate to inhibit the kinase activity. A synthetic

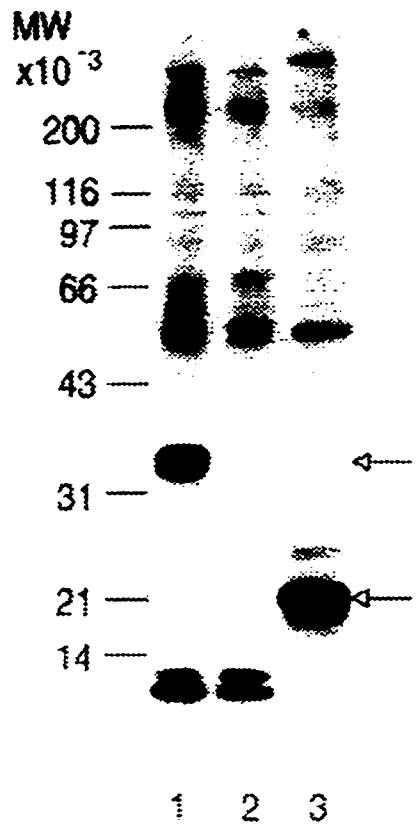

Figure 1.2. Autoradiograph of the phosphorylation of exogenous protein substrates. Cells were incubated with 5 μg/well α-casein (**Lane 1**), without any exogenous protein (**Lane 2**), or with 5 μg/well myelin basic protein (**Lane 3**). The top arrow indicates the position of α-casein, and the bottom arrow indicates the position of myelin basic protein. Molecular weights are shown on the left (Hogan et al., 1996).

peptide composed of amino acids 19 to 31, in which a serine substitutes an alanine at position 25 was used. It is hypothesized that the synthetic peptide as a specific substrate of PKC will compete with the endogenous substrates, resulting in inhibition of phosphorylation. **Figure 1.3** shows that addition of this synthetic PKC substrate indeed inhibits the phosphorylation of 12K and 13K proteins.

Antibodies generated against PKC were further used to confirm the above finding. Monoclonal antibody 1.9 (MAb.1.9) generated against the conserved PKC catalytic region was applied to the cells, and the result shows that MAb1.9 has an inhibitory effect on 12K and 13K protein phosphorylation (**Figure 1.4**). PKC exists as different isozymes and accounts for a diverse functions. Atypical PKC isozymes with discrete properties have kept emerging from a variety of species (Hug et al., 1993; Wang et al., 1993; Johannes et al., 1994; Hunter et al., 1995). Here antibodies generated against some typical and atypical PKC isozymes were used and their effects on 12K and 13K protein phosphorylation were accessed. The anti-PKC isozymes antibodies tested include alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε), zeta (ζ), eta (η), and theta (θ). Among them, only the anti-PKC δ antibody showed an inhibitory effect. Further investigation revealed that anti-PKC δ-V_3 antibody, which is against the third variable region of PKC δ, does not inhibit the phosphorylation of the 12K and 13K proteins. It is the anti-PKC δ-COOH antibody that is responsible for the

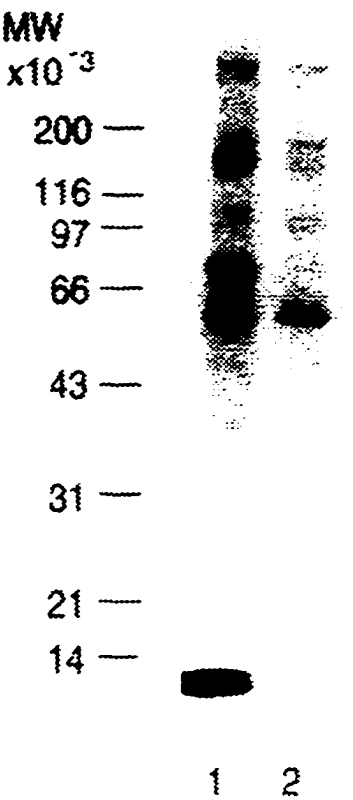

Figure 1.3. Inhibition of phosphorylations of 12K and 13K proteins by PKC serine substrate. Extracellular phosphorylation reactions were carried out without (**Lane 1**) or with (**Lane 2**) 10 μM PKC serine substrate (Hogan et al., 1996).

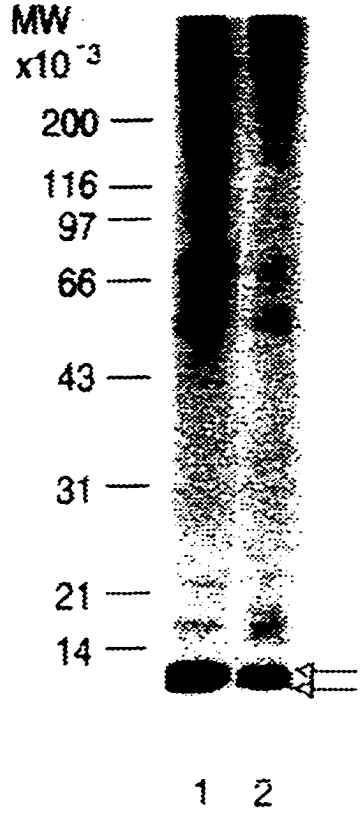

Figure 1.4. Inhibition of phosphorylations of 12K and 13K proteins by Monoclonal antibody 1.9. Reactions were carried out without (**Lane 1**) or with (**Lane 2**) 0.1 μg/well of anti-PKC monoclonal antibody 1.9 (Gibco). Arrows on the right indicate the position of the12K and 13K proteins (Hogan et al., 1996).

observed effect, which is against the last eight amino acids of the PKC δ carboxyl terminus (**Figure 1.5**). This result suggests that the anti-PKC δ-COOH antibody recognizes the ecto-protein kinase phosphorylating 12K and 13K proteins and that the interaction between the kinase and the antibody interferes with the kinase activity. Quantitation of the inhibition of 12K and 13K protein phosphorylation by PKC substrate, Mab1.9 and anti-PKC δ-COOH antibody is presented in **Table 1**.

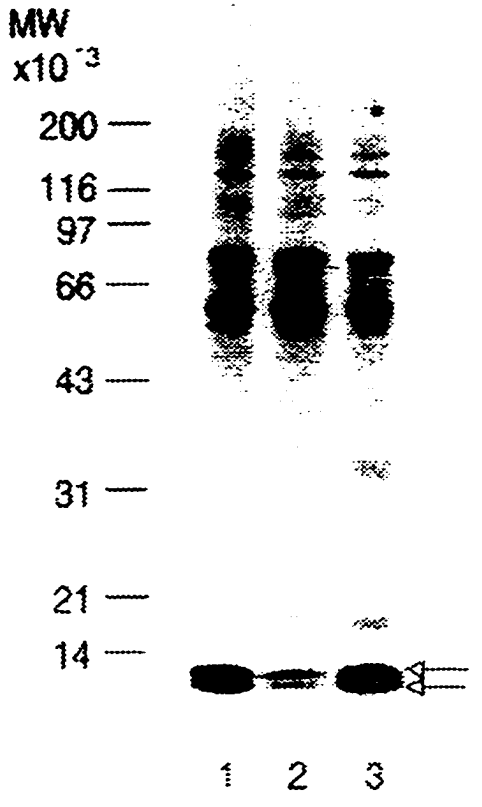

Figure 1.5. Inhibition of the phosphorylation of 12K and 13K proteins by anti-PKC δ antibodies. Lane 1: Reactions were carried out without any antibody. **Lane 2:** Reactions were carried out with anti-PKC δ-COOH antibody (1:300). **Lane 3:** Reactions were carried out with anti-PKC δ-V_3 (1:300). Arrows on the right indicate the positions of the12K and 13K proteins (Hogan et al., 1996).

M.W. of endogenous substrates	12K (% of inhibition)	13K (% of inhibition)
PKC serine substrate (19-31)	**84.3±0.9%**	**74.3±2.4%**
Monoclonal antibody 1.9 (MAb 1.9)	**44.0±5.6%**	**62.2±4.2%**
Anti-PKC δ-COOH	**55.9±5.1%**	**60.4±2.2%**

Table 1. Quantitation of the inhibition of ecto-PKC δ-like protein kinase by inhibitors of PKC. The results were obtained from three separate experiments, each experiment had two or three repeats.

1-3. Discussion

It has been found that in prokaryotes, extracellular phosphorylation regulates the cell's ability to invade host cells (Lester et al., 1990,1991; Hermoso et al., 1994; Assefa et al., 1995; Serrano et al., 1996; Sacerdoti-Sierra & Jaffe, 1997). In eukaryotes, it is involved in platelets aggregation (Korc-Grodzicki et al., 1988; Asch et al., 1993; Barbinska et al., 1996), growth factor/inhibitor phosphorylation (Skubitz et al., 1991a; Vilgrain et al., 1991; Kübler et al., 1992; Friedberg et al., 1995; Friedman et al., 1992), muscle contraction and myogenesis (Lamport-Vrana et al., 1991; Chen et al., 1991a,b), and formation of bone tissue (Satoyoshi et al., 1995; Golub, 1996; Suzuki et al., 1996; Zhu et al., 1997; Suzuki et al., 1998). In the nervous system, it has been associated with neurotransmitter uptake, synapse formation, inhibition of neurite extension, long-term potentiation, and Alzheimer's disease (Ehrlich et al., 1986b,1987, 1990, 1991; Hendley et al., 1988; Muramoto et al., 1988, 1994; Nagashima et al., 1991; Knops et al., 1993; Pawlowska et al., 1993; Wieraszko et al., 1994; Fujii et al., 1995; Chen et al., 1996; Hogan et al., 1996). However, the signal transduction pathways associated with extracellular phosphorylation by ecto-protein kinases are not well understood.

Could it be that some extracellular protein phosphorylation found so far are artifacts? Actually, contamination from intracellular protein kinases released from dead cells has been mentioned to argue against the existence of an ecto-protein kinase (Noland et al., 1986). Some proteins that are used as exogenous

substrates of ecto-protein kinases are known to induce damage to the cell membrane causing possible leakage of kinases (Kübler et al., 1982a). This possibility remains to be studied.

Using exogenous substrates as inducers, ecto-protein kinase activity can be recovered in the extracellular fluid (Kübler et al., 1983; Kübler et al., 1986; Korc-Grodzicki et al., 1988; Skubitz et al., 1991b). In some cases, the kinase is spontaneously shed off (Paas et al., 1995). The substrate-induced release of ecto-protein kinases have been so far observed for casein kinase 1 (CK1)- and casein kinase 2 (CK2)- like enzymes (Murakami et al., 1990; Bennett et al., 1993; Dalvletov et al., 1993; Angelov, 1994; Walter et al., 1996).

This is the first time a PKC δ-like ecto-protein kinase has been identified by applying the widely used anti-PKC antibodies and synthetic fragments of PKC (Mochly-Rosen et al., 1987,1988; Baudier et al., 1991; Toullec et al., 1991; Chen S. et al., 1993; Chen C. et al., 1993).

PKC is a well-known protein kinase (Nishizuka, 1986; House et al., 1987 Tanaka et al., 1994;Johnson et al., 1996). Immunoblotting of cultured fetal chicken neurons with anti- PKC α, β, γ, δ, and ε antibodies revealed that only PKC ε was recognized in the preparation (Heidenreich et al., 1990). These findings suggest that the ecto-PKC δ-like protein kinase might be a novel PKC, which is different from known PKC δ.

The factors which might affect extracellular phosphorylation warrant some discussion. Extracellular matrix is the site where intercellular communication is established. The medium surrounding the cells undoubtedly has some influence

on the cells. Sodium bicarbonate ($NaHCO_3$) and HEPES are two commonly used chemicals to maintain the pH of the medium, and their effects on cellular activities have been compared in different tissues (Oosterom et al., 1983; Lieu, 1990; Bell et al., 1991; Tornquist et al., 1995). $NaHCO_3$ is a physiological buffer. In addition to regulate intracellular pH, $NaHCO_3$ has been shown to regulate receptor binding, ion channels, sperm motility, and cytotoxicity (Kurioka et al., 1981; Liu et al., 1985; Okamura et al., 1985; Carroll et al., 1988; Ganz et al., 1989;Thomas, 1989; Allen et al., 1993). HEPES is not a physiological buffer, cells grown in HEPES tend to have lower extracellular pH. It has been reported that an acidified condition triggers neuroprotective effect, increase of catecholamine secretion, elevation of intracellular Ca^{++} concentration and elevation of endogenous sphingoid bases are also observed (Kaku et al., 1993; Fujiwara et al., 1994; Lavie et al., 1994; Gasalla-Herraiz et al., 1995).

In addition to the medium, ionic concentration will certainly affect extracellular phosphorylation. Manipulation of ionic concentration can be achieved by either providing media containing different ionic concentrations or using reagents to inhibit certain ion channels. High extracellular K^+ is known to affect resting membrane potential and Ca^{++} uptake (Wakade et al., 1986; Ichida et al., 1993). Several reagents tested by others on their effects on ecto-protein kinase activity, such as DIDS (4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid) and heparin, are known as inhibitors of ion transporters (Kinzel et al., 1986; Kübler et al., 1986; Visconti et al.,1990; Okamura et al., 1991; Spivak-Kroizman et al., 1994; Schachner et al., 1995). Though the12K and 13K proteins are

phosphorylated by an ecto-PKC δ-like protein kinase, regulation of the phosphorylation is far from clear. It will be of great interest to investigate the effect of media and ionic concentrations on the phosphorylation of 12K and 13K proteins, thus to shed light on the mechanism of regulation of extracellular phosphorylation in general.

The last issue is what might be the downstream effectors of extracellular phosphorylation. Phosphorylation of cytoskeletal and associated proteins has been shown to be developmentally regulated (Bennett et al., 1988; Go et al., 1989; Simkowitz et al., 1989; Dahl et al., 1992; Mandell et al., 1996). In the nervous system, a gradient distribution of a growth factor in the extracellular matrix can function as a guiding post for an extended neuron. Therefore, it is reasonable to speculate that one possible direct downstream effector of extracellular phosphorylation is the formation of a transmembrane gradient by a specific extracellularly phosphorylated protein. Differential phosphorylation of that protein may serve as a signal to initiate a series of intracellular events.

1-4. Conclusions

It is possible that extracellular protein phosphorylation is caused by the protein kinases released from dying cells. This includes probably the ecto-PKC δ reported earlier. Furthermore, it has recently been reported that PKC δ is involved in apoptosis (Denning et al., 1998; Reyland et al., 1999). Therefore, it will be important to determine how similar structurally and functionally the ecto-PKC δ that we have reported is to the apoptosis-induced intracellular PKC δ.

In my extracellular protein phosphorylation assays, there is a phosphoprotein with an apparent molecular weight of 17 kilodaltons (17K); whenever the phosphorylation of 12K and 13K proteins was inhibited by anti-PKC δ antibody, the phosphorylation of this 17K protein was stimulated (data not shown). Taking into consideration the result obtained from chapter 2, that is, Raf-1 forms complex with Bad, a proapoptotic agent, belonging to the Bcl-2 family of proteins with molecular weights ranging from15kD to 25kD, I would like to suggest that this 17K protein might be part of the Bad, Raf-1 complex. Further experiments to characterize the 17K protein will help to resolve the issue.

1-5. Materials and Methods

Neuronal cell culture. Primary embryonic chick neurons were prepared following the method of Pettmann et al. (1979) with modifications as described by Hogan et al. (1988) (Fig. 1). In brief, 48-well plastic dishes (Falcon, Cat # 2097) were coated with poly-L-lysine (Sigma, Cat # P-1274, molecular weight ~100,000) which was dissolved in boric acid (Sigma, Cat # B-0394) with pH8.4 at the concentration of 15mg/L. For cell plating, D-MEM (Gibco, Cat # 430-2800EL) containing 10% FBS (Gibco, Cat # 16140-022, heat-inactivated fetal bovine serum), and 1% PS (Gibco, Cat # 15140-122, final concentration at 100 U/ml penicillin G, and 100mg/ml streptomycin sulfate) was used. The medium was pre-warmed in a 37°C incubator. Fertilized White Leghorn chicken eggs (Hall's Brother Hatchery) were incubated at 37°C in a humid atmosphere (Circulated Air Incubator, manufactured by G.Q.F., Model # 1202). The telencephalons from 7-day chick embryos (stage 29 - 31, according to Hamburger et al (1951)) were aseptically removed, triturated, filtered through a sterile 40-mm nylon mesh (Falcon, Cat # 2340), and cells counted (counting chamber: Spotlite Hemacytometer, Cat # B3175) under microscope. Neurons were plated at a concentration of $22x10^4$ cells/well. After 24 hours, the medium was changed to a chemically defined medium (DMEM containing the ITS$^+$ supplement from Collaborative Biomedical Products, Cat # 40352, at the following final concentrations: insulin, 6.25 mg/ml; transferrin, 6.25 mg/ml, selenious acid, 6.25 ng/ml; bovine serum albumin, 1.25 mg/ml; and linoleic acid, 5.35 mg/ml). No

serum or antibiotics were present in the chemically defined medium. The neuronal cultures from 1day in vitro (DIV) to 6 DIV were shown in **Fig. 1.6**.

Extracellular protein phosphorylation. Phosphorylation reactions were performed with intact, attached cells between 2 to 6 DIV in a 37°C water bath as detailed by Ehrlich et al. (1986,1990) and Pawlowska et al. (1993) (**Fig. 1.7**). In brief, the medium was removed and the cells were rinsed gently 2X in warmed (37°C) Krebs-Ringer buffer (KRB; pH 7.4) containing 145 mM NaCl, 5 mM KCl, 1.28 mM $CaCl_2$, 1.20 mM $MgCl_2$, 20 mM glucose, and 25 mM HEPES, with 1mM Na_2HPO_4 (Sigma tissue quality reagents). To each well 120 ml KRB or conditioned medium was added. To start the ecto-phosphorylation reaction 5 ml of [γ-^{32}P]ATP (ICN Biomedicals, Cat # 35001X; 15 mCi, 0.1 mM final ATP concentration) was added to each well. To stop the reaction 26 ml of 6X sodium dodecyl sulfate (SDS) stop solution (1.2 g SDS, 1.2 ml □-mercaptoethanol, 3.6 g sucrose, 1.25 ml 3 M Tris-HCl, pH 6.75, 4 ml 150 mM EDTA, and 0.24 ml 0.5% bromophenol blue were diluted to 10 ml with water) was added, and then the samples were heated at 90°C for 5 min before electrophoresis.

Exogenous reagents added to test their effect on extracellular phosphorylation were prepared in KRB and added 30 sec before starting phosphorylation assays. The reagents tested included myelin basic protein

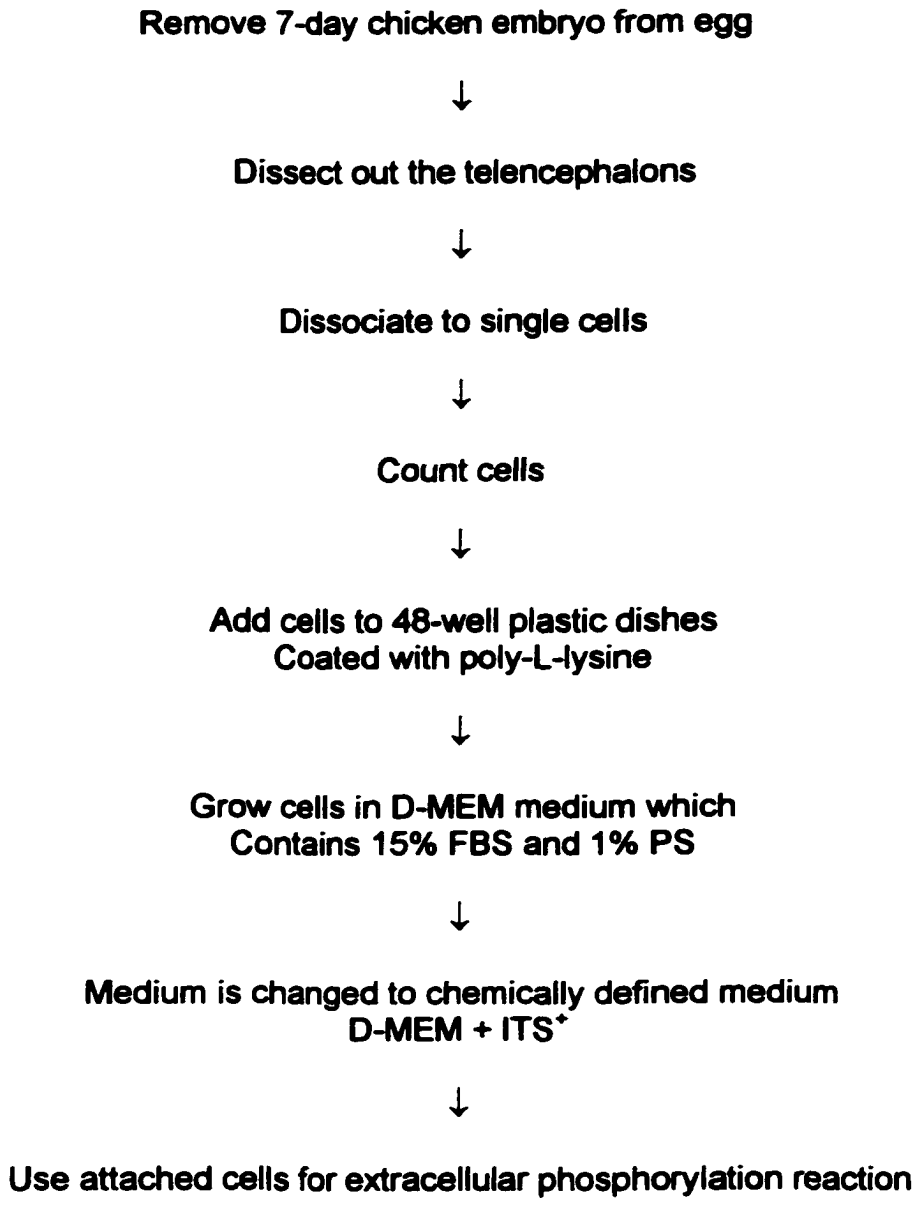

Figure 1.6. Preparation of cultured chicken telencephalons.

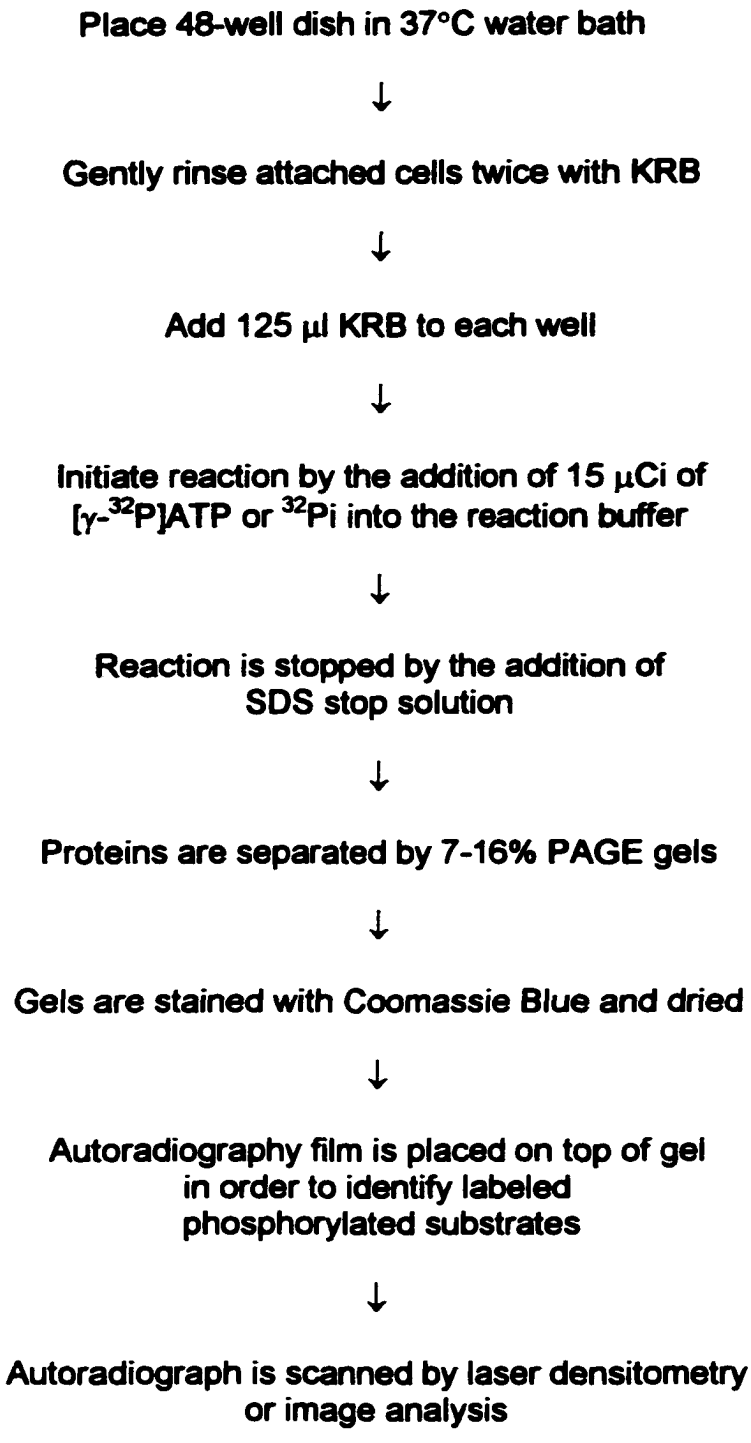

Figure 1.7. Extracellular phosphorylation reaction

(Gibco, Cat # 3228SA), α-dephosphorylated casein (Sigma, cat # C-8032), heparin (Sigma, Cat # H-3393), PKC (19-31) (Bachem, California, Cat. # PPHA114), PKC (19-36) (Gibco, Cat # 3121SA; Bachem, California, Cat # PPHA 11o), [ser 25] PKC (19-31) (Gibco. Cat # 3121SA; Bachem, California, Cat. # PPHA115), anti-PKC Mab1.9 (Gibco, Cat # 3101SA). Anti-δ -COOH and anti-δ-V3 were provided by Dr. Peter Parker from Imperial Cancer Research Fund, London; and all other anti-PKC isozymes were provided by Dr. N. P. Groome from Oxford Brookes University. The concentrations tested for each reagent are specified in figure legends.

SDS-PAGE gel electrophoresis and autoradiography. Proteins were separated by SDS-polyacrylamide gel electrophoresis (7-15% linear gradient gels) as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue, then destained and dried. Molecular weight standards and reagents for electrophoresis were obtained from BioRad. Autoradiograph was taken by incubating the dried gel in a cassette with an x-ray film (Kodak XRP films) on top of the gel (exposure time was usually 2 or 3 days).

Statistics. Autoradiographs were analyzed with Technology Resources Image Analysis System, which yielded the total volume (arbitrary density units) of each band after subtraction of background. Statistical analyses were performed using SigmaStat and SigmaPlot software from Jandel Scientific. p values and significance were determined using a two-tailed unpaired Student t- test.

CHAPTER TWO

RAF-1 PHOSPHORYLATION IN CELLS EXPRESSING SEROTONIN 1A RECEPTOR

2-1. Introduction

As discussed in the previous chapter, protein phosphorylation is an important regulatory mechanism. While extracellular protein phosphorylation is still a new territory rarely explored by scientists, knowledge of intracellular protein phosphorylation is at a much more advanced stage. The intracellular protein kinase that was studied in the second half of my thesis is the Raf-1 protein kinase.

Raf-1 protein is the product of a proto-oncogene, which indicates that Raf-1 kinase activity is constitutively on in transformed cells. Raf-1 is an important component of the MAP kinase pathway which is stimulated by both tyrosine kinase receptors and G protein-coupled receptors. Activation of this pathway leads to mitosis and transformation of cells.

It has been previously reported that the MAP kinase pathway is involved in serotonin-evoked cell protection. The hypothesis proposed in this study was that the function of Raf-1 is regulated by serotonin treatment and that Raf-1 interacts with other proteins to regulate apoptosis.

Specific aims:

The goal of this thesis is to investigate the relationship between Raf-1 phosphorylation and apoptosis. A hippocampus-derived hybrid neuroblastoma cell line, engineered to stably express the serotonin 1A receptor (HN2-5), was used in my study. Stimulation of serotonin 1A receptor has been shown to activate MAP kinase which causes protection of the HN2-5 cells against anoxia. Therefore, we hypothesized that agonist stimulation of serotonin 1A receptor (%-HT_{1A}-R) affects Raf-1 phosphorylation, and that the phosphorylated Raf-1 interacts with proteins that are known to be involved in regulating apoptosis.

We proposed to perform the following experiments to test the hypothesis. First, phosphorylation assays were performed on either non-treated or agonist-treated HN2-5 cells. Raf-1 protein was immunoprecipitated and the effects of a 5-HT_{1A} agonist on Raf-1 phosphorylation and Raf-1 kinase activity were evaluated. Second, Raf-1 phosphorylation assays were performed as before, but different antibodies were used for immunoprecipitation to identify the other proteins that are capable of forming immunocomplexes with Raf-1.

Background and significance:

Raf-1 protein kinase is a key player of the MAP kinase pathway as delineated in **Figure 2.1**. The MAP kinase pathway, which is involved in mitosis and transformation, is commonly stimulated by growth factor receptor tyrosine kinases. (Morrison, 1990; Kolch et al., 1991; Fabian et al., 1993; Avruch et al., 1994; Daum et al., 1994). Recently, receptors that couple to G proteins have been shown to also activate the MAP kinase pathway (Blumer & Johnson, 1994; Crespo et al., 1994; Yang et al., 1996; Dell Rocca et al., 1997;Yamazaki et al., 1997).

One major upstream regulator of the MAP kinase activity is the serine/threonine kinase, Raf-1. The mechanism of activation of Raf-1 is not completely understood yet. The widely accepted model states that Raf-1 is recruited to the plasma membrane and activated by a small GTP-binding protein, Ras. Upon activation, Raf-1 can phosphorylate and activate a MAP kinase kinase (MEK), which in turn can phosphorylate and activate MAP kinase.
Both Ras-dependent and Ras-independent activation of Raf-1 have been reported (Fabian et al., 1993; Leevers et al., 1994; Dent et al., 1995; Marais et al., 1995; Michaud et al., 1995; Morrison, 1995; Whitehurst et al., 1995; Marais et al., 1997; Roy et al., 1997; Stokoe et al., 1997; Marais et al., 1998). Protein kinase C (PKC) can also stimulate Raf-1 activity by direct or indirect phosphorylation in a Ras-dependent or -independent manner (Kolch et al., 1991; Sözeri et al., 1992; Wood et al., 1992; Kolch et al., 1993; Ueffing et al., 1997; Ho

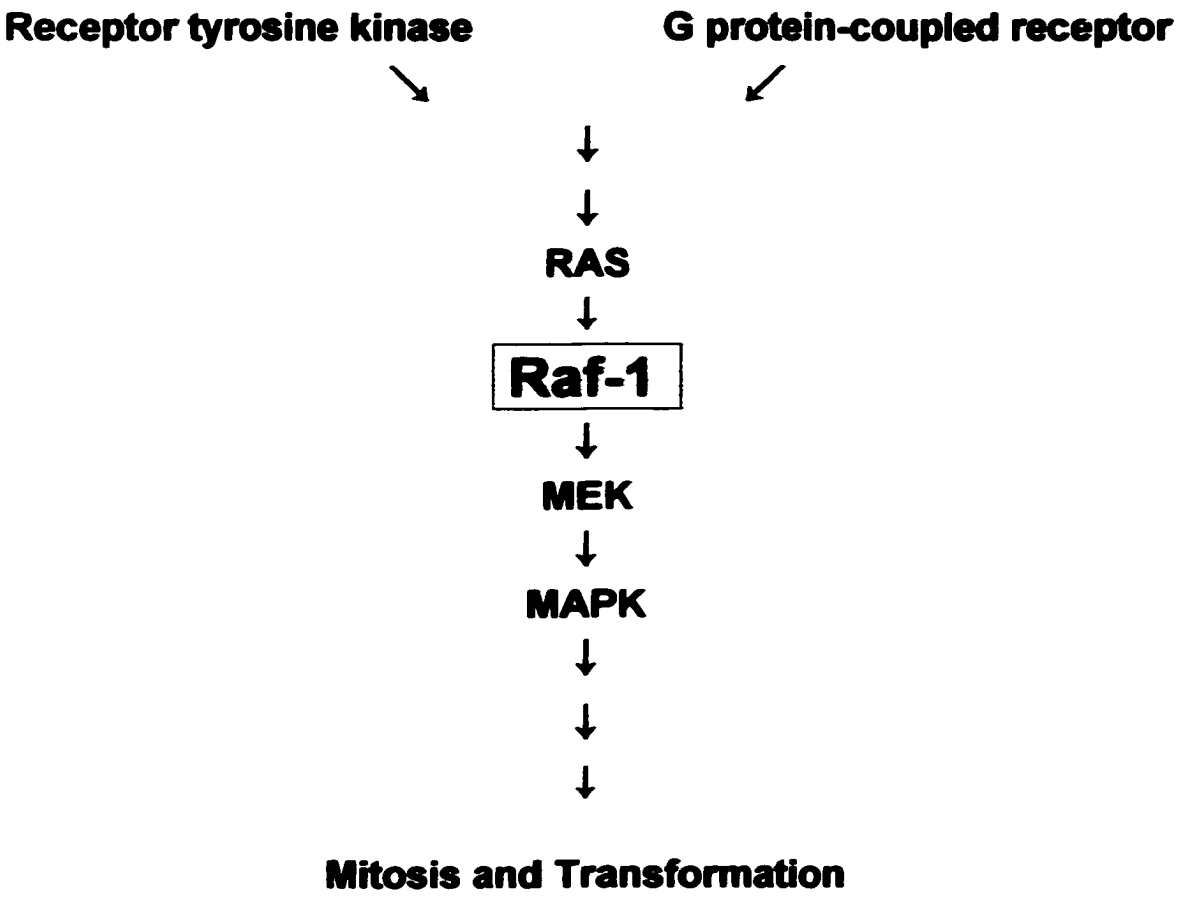

Figure 2.1. The Raf-1-mediated MAP kinase pathway in mitosis and transformation. Two types of receptors can trigger the MAP kinase pathway. There are tyrosine kinase-coupled receptors and G protein-coupled receptors. Upon receptor activation, a series of intracellular signals are transduced, which eventually lead to mitosis and transformation. Proteins outlined here are the major key plays in the signal transduction of MAP kinase pathway. Among them Raf-1 protein is the focus of my study.

et al., 1998; Marais et al., 1998). In the tumor necrosis factor α (TNFα)-initiated sphingomyelin pathway, ceramide-activated protein (CAP) kinase that is sensitive to the hydrolytic product of sphingomyelin (ceramide) can phosphorylate Raf-1. Increased Raf-1 kinase activity is observed with this type of phosphorylation (Yao et al., 1995). In addition, Raf-1 can be phosphorylated by cAMP-dependent protein kinase (PKA); consequently, a decrease in kinase activity is observed (Häfner et al., 1994; Hershenson et al., 1995; Mischak et al., 1996). Negative feedback phosphorylation by MEK has also been reported (Wartmann et al., 1997). Recently the AMP-activated protein kinase (AMPK), a member of a larger metabolite-sensing protein-kinase family, has been shown to phosphorylate Raf-1. However, the effect of this phosphorylation on Raf-1 kinase activity is still unknown (Sprenkle et al., 1997; Kemp et al., 1999).

The complexity lies in the Raf-1 protein itself. Raf-1 can be phosphorylated at several amino acid residues by different protein kinases, and phosphorylation plays a very important role in regulating its kinase activity (Morrison et al., 1988; Heidecker et al., 1992; Burgering et al., 1995; Morrison, 1995; Morrison & Cutler, 1997).

Phosphorylation on tyrosine 340 and 341, and threonine 269 have been reported to stimulate Raf-1 kinase activity (Morrison et al., 1989; Fabian et al., 1993a,b; Marais et al., 1995, 1997; Yao et al., 1995). Studies of phosphorylation at serine 43, 259, 499, 621, and 624 have yielded inconsistent results, probably due to the heterogeneity of protein kinases that phosphorylate Raf-1 (McGrew et

al., 1992; Kolch et al., 1993; Morrison et al., 1993; Daum et al., 1994; Mischak et al., 1996; Ferrier et al., 1997; Wartmann et al., 1997).

Activation of Raf-1 does not always proceed to the activation of MAP kinase. In hypoxia-induced activation of nuclear factor-κB (NF-κB) which is a transcription factor, Raf-1 pathway is stimulated without activation of the MAP kinase. Although MAP kinase can be activated by low oxygen conditions, it does not appear to be involved in regulating hypoxia-induced activation of NF-κB (Koong et al., 1994). In some cases, Raf-1 activation is not involved, even though the MAP kinase activity is induced by glutamate or norepinephrine receptors (Kurino et al., 1995; Sawada et al., 1997). Therefore, the Raf-1 protein may function as a branching point through which different receptors stimulate their respective signaling pathways (Apostolidis & Weiss, 1997).

We are interested in studying the role of Raf-1 protein phosphorylation in the serotonergic system. Serotonin, 5-hydroxytryptamine (5-HT), is an abundant molecule. It can be found in a variety of body tissues and serves a variety of functions. In the nervous system, the brain stem has an extensive serotonergic system, and the vast majority of serotonergic neurons are located within the raphe nuclei and adjacent nuclear groups. Projections of these neurons extend to the cerebral cortex and spinal cord. The function of serotonin depends on the type of receptors. Serotonin receptors belong to a group of G protein-coupled receptors which are classified according to the individual intracellular messenger with which each G protein is associated (Hoyer & Boddeke, 1993; Humphrey & Hoyer, 1993; Milligan, 1993; Martin et al., 1998). Serotonin 1 receptor (5-HT_1)

receptor is one of the subtypes, and there are fourteen different 5-HT receptors classified by their pharmacological properties (Beer et al., 1993; Hoyer & Martin, 1997).

Serotonin 1A (5-HT_{1A}) receptor has generated a lot of interest recently. Brain areas rich in 5-HT_{1A} receptors include septum, hippocampus, frontal and entorhinal cortices, and brain stem (Köhler et al., 1986; Kia et al., 1996). A transient expression of 5-HT_{1A} receptor has been also found in the cerebellum (Miquel et al., 1994). Electrophysiological studies indicate that 5-HT_{1A} receptor-mediated hyperpolarization is observed in the hippocampal CA1 neurons (Wu et al., 1988) and mesopontine raphe neurons (Johnson, 1994), and inhibition of Ca^{2+} current is demonstrated in the dorsal raphe neurons (Penington et al., 1994).

5-HT_{1A} receptor agonists are potent anxiolytic drugs and antidepressants (Cervol & Samanin, 1987; Carli & Samanin, 1988; Feighner & Boyer, 1989; Blier et al., 1990; Artigas et al., 1996; Davids & Lesch, 1996; Stahl, 1997; Stockmeier et al., 1998). The involvement of 5-HT_{1A} receptor in stress and schizophrenia has been also described (Alexander et al., 1995; Glavin et al., 1995; Saphier and Welch, 1995; Singh et al., 1996; Burnet et al., 1997). In addition, 5-HT_{1A} receptor is said to be neurotrophic during development (De Vitry et al., 1986; Hillion et al., 1993; Azmitia et al., 1995), and expression of the receptor is associated with neuronal differentiation (Charest et al., 1993). When cells are under an assault, treatment with the agonists can improve survival rate (Suchanek et al., 1996, Adayev et al., 1999).

The agonist activation of the 5-HT_{1A} receptor results in the dissociation of the coupled G_i protein into α and $\beta\gamma$ subunits. The α subunit has been shown to inhibit adenylate cyclase activity and to stimulate Na^+/H^+ exchange, the $\beta\gamma$ complex can initiate the MAPK pathway (Clarke et al., 1987; Garnovskaya et al., 1996; 1997; 1998).

As mentioned earlier, 5-HT_{1A} receptors may be involved in cell protection. In other words, they may mediate antiapoptotic signals. Apoptosis describes programmed cell death that occurs naturally during development, and it can be induced when cells are damaged. Common features of cells undergoing apoptosis include shrinkage of cytoplasm, membrane blebbing, nuclear condensation, and DNA fragmentation. The intracellular events responsible for apoptosis have just begun to be understood. Activation of a family of cysteine proteases called caspases during apoptosis results in cleavage of signaling proteins and irreversible commitment to cell death (Widmann et al., 1998). The mechanism of caspase activation is still vigorously pursued, caspase prodomains, adaptors recruited by caspases, and oligomerization of adaptors and procaspases have all been discussed (Kumar & Colussi, 1999).

The proto-oncogene products of the Bcl-2 family can function as upstream regulator of caspases through yet unknown mechanisms (Chinnaiyan et al., 1996; Shimizu et al., 1996a). The degree of mitochondrial dysfunction is also a crucial factor that decide the mode of cell death (Kluck et al., 1997; Yang et al., 1997; Abbracchio et al., 1999).

In the Bcl-2 family, Bcl-2 and Bcl-xL are known to be antiapoptotic, and Bcl-xL/Bcl-2-associated death promoter (Bad) is proapoptotic (Haldar et al., 1994; Reed, 1994; 1996; 1997; Yang et al., 1995; McDonnell et al., 1996; Zha et al., 1996; Hsu et al., 1997; Kaipia et al., 1997; Kelekar et al., 1997; Ottilie et al., 1997; D'Agata et al., 1998;Kitada et al., 1998). By forming a heterodimer complex at the mitochondrial membrane, Bad inactivates Bcl-2/Bcl-xL, causing cells to die. Phosphorylation of Bad results in dissociation of the complex, and free phosphorylated Bad can then form a new complex with a cytosolic protein, 14-3-3, a group of evolutionarily conserved proteins essential for signal transduction and cell cycle progression, thus allowing Bcl-2/Bcl-xL to function as an antiapoptotic agent. Protein kinases known to phosphorylate Bad include PI 3-kinase-dependent protein kinase B (PKB), also known as Akt; and mitochondria-anchored PKA (Gajewski et al., 1996; Zha et al., 1997; Datta et al., 1997; Franke et al., 1995,1997a, 1997b; Peso et al., 1997; Harada et al., 1999).

Bcl-2 has been shown to assist in the targeting of Raf-1 to the mitochondrial membrane, Bag-1, a Bcl-2 binding protein, then activates Raf-1, and phosphorylation of Bad ensues (Wang et al., 1994,1996a, 1996b, 1998). Nevertheless, there is no direct evidence that Raf-1 can phosphorylate Bad. Activation of PI3-kinase and Akt is necessary for Ras-induced antiapoptosis, and it could be a Ras-dependent pathway distinctive from Raf-1-MEK-MAP kinase signaling transduction (Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Marte et al., 1997; Downward, 1998). It is interesting to know that both Ras and Raf-1 have been reported to be proapoptotic as well as antiapoptotic, since decrease

and increase in Raf-1 kinase activity have been associated with Raf-1-induced apoptosis (El-Ashry et al., 1997; Parrizas et al., 1997; Weissinger et al., 1997). It seems that Raf-1 kinase activity is correlated with phosphorylation of Bcl-2, and which is responsible for Bcl-2 inactivation and subsequent apoptosis (Chen & Faller, 1996; Blagosklonny et al., 1996).

The purpose of my research is to investigate the role of Raf-1 phosphorylation in 5-HT_{1A} receptor-mediated signal transduction pathways in a transfected hippocampal x neuroblastomal cell line stably expressing 5-HT_{1A} receptors (HN2-5 cells) (Hammond et al., 1986; Lee et al.,1990; Singh et al., 1996). HN2-5 cells pretreated with the 5-HT_{1A} receptor-specific agonist 8-hydroxy-N,N-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT) have shown less degree of cell death when subjected to hypoxia and reoxygenation, and this effect seems to involve the MAP kinase pathway (Adayev et al., 1999). A simplified model of 5-HT_{1A} receptor-mediated cell protection in HN2-5 cells is shown in **Figure 2.2.**

The experiments performed in my study are to address three questions. The first question is whether the 5-HT_{1A} receptor agonist, 8-OH-DPAT, affects Raf-1 phosphorylation. The second question is if the agonist stimulates Raf-1 kinase activity. The last question is whether Raf-1 protein can be co-immunoprecipited with antibodies generated against other proteins involved in apoptotic pathway. Thus association of Raf-1 with Bcl-2, Bad, and caspase 8 has been tested in this study.

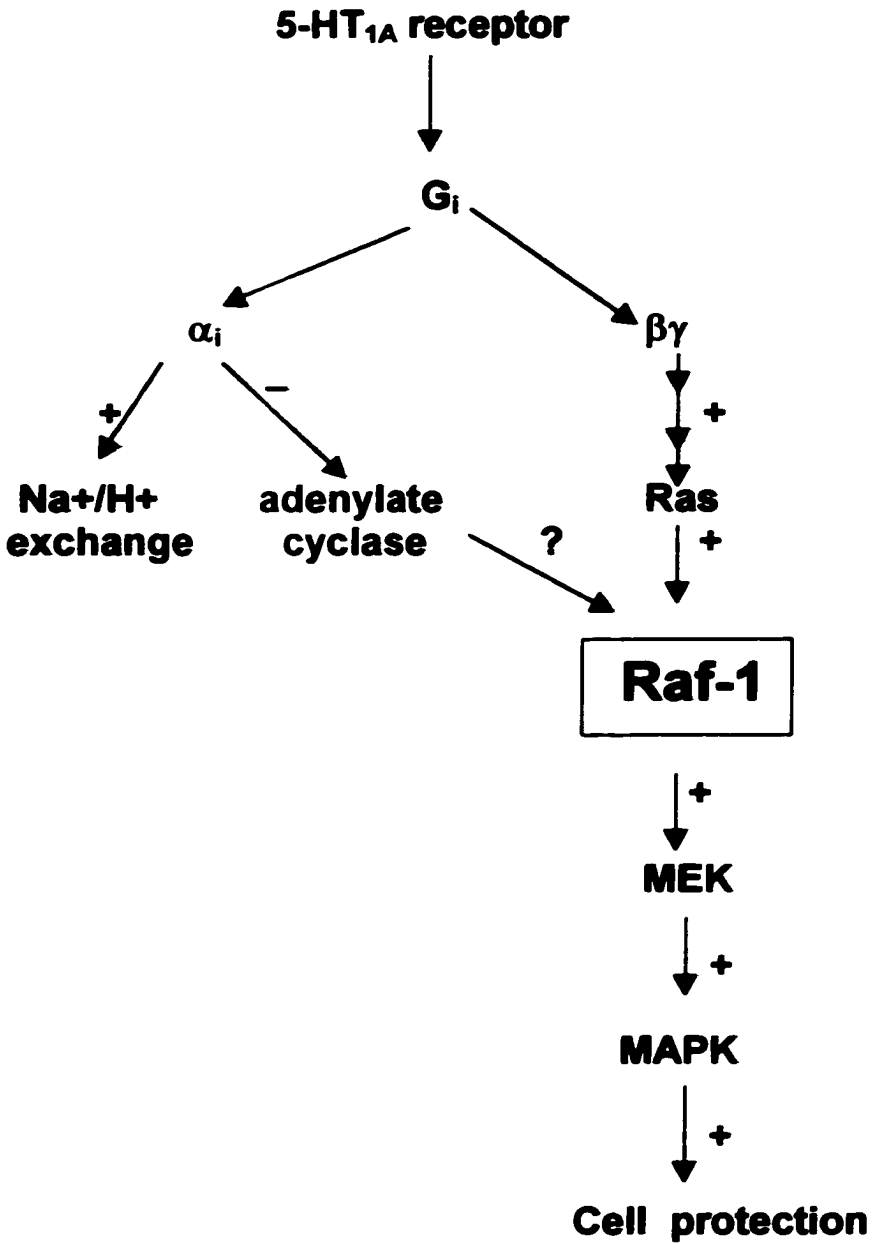

Figure 2.2. The model for the serotonin 1A receptor-induced and Raf-1-mediated cell protection. Serotonin 1A ($5\text{-}HT_{1A}$) receptor is coupled to a trimeric G_i protein composed of α, β, and γ three subunits. Upon receptor binding, the three subunits separate themselves to perform different tasks. The α subunit reportedly can stimulate Na^+/H^+ exchange and inhibit adenylate cyclase activity; the βγ subunits activate the MAP kinase pathway, resulting in cell protection. The plus sign (+) indicates a stimulation or an activation; the minus sign (-) indicates an inhibition.

2-2. Results

Increased Raf-1 phosphorylation is induced by serotonin 1A receptor agonist, 8-hydroxy-N,N-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT)

The primary goal of my study is to determine the role of Raf-1 in serotonin 1A receptor-activated MAP kinase pathway. Hippocampal and neuroblastomal hybrid cell line was transfected with the serotonin 1A receptor gene. A cell line stably expressing serotonin 1A receptors on the cell surface was selected and named HN2-5 cell. HN2-5 cells can be elicited to express neuronal properties such as the growing of processes by way of differentiation. Differentiation was induced by treating the cells overnight with Kreb's Ringers Buffer. The next day the Kreb's Ringers Buffer was removed. Cells were placed in phosphate-free D-MEM medium and incubated with radioactive phosphate for three hours. Differentiated HN2-5 cells received one of the following treatments. There were carrier-treated, agonist (8-OH-DPAT)-treated, and agonist-antagonist-treated (8-OH-DPAT and WAY) groups.

The initial result on the first dimensional SDS-PAGE gel that separates the proteins by their molecular weights was showed no change from carrier-treated cells (**Figure 2.3a**). Western blot indicates that the phosphorylated protein is indeed the Raf-1 protein as it can be recognized by monoclonal anti-Raf-1 antibody (**Figure 2.3b**). 8-OH-DPAT did not stimulate the Raf-1 kinase

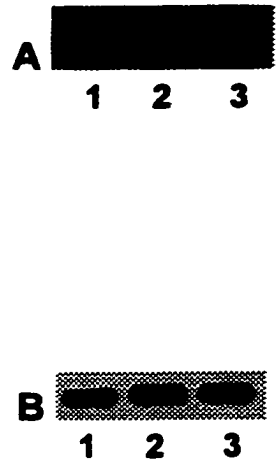

Figure 2.3. (A) Autoradiograph of Raf-1 phosphorylation, and (B) Westernblot of Raf-1 protein. (A) HN2-5 cells were labeled with $^{32}PO_4^{-3}$ and immunoprecipitated with rabbit anti-Raf-1 antibody. (B) Raf-1 protein was probed and visualized with mouse anti-Raf-1 antibody as primary antibody and horseradish peroxidase conjugated goat anti-mouse antibody as secondary antibody. **Lane 1**: non-treated cells. **Lane 2**: 5-HT_{1A} receptor agonist, 8-OH-DPAT, treated cells. **Lane 3**: 5-HT_{1A} receptor antagonist, WAY-100635, treated cells.

activity either, both non-treated and agonist- treated cells display minimal amounts of Raf-1 activity as compared to the positive control (**Figure 2.4**). However, further separation of the proteins with the two-dimensional gel shows that there was an increase of Raf-1 phosphorylation upon agonist stimulation of receptors (**Figure 2.5a&b**).

Antibodies against Bad and caspase 8 immunoprecipitate phosphorylated Raf-1 in HN2-5 cells treated with serotonin 1A-receptor agonist (8-OH-DPAT).

Since the serotonin 1A-receptor activated MAP kinase pathway is known to be involved in cell protection, we would like to determine what role Raf-1 plays here. Our approach to this question was to conduct a series of immunoprecipitation studies using antibodies against those proteins that are important in apoptotic pathways. Three antibodies directed against Bad, Bcl-2, and caspase 8 were used. Bad and Bcl-2 belong to the Bcl-2 family proteins. Bcl-2 is antiapoptotic by suppressing the releases of cytochrome c and sequestered matrix Ca^{2+}. Bad is pro-apoptotic by inactivating Bcl-2. Bcl-2 has been shown to target Raf-1 to the mitochondrial membrane, resulting in the activation of Raf-1 and phosphorylation of Bad (Wang et al., 1994, 1996a, 1996b, 1998). However, direct interaction between Raf-1, Bcl-2, and Bad has never been shown. Our results demonstrate that phosphorylated Raf-1, which is induced by agonist, is co-immunoprecipitated with Bad antibody (**Figure2.5c&d**).

Bcl-2 antibody did not immunoprecipitate with phosphorylated Raf-1 (**Figure2.5g&h**).

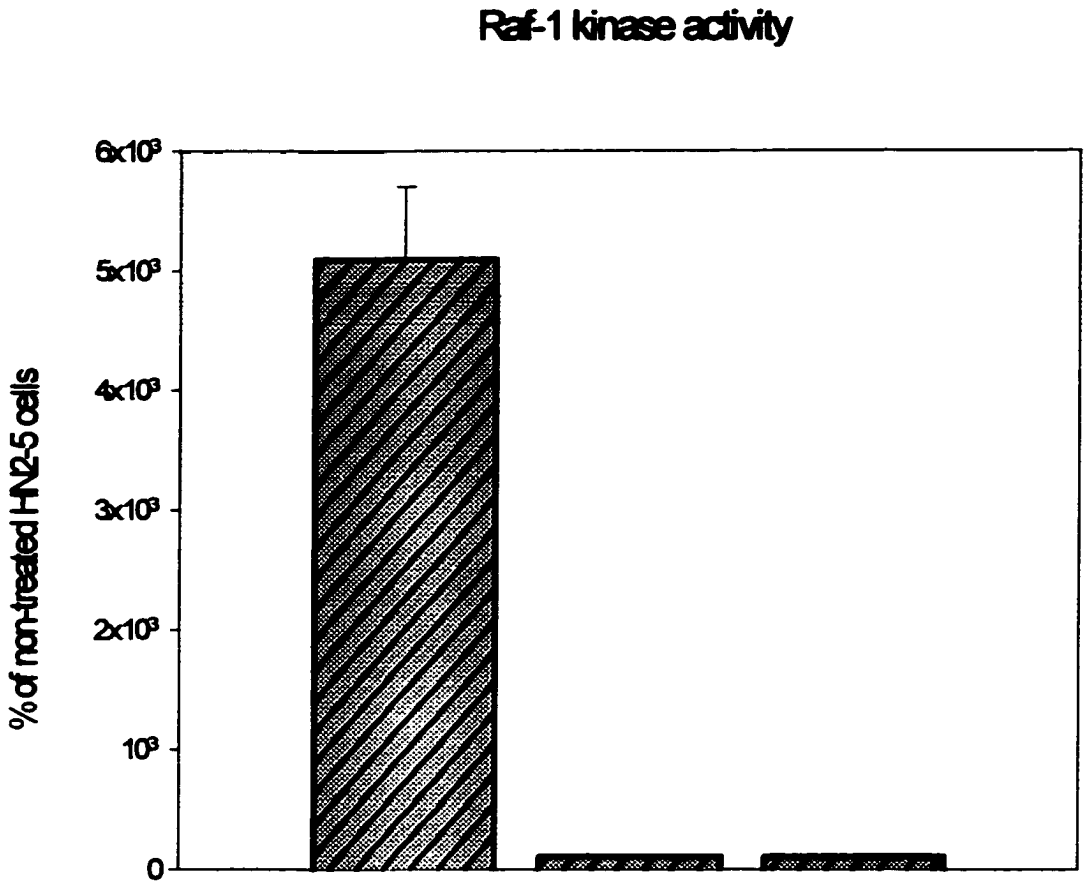

Figure 2.4. The Raf-1 kinase activity. HN2-5 cells were treated with carrier or the 5-HT_{1A} receptor agonist, 8-OH-DPAT, for 30 minutes. Bar I: positive control showing an activated Raf-1 kinase as provided by the assay kit. Bar II: non-treated cells. Bar III: cells treated with 8-OH-DPAT.

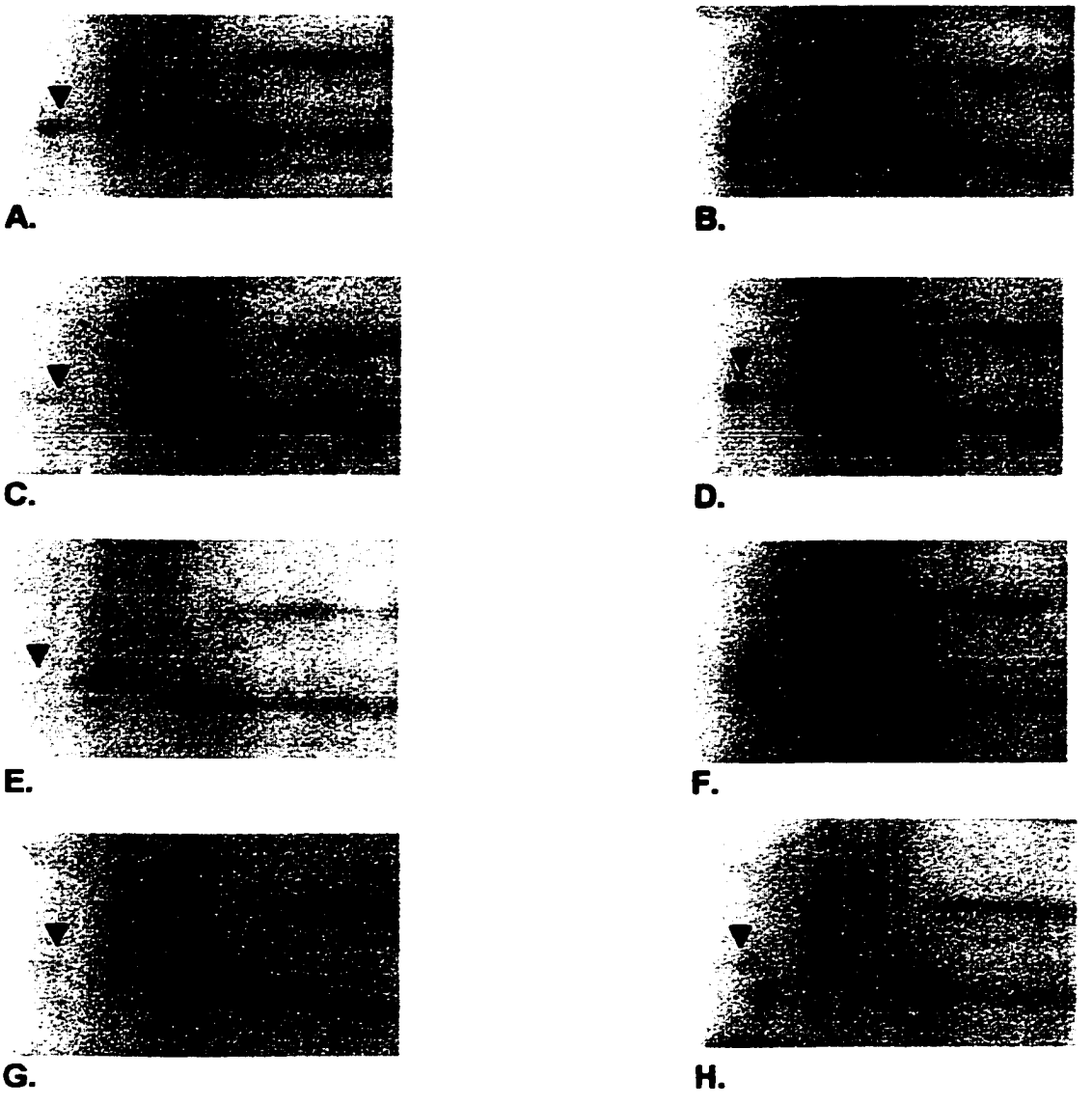

Figure 2.5. The autoradiographs of two-dimensional gels from immunoprecipitations. HN2-5 cells were either non-treated (**A**, **C**, **E**, **G**) or treated with 8-OH-DPAT (**B**, **D**, **F**, **H**). **A** and **B** are immunoprecipitations with Raf-1 antibodies. **C** and **D** are immunoprecipitations with Bad antibodies. **E** and **F** are immunoprecipitations with caspase 8 antibodies. **G** and **H** are immunoprecipitations with Bcl-2 antibodies. Arrows (↑) indicate the position of phosphorylated Raf-1.

Caspase 8 belongs to a family of cysteine proteases with aspartic acid specificity. Caspase 8 is activated by death receptors such as CD95 (also called Fas or Apo1) and tumor necrosis factor receptor 1 (TNFR1, also called p55 or CD120a) (Muzio et al., 1996,1998; Medema et al., 1997). Unlike effector caspases degrading DNA, caspase 8 is an initiator caspase, which means it activates other effector caspases, it is one of the early signals in apoptotic pathway. Procaspase 8 has been shown to form a complex with p28 Bap31, and Bcl-2/Bcl-xl (Ng et al., 1997). Since Raf-1, Bad, and Bcl-2 all are involved in the regulation of the apoptotic pathway, we would like to investigate whether Raf-1 also forms a complex with caspase 8. Our result indicates that caspase 8 antibody immunoprecipitates agonist-induced phosphorylated Raf-1 (**Figure 2.5e&f**).

2-3. Discussion

Two sets of results have been obtained from my experiments. One is that increased Raf-1 phosphorylation is induced by the serotonin 1A receptor agonist, and the other is that antibodies to both Bad and caspase 8 co-immunoprecipitate phosphorylated Raf-1 from cells stimulated with serotonin1A receptor agonist. This is the first evidence showing that the level of Raf-1 phosphorylation is associated with the binding specificity of Raf-1. When the cells were not stimulated with the serotonin 1A receptor agonist, the level of Raf-1 phosphorylation is low. When cells were stimulated with the agonist, the highly phosphorylated Raf-1 then complexed with Bad and caspase 8. These results suggest that the phosphorylation of Raf-1 not only regulates its kinase activity, but it also regulates its binding capacity.

Furthermore, the serotonin 1A receptors in HN2-5 cells mediate antiapoptotic signal, and Bad and caspase 8 are proteins associated with the induction of apoptosis. Bad associates with Bcl-2 to inactivate it, thus resulting in increased release of cytochrome c and Ca^{++} from mitochondria.

Caspase 8, also called FLICE, is activated in neurons of the rat receiving focal stroke induced by permanent middle cerebral artery occlusion (Velier et al., 1999). The treatment of focal stroke used to induce apoptosis in rat neurons is similar to our anoxia treatment on HN2-5 cells. It is possible that caspase 8 is activated in the oxygen-deprived HN2-5 cells. Another function of caspase 8 is that it induces cytochrome c release from mitochondria (Bossy-Wetzel & Green,

1999). This finding raises the possibility that Bad and caspase 8 might interact with each other to induce apoptosis. Therefore, it is reasonable to speculate that, by forming complexes with Bad and caspase 8, Raf-1 might inactivate Bad and caspase 8 and rescue cells from death. A model is proposed to illustrate the role of phosphorylated Raf-1 in serotonin 1A receptor-mediated signaling pathway (**Figure 2.6**). At present, we do not know what kinase phosphorylates Raf-1 and what molecular mechanisms could account for the complex formation. Further experiments will shed light on this issue.

The result that 5-HT_{1A} receptor agonist, 8–OH-DPAT stimulates Raf-1 phosphorylation, but does not stimulate the Raf-1 kinase activity, suggests that a Raf-1-independent pathway may be required to activate MAP kinase. The question is what could be the Raf-1 independent activation of MAP kinase in serotonin 1A receptor-mediated pathway?

Recently it has been reported that 5-HT_{1A} receptor-mediated MAP kinase activation requires calcium/calmodulin-dependent receptor endocytosis (Della Rocca et al., 1999). Proteins involved in initiating endocytosis seem to play crucial roles in both activation and termination of the signal transduction (Barak et al., 1997; Daaka et al., 1998; Pitcher et al., 1998). However, in this serotonin 1A receptor-mediated and endocytosis-activated MAP kinase pathway, Ras and Raf-1 are also involved. Furthermore, the HN2-5 cells used in my study do not

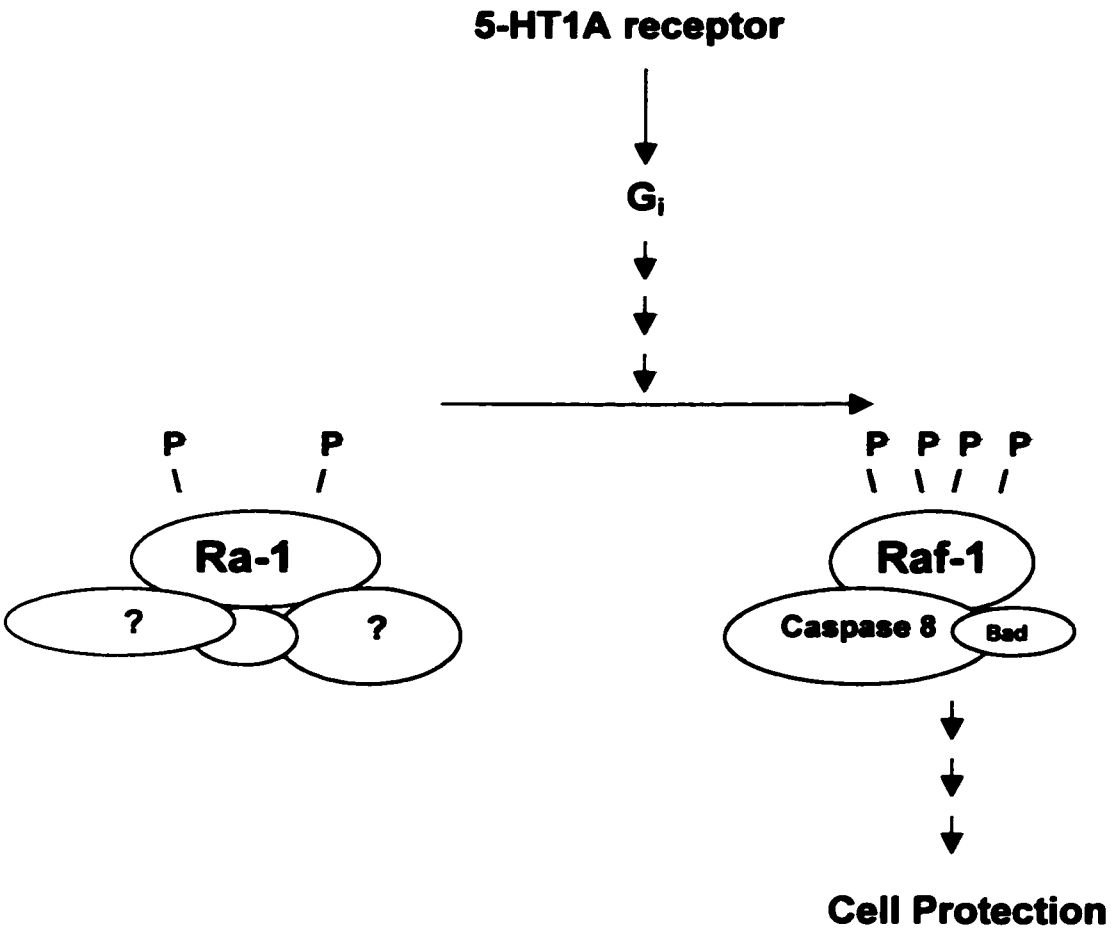

Figure 2.6. The model for the functions of serotonin 1A receptor-induced hyperphosphorylation of Raf-1. Serotonin 1A (5-HT1A) receptor is coupled to a trimeric Gi protein composed of α, β, and γ three subunits. Upon receptor stimulation, Raf-1 phosphorylation is increased. The resulting Raf-1 then binds to caspase 8 and Bad, and eventually leads to cell protection.

display any detectable voltage-gated calcium current (Adayev, et al., 1999). The mechanisms that HN2-5 cells use to perform endocytosis are not well understood yet. Therefore, it is not known whether the activation of MAP kinase in HN2-5 cells involves endocytosis or not. It is likely that two different pathways exist for the serotonin1A receptor-mediated activation of MAP kinase, depending on the type of cells the receptors are found. In some cells, activation of MAP kinase requires endocytosis and involves Ras and Raf-1, and in HN2-5 cells, activation of MAP kinase may not require endocytosis, thus can bypass Raf-1 activation.

Another protein that has been indicated to activate MAP kinase through G protein-coupled receptors is phosphatidylinositol 3-kinase (PI3-K). PI3-K and its downstream effector protein kinase B (PKB, or Akt) are known to be an important part of the G protein-coupled receptor-mediated cell survival (Kennedy et al., 1997; Kulik et al., 1997; Crowder et al., 1998; Murga et al., 1998). PI3-K is a lipid kinase, and PKB is a serine-threonine protein kinase. The PI3-K has been shown to be an upstream regulator of MAP kinase pathway (Yamauchi et al., 1993; Cowen et al., 1996; King et al., 1997; Lopez-Ilasaca et al., 1997; Berra et al., 1998). Though PI3-K seems to be associated with Ras-Raf-1-MAP kinase pathway, there are exceptions. Detachment of epithelial cells from the extracellular matrix causes apoptosis which can be inhibited by a Raf-1-independent and Ras-dependent PI3-K-mediated pathway (Khwaja et al., 1997). Another example was found in the T-lymphocytes, in which interleukin-2 receptor-mediated MAP kinase activation in cell cycle regulation is PI3-K-

dependent and Raf-1-independent (Brennan et al., 1997). It seems that PI3-K could activate MAP kinase without Raf-1's involvement. However, this is not the case in HN2-5 cells. Wortmannin, a PI3-K inhibitor, does not reverse the effect of MAP kinase-mediated inhibition of anoxia-induced apoptosis in HN2-5 cells, suggesting that the activation of MAP kinase in HN2-5 cells is probably not mediated by PI3-K. (Adayev et al., 1999).

Raf-1, as shown in **Figure 2.1,** does not phosphorylate MAP kinase directly. In between is are a group of kinases called MAP kinase kinase (MEK). MEK can be phosphorylated and activated by another group of serine-threonine protein kinases called MEK kinases (MEKKs), which are different from Raf-1 kinase (Blumer & Johnson, 1994; Winston et al., 1995; Kinane et al., 1997). We need to point out that the MAP kinases activated by Raf-1 are extracellular signal-regulated kinases (Erk1/Erk2). In addition to Erk1/Erk2, MEKKs can activate other pathways as well, such as the stress-activated protein kinase (SAPK) pathway and the p38 pathway; the last two are associated with apoptosis (Minden et al., 1994; Xia et al., 1995; Johnson et al., 1996). MEKK1 and MEKK 3 have been mentioned to activate both Erk1/Erk2 and SAPK pathways, depending on the types of stimuli cells receive (Ellinger-Ziegelbauer et al., 1997; Yujiri et al., 1998; Gibson et al., 1999).

The molecular mechanisms responsible for the activation of MEKK are not well understood. Studies in yeast have shown that osmotic pressure can directly activate a MEKK family member, which in turn activates a yeast homologue of the p38 pathway (Han et al., 1994). Though the p38 pathway is different from the

Erk1/Erk2 pathway, the latter has been shown to be activated by osmotic swelling in human intestine cells (Van der Wijk et al., 1998). Since it is known that the α subunit of G_i protein can stimulate the Na^+/H^+ exchange, which might have some effect on the osmolarity of the cells, a hypothesis has to be tested immediately that osmotic pressure-induced activation of MEKK could be responsible for the activation of serotonin 1A receptor-mediated Erk1/Erk2 pathway.

Osmotic pressure is one way to activate MEKK; Ras is another way. Ras has been reported to interact and activate MEKK, suggesting a direct activation of Erk1/Erk2 by MEKK independent of Raf-1 (Lange-Carter & Johnson, 1994; Russell et al., 1995). Therefore, a revised model based on my findings is proposed to delineate the pathway involved in 5-HT_{1A} receptor-mediated cell protection against anoxia-induced apoptosis (**Figure 2.7.**). In the model, MAP kinase is activated by MEKK, which in turn can be activated either by Ras protein or by G protein-induced osmotic pressure.

The next issue that will be discussed is what factors determine the active or inactive state of Raf-1 kinase. Raf-1 is composed of 638 amino acids. Functionally, Raf-1 contains two domains: the regulatory domain at the amino terminal (N-terminal), and the catalytic domain at the carboxyl terminal (C-terminal). The regulatory domain has the Ras binding site, and the catalytic domain has the ATP binding site. Raf-1 can be phosphorylated by a variety of protein kinases at specific sites, thus conveying a variety of cellular responses.

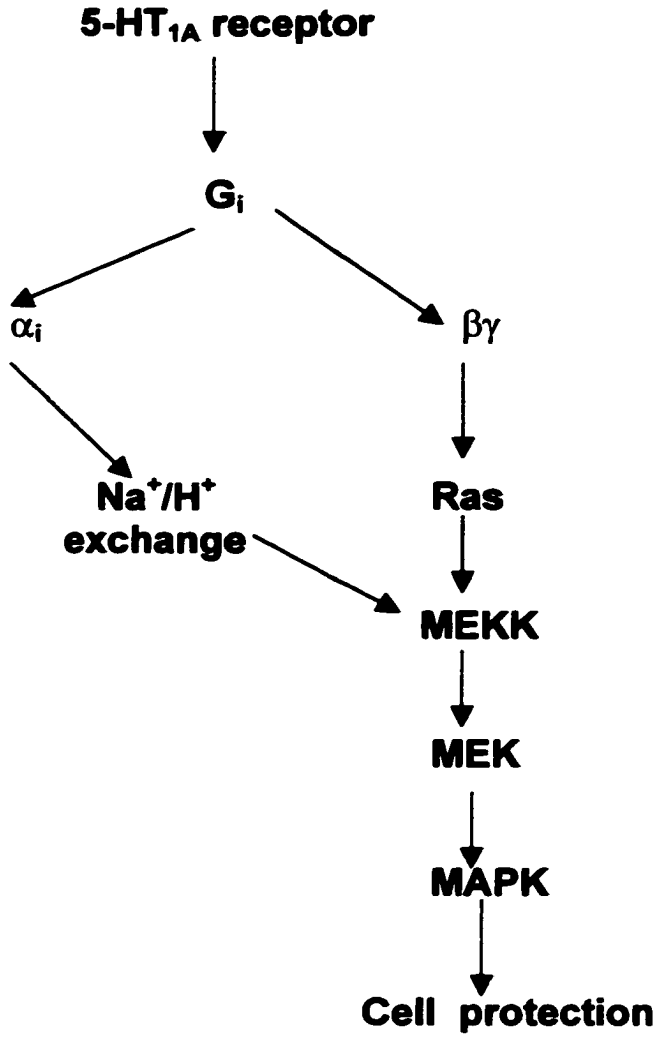

Figure 2.7. The revised model for the serotonin 1A receptor-mediated MAP kinase pathway Serotonin 1A ($5\text{-}HT_{1A}$) receptor is coupled to a trimeric G_i protein composed of α, β, and γ three subunits. Upon receptor binding, the three subunits separate themselves to perform different tasks. The α subunit reportedly can stimulate Na^+/H^+ exchange. MAP kinase kinase (MEKK), replacing Raf-1, is proposed to activate the MAP kinase, resulting in cell protection.

Phosphorylation, that has been shown to negatively regulate Raf-1 kinase activity, includes PKA phosphorylation at serine 43 and 621 (Daum et al., 1994; Mischak et al., 1996). However, this does not seem to be the case in cells expressing 5-HT_{1A} receptor, because activation of receptor is known to inhibit adenylate cyclase, the enzyme that is responsible for the activation of PKA.

Studies using phosphatases have yielded inconsistent results. In Ras-mediated Raf-1 activation, phosphorylation of Raf-1 at both tyrosine and serine-threonine sites is required for Raf-1 activity, addition of protein phosphatases inactivate Raf-1 (Dent et al., 1995b). During Drosophila photoreceptor development, a reduction in protein phosphatase 2A production stimulates signaling from Ras, but impairs signaling from Raf-1, suggesting that hyperphosphorylation of Raf-1 might result in inactivation of Raf-1 protein kinase (Wassarman et al., 1996; Millward et al., 1999).

In addition to phosphorylation and dephosphorylation, forming complexes with other proteins can regulate Raf-1 activity. Various proteins have been shown to directly interact with Raf-1 and consequently activate or inactivate Raf-1 (Cleghon & Morrison, 1994; Pumiglia et al., 1995; Michaud et al., 1997; Coss et al., 1998). It is important to differentiate those mechanisms that inactivate an active Raf-1 protein kinase and those prevent Raf-1 to become active. A 14-3-3-Raf-1 protein complex can illustrate this. An isozyme of 14-3-3 protein has been reported to negatively regulate Raf-1 kinase activity (Clark et al., 1997). Further investigation reveals that in unstimulated cells Raf-1 binds to a monomer of 14-3-3 protein, thus yielding an inactive form of Raf-1; and by binding to a 14-3-3

protein dimer, Raf-1 kinase can be stably activated (Tzivion et al., 1998). In non-transformed cells, Raf-1 kinase is not usually activated, and 5-HT_{1A} receptor agonist-induced signaling alone is probably not sufficient to turn on the kinase activity. The Raf-1 kinase activity could remain inactive by forming a protein complex with other proteins.

We propose two possibilities to explain the observed lack of Raf-1 kinase activity in cells stimulated with serotonin 1A receptor agonist. One possibility is that hyperphosphorylation of Raf-1 prevents it from being activated, and the other is that Raf-1 is in complex form with other proteins, thus cannot be activated.

In summary, according to the models proposed in **Figure 2.6** and **Figure 2.7**, the serotonin 1A receptor-mediated cell protection may be achieved by the following mechanisms: there are a Raf-1-independent activation of MAP kinase pathway mediated by osmotic pressure-sensitive MEKK, and a Raf-1 phosphorylation-dependent pathway mediated by Raf-1-Bad-caspase 8 complex.

2-4. Conclusions

First, we have demonstrated that an agonist-induced Raf-1 phosphorylation was detected on 2-D autoradiograph. However, we failed to demonstrate any Raf-1 kinase activity associated with this agonist-induced Raf-1 phosphorylation. Knowing that Raf-1 phosphorylation is closely associated with Raf-1 kinase activity, it will be of great interest to determine whether or not the level of Raf-1 phosphorylation observed in our studies in the absence of agonist stimulation is enough to shut down the Raf-1 kinase activity, therefore any further increase in the phosphorylation of Raf-1 does not affect its kinase activity. Future experiments using phosphorylation inhibitors or phosphatases should be performed to answer the question.

Second, we have demonstrated that inactive Raf-1 forms complex with caspase 8 and Bad. However, we do not have direct evidence showing that the complex formation is indeed anti-apoptotic. Therefore, it is important to determine whether Raf-1-Bad complex interferes with the proapoptotic properties of Bad or not, and whether Raf-1-caspase 8 complex interferes with the enzymatic activity of caspase 8 or not. Furthermore, it will be interesting to see whether Raf-1 kinase activity is involved in regulating the complex formation or not. By eliciting the Raf-1 kinase activity, we can investigate the effects of Raf-1 kinase activity on complex formation.

2-5. Materials and Methods

Cell culture. HN2-5 cells were cultured to about 80-90% confluence in D-MEM containing 10% fetal bovine serum and 1% Pen-Strep in poly-L-lysine coated plates. Overnight treatment with phosphate-free Kreb's Ringer buffer (KRB: 145 mM NaCl, 5 mM KCl, 1.28 mM $CaCl_2$, 1.20 mM $MgCl_2$, 20 mM glucose, and 25 mM HEPES) was used to induce cell differentiation. This process of differentiation confers to the cells neuronal morphology and strong immunoreactivity to antibodies raised against neuron-specific neurofilament protein (Singh et al., 1996).

Intracellular phosphorylation. Differentiated HN2-5 cells were cultured in phosphate- and serum-free D-MEM, and treated with 0.5mCi/ml of $^{32}PO_4^{-3}$ for at least 3 hours to label the intracellular ATP pool. Cells that received serotonin 1A receptor agonist were treated with 8-OH-DPAT for 30 minutes. Cells that received both antagonist and agonist were first treated with the antagonist, WAY-100635, for 15 minutes, and then treated with the agonist for 30 minutes. Phosphorylation reactions were terminated by removing the $^{32}PO_4^{3-}$ –containing D-MEM from the cells, and adding cold 1x RIPA buffer to the cells (PBS: 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS. Protease inhibitor cocktail (Boehringer Mannheim), 0.5 mM PMSF and 1mM Na_3VO_4 were added to the RIPA buffer prior to use.

Immunoprecipitation. HN2-5 cells were solublized in cold RIPA buffer and DNA was disrupted by repeated aspiration through a 21-gauge needle and syringe. Cell lysates were precleared with normal rabbit serum (1: 500) and 20 μl of protein A sepharose beads for 30 minutes in 4°C. Samples were then centrifuged at 14,000 rpm for 5 minutes (Eppendorf, Centrifuge 5415C). Supernatant was collected, 1 μg/ml rabbit anti-Raf-1 antibody (Santa Cruz Biotechnology, Raf-1(C-12)) was added to each sample, and the samples were incubated for 1 hour at 4°C. Protein A sepharose beads 20 μl was further added to each sample for overnight incubation at 4°C. The next day, samples were centrifuged at 14,000 rpm for 5 minutes, supernatants were discarded, and pellets were washed 4 times with RIPA buffer, each time the same centrifugation step was repeated. After washing, the samples were ready for further analysis.

Gel electrophoresis, protein transfer, and autoradiography. The electrophoresis was performed using the BioRad minigel system. The immunoprecipitates of HN2-5 cells were dissolved in SDS-PAGE treatment buffer (62.5mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-β-mecaptoethanol) and placed in boiling water for 5 min. The beads were spun down and supernatants loaded on a 8.5% acrylamide gel. Proteins separated on the SDS-PAGE gels were transferred to a nitrocellulose membrane using BioRad minigel transfer apparatus (transfer buffer: 25 mM Tris, 190 mM glycine, 20% MeOH). The immunoblot was wrapped in a plastic wrap and put into a cassette with an X-

ray film on top of it. Autoradiographs were obtained to determine the level of Raf-1 phosphorylation.

Immunoblotting. The nitrocellulose membrane was incubated for 1 hour at room temperature in blocking buffer (5% non-fat dry milk, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20). The primary antibody, Raf-1 monoclonal antibody (Transduction Laboratories: Cat # R19120), was diluted in blocking buffer (1: 1000). The secondary antibody, goat anti-mouse IgG antibody conjugated to horse peroxidase, was diluted the same way as the primary antibody was. At the end of the first incubation time, the blocking buffer was removed, and the blot was placed in the primary antibody solution for overnight at 4°C. The next day, the blot was washed for 30 minutes with agitation, washing buffer (1XTBS with Tween 20: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) was changed every 3-5 minutes. After washing, the blot was put into the secondary antibody solution for 1 hour at room temperature. Finally, the same washing procedure was repeated, and the blot was ready for chemiluminescent detection of Raf-1 protein (Pierce, SuperSignal Substrate, Cat # 34080).

Raf-1 kinase activity assay. The assays were performed using the Raf-1 kinase cascade assay kit (Upstate Biotechnology: Cat # 17-172). In summary, the assay was composed of two parts: one part is a Raf-1-dependent activation of MEK1 and MAP kinase 2/Erk2, and the other part is a phosphorylation of myelin basic

protein (MBP) by MAP kinase2/Erk2. At the first stage, unactivated MEK1 and unactivated MAP kinase 2/Erk 2 were incubated together with the Raf-1 immunoprecipitates from HN2-5 cells at 30°C in a shaking incubator for 30 min. An aliquot of the sample was removed and added to a mixture of MBP and [γ-^{32}ATP]. The phosphorylation of MBP was quantitated by scintillation counter.

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