An ErbB4 Regulating Novel Acid Phosphatase Implicated in Neuronal Development

by

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Abstract

Maintaining homeostasis or responding to cellular stimuli requires an intricate network of proteins that are capable of communicating and working in concert. Kinases and phosphatases are key participants in this network. Most frequently functioning as "on" and "off" switches for signaling cascades, they are regulators of physiological processes that determine the phosphorylation state of receptors, phospholipids, metabolites and other proteins. Histidine acid phosphatases comprise a family of enzymes that achieve optimal catalytic activity at an acidic pH, *in vitro*. They include prostatic (PAP), lysosomal and lysophosphatidic acid phosphatases.

In this study, a novel histidine acid phosphatase (ACPT), that is highly homologous to PAP, was cloned and characterized. RT-PCR studies indicated that ACPT has a wide tissue distribution and within the brain, it is highly expressed in the cortex, hippocampus and striatum, as indicated by antibodies generated specifically against ACPT. This distribution pattern is notably different from PAP in that it is present in the brain, whereas PAP is absent. ACPT was found to be enriched at post-synaptic sites and immunocytochemical studies of hippocampal neurons revealed coexpression of the enzyme with synaptic proteins.

Recently, PAP has been shown to regulate the function of ErbB2, a member of the epidermal growth factor (EGF) family of receptor tyrosine kinases. Using immunoprecipitation studies, it was found that ACPT associates with ErbB4, a member of the EGF receptor family that is enriched in the brain. It was also demonstrated that this interaction results in regulation of ErbB4 function when ACPT acts as a tyrosine phosphatase and consequently influences receptor cleavage. The differentiation of PC12 cells induced by

ErbB4 activation can be prevented by the expression of ACPT. Although this neurite outgrowth is mediated by the same MAP kinase pathway which is responsible for nerve growth factor (NGF) induced-trk-dependent differentiation, the phosphatase regulation is ErbB4 specific. Therefore, ACPT may participate in neuronal differentiation through its regulation of the ErbB4 receptor.

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CHAPTER I

Introduction

1.1 Altering the Activity of Biological Proteins by Phosphorylation

After synthesis on ribosomes, nearly all proteins can be chemically altered within a cell. Depending on the nature of the alteration, whether it be categorized as a chemical modification or processing, these modifications can impact on activity, life span or cellular location of proteins. Processing involves the removal of a peptide segment most commonly by proteolytic cleavage. In contrast, chemical modifications involve the linkage of a chemical group to the terminal amino or carboxyl groups of the backbone or to reactive groups in the side chains of internal residues. This can be achieved by acetylation, fatty acid acylation, glycosylation or phosphorylation.

Phosphorylation is an energetically favourable process involving the transfer of a phosphoryl group $(-PO_3^2)$ to the hydroxyl group of a molecule to form a phosphoester bond (fig. 1-1A). This includes reactions such as the substitution of a phosphate group for a hydroxyl group on serine, threonine and tyrosine residues (fig. 1-1B) (Sparks and Brautigan, 1986; Burke and Zhang, 1998). It is an easily reversible reaction, which serves as a mechanism for regulating cellular activity. The addition of a phosphate group, acquired from the hydrolysis of phosphoanhydride bonds in adenosine triphosphate or ATP, is carried out by kinases, and removal of the phosphate group is catalyzed by phosphatases (Sparks and Brautigan, 1986). In general, the addition and removal of a group from an amino acid residue involves a pair of enzymes and their counteracting activities provide cells with a "switch"

A.

 $R_1 - O - PO_3^{2-} + R_2 - OH \implies R_1 - OH$

 $+ R_2 - O - PO_3^{2-}$



Figure 1-1. Schematic of Phosphoryltransfer Reactions. *A*. A phosphoester bond is formed when the hydrogen from the hydroxyl group of molecule R_2 is substituted by a phosphate group from molecule R_1 in a hydrolysis reaction. *B*. A tyrosine residue is phosphorylated to become phosphotyrosine.

by which the function of various proteins can be turned up or down. However, it should be noted that the balancing of kinase-induced activity can often be accomplished by one or more phosphatases (Sparks and Brautigan, 1986).

The significance of phosphorylation in the regulation of protein function is suggested by the large number of total kinases present in the human genome. There are approximately 518 protein kinases, constituting approximately 1.7% of all human genes, making them one of the largest families of genes in eukaryotes (Manning et al., 2002). Intense research on protein kinases has discovered a role for these enzymes in the control of cellular processes such as metabolism, transcription, cell cycle progression, cytoskeletal rearrangement, cell movement, apoptosis, differentiation, intracellular communication most notably during development, physiological responses, homeostasis and in the functioning of the nervous and immune systems. Mutations and dysregulation of protein kinases has been found to be a contributing factor in human disease, thereby making them ideal targets for the development of agonists and antagonists for use in disease therapy. As the counteractive force to kinases, phosphatases can also be considered as biological control points, making them attractive targets for disease therapy as well.

Kinases and phosphatases influence a wide spectrum of activities and functions because of the large diversity of substrates that they act upon. They not only add and remove phosphate groups from proteins, but from lipids and metabolites as well. Lipids such as sphingosine 1-phosphate (S1P), ceramide 1-phosphate, phosphatidic (PA) and lysophosphatidic (LPA) acid are only a few of the bioactive lipids that are modified by kinases and phosphatases (Brindley et al., 2002; Sciorra and Morris, 2002; Wu and Cunnick, 2002). They are not only components of the lipid bilayer of the cell membrane, but are

present in biological fluids and serve as mediators of signal transduction. For example, LPA and S1P can regulate cells through stimulation of their respective G-protein-coupled receptors (Lee et al., 1998; Fukushima and Chun, 2001; Kranenburg and Moolenaar, 2001; Brindley et al., 2002). LPA stimulates fibroblast division, is important in wound repair and is active in maintaining the growth of ovarian cancers (Guo and Donner, 1996; Hecht et al., 1996; Fukushima et al., 1998). S1P stimulates chemotaxis, proliferation and differentiation of vascular endothelial and smooth muscle cells (Goetzl and An, 1998; Lee et al., 1998). PA and S1P are believed to act primarily inside the cell where they facilitate vesicle transport (Bajjalieh et al., 1989; Shinghal et al., 1993; Huijbregts et al., 2000; Zimmerberg, 2000).

As modifiers of lipid phosphates, phosphatases can dramatically alter the signaling balance between phosphate esters and their dephosphorylated products. Often, the dephosphorylated molecules have biological activity (Sciorra and Morris, 2002). In this manner, lipid phosphatases can change signals that the cell receives. Additional activity of lipid phosphatases includes "ecto-activity". When functioning as "ecto-enzymes", lipid phosphatases degrade lipid phosphate substrates in both the extracellular space and outer leaflet of the plasma membrane (Sciorra and Morris, 2002). This results in the regulation of circulating and the locally effective concentration of various lipids such as LPA and S1P.

The influence of kinase and phosphatase activity on metabolites is similar to their effect on lipids, in that they can modify cell signals. The inositol polyphosphate family is an example of a group of substrates that can yield metabolites with the potential to continue intracellular signaling upon phosphorylation or dephosphorylation (Majerus, 1992; Menniti et al., 1993; Fukuda and Mikoshiba, 1997). In general, cells contain a variety of inositol polyphosphates with four or more phosphate groups. Structurally they are composed of a six

carbon inositol ring (Ins) and one or more phosphate groups (P_x , where x=the number of phosphate groups added). As second messengers, they are key intermediates in translating extracellular signals to the cytoplasm and nucleus of eukaryotic cells.

The complexity of this intricate network of inositol lipids is well exemplified by inositol-1,4,5-triphosphate (Ins(1,4,5)P₃). In 1983, it was discovered that Ins(1,4,5)P₃ acted as a Ca²⁺-releasing second messenger (Streb et al., 1983). By binding to Ca²⁺ channels located on the endoplasmic reticulum, Ins(1,4,5)P₃ was able to release stored Ca²⁺ (Streb et al., 1983). Dephosphorylation of this inositol polyphosphate upon cell stimulation also produced Ins(1,4)P₂ and InsP which coincided with an increase in two metabolites, Ins(1,3,4,)P₃ and Ins(1,3,4,5)P₄ (Irvine et al., 1984; Batty et al., 1985). These metabolites are now viewed as rapidly synthesized metabolites of Ins(1,4,5)P₃ which can impact on cellular function. Their production has been linked to coincidence detection at the post-synaptic sites of dendritic spines of neurons (Irvine, 2001). This is a process whereby a cell responds differently to a signal if another signal is received simultaneously and is central to long-term potentiation.

Research has made it clear that the widespread activity of kinases and phosphatases can initiate a cascade of responses whose influence can be felt throughout the cell. The diversity and range of hydrolyzing activity is complicated. The variety of substrates alone reflects this, and is further highlighted when analyzing the resulting products. It will take many years of continued research to determine the exact nature of kinase and phosphatase function, activity and impact on cellular function and homeostasis.

1.2 Acid Phosphatases

Phosphatases comprise a large family of diverse enzymes that are often grouped according to function, mechanism of action and homology (Guan and Dixon, 1991; Vincent et al., 1992). A group that hydrolyzes phosphomonoesters with an acid pH optimum are referred to as acid phosphatases (Moss et al., 1995). Members of the acid phosphatase family can be further sub-classified as histidine acid phosphatases on the basis of the consensus sequence, RHGxRxP (Van Etten, 1982; Vincent et al., 1992; Ullah and Dischinger, 1993). This consensus sequence contains an invariant Arg-His-Gly tripeptide that harbours the histidine residue essential for the formation of the catalytic intermediate (Ostanin et al., 1992). Histidine acid phosphatases can additionally be referred to as high molecular weight acid phosphatases because of their ~50 kDa subunits. This is in contrast to the ~20 kDa low molecular weight acid phosphatases (Heinrikson, 1969; Ramponi and Stefani, 1997).

The known members of this sub-family of enzymes includes lysosomal (LAP), prostatic (PAP) and lysophosphatidic (LyAP) acid phosphatases. The structural and functional similarities between the genes coding for the various histidine acid phosphatases suggest that these enzymes belong to a multi-gene family originated from an ancestral gene during the course of evolution (Sharief et al., 1989). Although PAP shows as much as 28.5% amino acid sequence homology to LyAP, similarities are particularly striking between PAP and LAP which share 50% amino acid homology (Peters et al., 1989; Hiroyama and Takenawa, 1999). Each has putative glycosylation sites, the precursor proteins contain hydrophobic signal peptides (Pohlmann et al., 1988; Vihko et al., 1988) and both PAP and LAP are sensitive to L-tartrate (Lin et al., 1983; Pohlmann et al., 1988). The sensitivity of LyAP to L-tartrate has not yet been reported.

In spite of the significant degree of homology displayed by histidine acid phosphatases, the members of this family of enzymes are fairly diverse. As an enzyme bound to the lysosomal membrane, LAP is expressed by all cells (Pohlmann et al., 1988; Geier et al., 1989). In contrast, PAP is a soluble, non-membrane-bound protein that is expressed by prostatic epithelial cells and is hormone regulated (Lin and Meng, 1996; Meng et al., 2000). LyAP is located in the mitochondria and it has been suggested that it functions as a regulator of lipid metabolism (Hiroyama and Takenawa, 1999). It is evident that these three enzymes, although evolutionarily connected, serve functions that are distinct from each other.

The defining characteristic of this group of enzymes is two catalytic motifs, including the RHGxRxP consensus present at the N-terminus and a second motif of HD near the Cterminus (Van Etten et al., 1991; Ostanin et al., 1992). Both of these motifs are essential for the formation of the catalytic intermediate that drives the phosphoester hydrolysis reaction characteristic of this family of enzymes (Van Etten, 1982; Vincent et al., 1992). The histidine within the invariant tripeptide of the N-terminal catalytic motif acts as a nucleophilic phospho acceptor, whereas the histidine within the HD motif may act as a proton donor (Van Etten, 1982; Vincent et al., 1992). The aspartic acid residue is also believed to participate as a proton donor to the substrate-leaving group (Ostanin et al., 1992; Caffrey et al., 1999). In combination, these two motifs work to form an intermediate identified by the phosphorylation of the nucleophilic histidine residue within the N-terminal catalytic motif (McTigue and Van Etten, 1978; Vincent et al., 1992; Ullah and Dischinger,

1993). All members of the histidine acid phosphatase family contain these two motifs and are believed to use this mechanism to achieve catalytic activity (Chi et al., 1999; Hiroyama and Takenawa, 1999).

In addition to the catalytic motifs, the full-length gene for all three enzymes codes for an N-terminal signal sequence and specific cysteine and proline residues. Conservation of these specific amino acids implies marked tertiary structural similarities amongst the histidine acid phosphatases (Peters et al., 1989). Sequence variation beyond this point suggests a diverse set of possible functions for these enzymes. Unfortunately, little has been accomplished in elucidating the distinct function of each of these enzymes. Currently, experimental data based on *in vitro* studies has shown that these phosphatases can act as nonspecific orthophosphate monoesterases with an acidic pH optimum (Moss et al., 1995). As nonspecific orthophosphate monoesterases, these enzymes are able to remove a phosphate from a phosphate ester bond on a large range of substrates such as guanosine monophosphate, glycerophosphate, thiamine monophosphate, etc. (Silverman and Kruger, 1988). However, the implied true function of each enzyme rests on hypotheses based on tissue localization since *in vivo* data is limited.

1.2.1 Lysophosphatidic Acid Phosphatase

Lysophosphatidic acid phosphatase (LyAP) is the most recently cloned and characterized member of the histidine acid phosphatase family. The full-length protein is comprised of 421 amino acids with a subunit molecular weight of 45 kDa (Hiroyama and Takenawa, 1998, 1999). As is typical of members of this family, LyAP has a putative signal

sequence, conserved cysteine and proline residues and functions as a homodimer (Hiroyama and Takenawa, 1999). It is expressed in all tissues, but has marked expression in kidney, heart, small intestine, muscle and liver (Hiroyama and Takenawa, 1999). Subcellularly, LyAP is localized to the mitochondria by the signal peptide at its amino terminus where it is believed to regulate the biosynthesis of mitochondrial lipids by hydrolyzing lysophosphatidic acid (LPA) to monoacylglycerol (Hiroyama and Takenawa, 1999). It is one of two known LPA-hydrolyzing pathways used to regulate LPA activity. As a LPA-specific phosphatase, LyAP plays an important role in phospholipid metabolism. LPA is a key intermediate for the biosynthesis of glycerolipids and exerts its biological activities such as wound healing, regeneration and neurite retraction via G-protein-coupled LPA receptors (Bell and Coleman, 1980; Bishop, 1988).

Phospholipid analysis of cells where LyAP is over-expressed show reduction of two mitochondrially synthesized lipids, cardiolipin and phosphatidyl glycerol (PG) (Hiroyama and Takenawa, 1999). In contrast, phosphatidylethanolamine and phosphatidylinositol increase (Hiroyama and Takenawa, 1999). It has been suggested that expression of LyAP causes a reduction of cardiolipin and PG through the removal of the LPA precursor lipid from mitochondria. The increase in phosphatidylethanolamine and phosphatidylinositol may be caused by a disturbance of phospholipid metabolism through lack of LPA in mitochondria. In this manner, LyAP is believed to hydrolyze LPA synthesized in mitochondria and regulate mitochondrial lipid biosynthesis, thereby regulating mitochondrial functions.

1.2.2 Lysosomal Acid Phosphatase

Lysosomal acid phosphatase (LAP) was crucial in the discovery of lysosomes, and as such, is used as a biochemical marker for them. It has uniform expression in all organs, with the exception of brain and testis, where its expression is increased (Waheed et al., 1985; Pohlmann et al., 1988). It is due to its expression in all tissues that LAP is believed to be a housekeeping gene (Waheed et al., 1985).

As an acid phosphatase, this enzyme has the typical catalytic domains, conserved glycosylation sites and proline and cysteine residues, a putative signal sequence and functions as a homodimer (Pohlmann et al., 1988; Geier et al., 1989; Roiko et al., 1990). Until recently, LAP was considered to be a unique member of the acid phosphatase family because it possesses a transmembrane domain. As a type I glycoprotein, the transmembrane domain of LAP is oriented so that the densely glycosylated amino terminal is located within the lumen of the lysosomes (Waheed et al., 1988; Peters et al., 1989). Both LyAP and the clinically relevant isoform of PAP lack the transmembrane domain found at the C-terminus of LAP. This enzyme is transported to lysosomes as a membrane-bound precursor, where the soluble mature form of LAP is generated by limited proteolytic cleavage which is dependent on an acidic pH (Pohlmann et al., 1988; Waheed et al., 1988; Gottschalk et al., 1989).

Although the *in vivo* substrates and function of LAP are unknown, studies of LAP knockout mice has provided some insight into possibilities. Examination of LAP-deficient mice has shown that the kidneys and central nervous system develop lysosomal storage disease, a phenomena which results in the interruption of the natural recycling function of lysosomes in the absence of the enzyme, leading to various materials being inappropriately

stored in the cell (Saftig et al., 1997). These mice also exhibit an increased disposition towards epileptic seizures, and as they mature (6 months +) progressive skeletal abnormalities develop. It is therefore evident that LAP is critical for the catabolism of distinct substrates.

1.2.3 Prostatic Acid Phosphatase

Prostatic acid phosphatase (PAP) is the best characterized member of the histidine acid phosphatase family, largely due to its past clinical use as a marker and prognosticating tool for prostate cancer (Lin and Clinton, 1986). It is a dimeric glycoprotein of a molecular mass of 100kDa, with each subunit being ~50kDa (Kuciel et al., 1990; Luchter-Wasylewska, 2001). Its expression is limited to the prostatic epithelium where is exists in both an intracellular and secreted form (Vihko, 1979; Morris et al., 1989). These two forms of the enzyme may be the consequence of post-translational modifications of the enzyme. A glycosylated and non-glycosylated form of PAP have been shown to be present endogenously (Lam et al., 1980).

Expression of glycosylated PAP which is the clinically significant isoform, is generally confined to the prostate. It is synthesized under androgen regulation by prostatic epithelial cells and secreted into seminal fluid (Sharief et al., 1989). In contrast, the nonglycosylated form is expressed in other tissues, including platelets, heart, bowel and skeletal muscle (Lam et al., 1980; Epstein et al., 1986; Kimura and Sasano, 1986; Drenckhahn et al., 1987).

Until recently, PAP was thought to lack a transmembrane domain at the C-terminus that can be found in LAP (Pohlmann et al., 1988; Geier et al., 1989). It was even suggested that this domain was lost by deletion in PAP during evolution or that it was added after duplication into LAP. Additionally, an alternative amino acid sequence for the C-terminus of PAP that was recently sequenced (Genbank accession # XM 039822) indicates a possible variant of the enzyme that includes a putative transmembrane domain. The significance of this novel isoform has yet to be determined.

1.3 Prostatic Acid Phosphatase as a Tyrosine Phosphatase

Insight into the *in vivo* function of histidine acid phosphatases eluded scientists for many years due in part to the fruitless search for an endogenous substrate for any of the members of this family of enzymes. Discovery of just one *in vivo* substrate for a member of the histidine acid phosphatase family could guide the search for other substrates.

Precluding any knowledge of its biological function, PAP was extensively studied because of its importance in the diagnosis and management of prostate cancer (Sharief et al., 1989). Initial *in vitro* studies using artifically phosphorylated exogenous protein substrates suggested a specific activity of the enzyme towards phosphotyrosine-containing proteins (Lin and Clinton, 1986; Chevalier et al., 1988; Nguyen et al., 1990; Lin et al., 1994). However, to determine the true physiological role of PAP, endogenous protein substrates are of greater value. Extensive research of prostate cancer cells revealed a possible *in vivo* substrate. It was discovered that the cellular form of PAP is able to regulate the phosphotyrosine level of a 185 kDa phosphoprotein later identified as a member of the ErbB family of receptor

tyrosine kinases, ErbB2 (Lin and Meng, 1996; Meng and Lin, 1998). ErbB2 activity was found to be inversely correlated with the cellular activity of PAP (Lin et al., 1994; Zhang et al., 2001). Dephosphorylation of ErbB2 by PAP reduces the tyrosine kinase activity of the receptor, resulting in the down-regulation of the signal transduction cascade activated by ErbB2 stimulation. By exerting enzymatic action on ErbB2, PAP is believed to function as a regulator of cell proliferation (see section 1.4).

The tyrosine phosphatase function of PAP indicated that the phosphatase belongs to a large family of molecules referred to as protein tyrosine phosphatases important in signal transduction pathways within eukaryotic cells. PAP lacks the usual signature motif of [CXXXXR(S/T)] found in enzymes of this family (Burke and Zhang, 1998; Arregui et al., 2000), suggesting that it represents a distinct subtype of protein tyrosine phosphatase.

1.4 Epidermal Growth Factor Receptors

The epidermal growth factor (EGF) receptors are membrane-bound receptors important for the mediation of cell growth and differentiation (Ullrich and Schlessinger, 1990). The four members of the family, EGFR (ErbB1), ErbB2, ErbB3 and ErbB4, and the multiple ligands which they are capable of binding form an interactive system with a large potential for signal diversification (Pinkas-Kramarski et al., 1998; Hackel et al., 1999).

The EGF receptors mediate cell signal transduction via tyrosine receptor kinase activity. They are structurally related receptors consisting of a glycosylated extracellular ligand-binding domain, a single transmembrane domain and an intracytoplasmic tyrosine kinase domain (Walker, 1998; Novak et al., 2001). The network through which the EGF receptors signal is initiated when ligand induced hetero- or homodimerization occurs. This activates the intrinsic protein tyrosine kinase domain enabling the receptor to autophosphorylate several tyrosine residues within the cytoplasmic domain (Heldin and Ostman, 1996; Novak et al., 2001). Consequently, docking sites are generated (van der Geer et al., 1994; Massague, 1996), allowing proteins containing SH2 domains to interact and initiate a cascade of several tyrosine and serine-threonine kinases (Moghal and Sternberg, 1999; Novak et al., 2001). This simultaneous recruitment of several second messenger pathways can lead to activation of transcription factors and ultimately to the induction of cell proliferation or of specific cellular functional responses. These responses tend to be short-lived as the receptors are regulated or degraded to allow the cell to rapidly return to its basal state.

A pathway stimulated by all ligand-activated members of the EGF receptor family, regardless of the dimerization partner, is the mitogen-activated kinase (MAPK) pathway (Graus-Porta et al., 1995; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996). Although the same signal transduction pathway is generally activated, the differential expression of the receptors and of the ligands, as well as their isoform-specific properties mediates different effects *in vivo* (fig. 1-2).



Figure 1-2. Members of the Epidermal Growth Factor Receptor Family and Their Ligands. Each receptor has an extracellular domain with two cyseine-rich domains (cys, yellow), a single transmembrane domain (black), and a cytoplasmic region consisting of a tyrosine kinase domain (green) or tyrosine kinase dead domain (red) and a carboxyterminal domain. PM = plasma membrane.

1.4.1 Isoform Properties and Distribution Patterns

ErbB1, more commonly referred to as EGFR, was the first member of the EGF receptor family to be cloned. Its close homology to the transforming gene of the avian erythroblastosis virus (v-erbB) led to the proposal that the v-erbB gene is the oncogenic version of the EGF receptor gene and gave the EGFR gene the synonym ErbB1 (Lin et al., 1984). The ErbB1 protein is expressed in many normal tissues, most notably in epithelial cells of the breast, lung, the gastrointestinal tract and peripheral nervous system (Gusterson et al., 1984; Damjanov et al., 1986). It has high affinity for several ligands which all have sequence and structural homology, and characteristic positioning of six cysteine residues (Falls et al., 1993). Ligand-binding induces dimerization of ErbB1 and although this receptor is capable of homodimerization, the most active receptor constitutes a heterodimer of ErbB1 and ErbB2 (Yarden and Schlessinger, 1987a, b; Goldman et al., 1990). The generation of ErbB1 knockout mice identified a role for the receptor in development and physiology (Sibilia and Wagner, 1995; Threadgill et al., 1995; Sibilia et al., 1998). Strain-dependent phenotypes exhibited defects in placenta, skin and lung and a progressive neurodegenerative disorder that was lethal within 3 weeks of birth was developed by all strains.

The unique properties of the ErbB2 isoform render it incapable of binding any EGFlike factor with high affinity, it is generally the preferred hetero-dimerization partner for all of the other members of the EGF receptor family (Horan et al., 1995; Tzahar et al., 1997). Its precise pattern of expression has not yet been conclusively established. Data suggesting that expression is limited to the digestive tract, urinary tract, skin and reproductive system (Press et al., 1990) has been contradicted by an alternative source indicating additional ubiquitous

expression within the brain (Gerecke et al., 2001). A role for ErbB2 in development was evident in mice lacking the ErbB2 gene. These mice survived to day 10 at which point they died due to deficient cardiac ventricular myocyte development. Mice with restored ErbB2 expression under a myocardium specific promoter (Woldeyesus et al., 1999) survived to term, however, they lacked Schwann cells and their motorneurons were disorganized and poorly fasciculated.

The limitation of the ErbB3 receptor is its cytoplasmic domain which is devoid of tyrosine kinase activity (Guy et al., 1994), therefore making heterodimerization a mandatory requirement towards achieving enzymatic activity. ErbB3 protein is expressed in the digestive tract, urinary tract, respiratory tract, circulatory system, skin, endocrine system, nervous system (brain and peripheral nerves), placenta and reproductive system (Prigent et al., 1992). Even though ErbB2 is the preferred heterodimerization partner for ErbB3, this receptor has been observed to dimerize with ErbB4 in the absence of the more favourable alternative.

In contrast to ErbB2 and ErbB3, ErbB4 does not have limitations in its ligand-binding affinity nor its catalytic activity. It is therefore capable of heterodimerization with the other EGF receptor isoforms or homodimerization. This receptor has been found to be widely expressed in many adult and fetal tissues, including the lining epithelia of the gastrointestinal, urinary, reproductive and respiratory tracts, as well as the skin, skeletal muscle, circulatory, endocrine and nervous systems (brain, but not the peripheral nerves) (Srinivasan et al., 1998). Such a distribution pattern indicates that within the brain, the only likely functional EGF receptors are composed of either an ErbB3/ErbB4 or ErbB2/ErbB4 heterodimer or an ErbB4 homodimer.

1.5 The Influence of Expression, Structure and Ligand-binding on ErbB4 Function

As a member of the epidermal growth factor receptor family, ErbB4 is a receptor tyrosine kinase. Experimental evidence indicates that its activity leads to cell proliferation, chemotaxis or differentiation via the activation of specific signal transduction cascades (Chen et al., 1996; Zhang et al., 1996; Elenius et al., 1997a). It is particularly important during development where the absence of its activity, as seen in homozygous knockout mice, leads to severe abnormalities in the heart and brain. These mice die at embryonic day 10-11 due to the defective development of myocardial trabeculae in the heart ventricle (Gassmann et al., 1995). Examination of these mice has also revealed deficient axon guidance of cranial sensory ganglion afferent axons in the hindbrain which leads to the mistargeting of trigeminal and facial/acoustic ganglion axons, supporting a role for ErbB4 in neuronal development. Additionally, the receptor has been implicated in myelination, and primary radial glial cells expressing a dominant-negative form of ErbB4, when co-cultured with dissociated cerebellar granule cells display a reduction in neuronal migration indicating that ErbB receptor signaling in glia is critical for the neuronal induction of radial glial morphology (Rio et al., 1997; Steiner et al., 1999). The various avenues by which ErbB4 is able to influence development requires signaling diversity while simultaneously maintaining functional specificity. Receptor expression, structural alternatives and multiple ligands are the factors required to achieve this.

Protein expression patterns of the receptor support a potential role for ErbB4 in neuronal development. Immunohistochemistry indicates low levels of ErbB4 protein expression within the adult nervous system, whereas high levels of protein are evident in

developing neurons in the fetal nervous system (Srinivasan et al., 1998). This expression pattern coincides well with mRNA levels.

Examining the structure of the receptor tyrosine kinase can reveal clues pertaining to the method by which ErbB4 may contribute to neuronal development. At the carboxy terminus, ErbB4 contains a sequence that is unique among the EGF receptors. The three amino acid residues (T-V-V) that make up this sequence conform to the consensus sequence necessary for the interaction of the receptor with a PDZ domain. It has been shown both in vitro and in vivo that ErbB4 directly interacts with the PDZ-domain-containing protein PSD-95 (Garcia et al., 2000; Huang et al., 2000). This protein is implicated in the localization of various PDZ-binding proteins to the synaptic density because it acts as a scaffold. This interaction is thought to couple neural activity to synaptic plasticity. In this manner, the interaction of the ErbB4 carboxy terminal with the PDZ-motif of PSD-95 has been reported to be responsible for the sorting and delivery of ErbB4 to the cell surface of the post-synaptic density (Garcia et al., 2000; Huang et al., 2000). Not only is the association of ErbB4 with PSD-95 implicated in the sorting and delivery of the EGF receptor, but it also potentiates the response of ErbB4 to the binding of its ligand, perhaps by facilitating the dimerization of two receptor molecules (Huang et al., 2000).

Unlike the other EGF receptors, ErbB4 mRNA can be alternatively spliced to yield two functionally distinct isoforms (fig. 1-3) (Zhou and Carpenter, 2002). The splicing leads to the insertion of either 23 (JM-a) or 13 (JM-b) alternative amino acids in the proximal extracellular domain just N-terminal to the transmembrane domain referred to as the juxtamembrane domain (Elenius et al., 1997b). The additional residues in JM-a make it susceptible to proteolytic cleavage within the juxtamembrane domain, while JM-b is



Figure 1-3. Alternative Isoforms of ErbB4. A schematic of erbB4 with the deduced amino acid sequences of the two alternative juxtamembrane domains, JM-a and JM-b (in bold). cys = cysteine rich domain; TM = transmembrane domain; TK = tyrosine kinase domain.

insensitive (Elenius et al., 1997b; Zhou and Carpenter, 2002). Although both ErbB4 isoforms are usually expressed in cell lines, there are differences in their expression patterns, particularly within neural tissues, heart and kidney (Elenius et al., 1997b; Carraway and Sweeney, 2001). They have overlapping expression within the cerebellum and spinal cord. In contrast, only JM-a is found in the kidney and JM-b in the heart (Elenius et al., 1997b; Dixon and Lumsden, 1999).

Additional splice variation leads to CYT-1 (CYT-a) and CYT-2 (CYT-b). The splicing results in the deletion of 48 base pairs encoding amino acids 1046-1061 in the carboxy terminal of CYT-2 (Sawyer et al., 1998; Elenius et al., 1999; Kainulainen et al., 2000). This region of the C-terminus contains the PI-3 kinase binding site. CYT-1 is the dominant isoform in heart, breast, uterus and abdominal aorta. CYT-2 is predominant in kidney and neural tissues such as cerebellum, cerebral cortex, spinal cord and medulla oblongata (Moscoso et al., 1995; Elenius et al., 1999). The various isoforms and their expression patterns within tissues increase the signaling diversity and sensitivity of ErbB4 receptor signaling.

Activation of EGF receptors is the result of binding of a ligand to the N-terminal, extracellular domain. These receptors are atypical in that the activation site does not display a high degree of specificity; rather they are capable of binding a number of members of the neuregulin growth factor family thereby adding to the complexity of signaling specificity and diversity. In the case of ErbB4, this includes neuregulins (NRG-1,2,-3,-4) (Ben-Baruch and Yarden, 1994; Jones et al., 1999), betacellulin (Beerli and Hynes, 1996; Riese et al., 1996), heparin-binding EGF-like growth factor (Elenius et al., 1997a) and epiregulin (Komurasaki et al., 1997), all of which contain an EGF-like domain that is essential and sufficient for ErbB4

binding and activation (Jones et al., 1998; Jones et al., 1999). Although each ligand can bind to this one receptor, the subsequent autophosphorylation signal that is activated follows a distinct pattern for each ligand, thereby rendering the receptor with different propensities to recruit downstream signaling molecules (Sweeney et al., 2000) and allowing for a wide diversity of responses. Signaling pathways common to other receptor tyrosine kinases such as the mitogen-activated kinase (MAPK), STAT and PI-3 kinase are often involved. Binding of NRG- β 1, the ligand with the highest affinity for ErbB4, strongly stimulates the association of both Shc and p85 subunit of PI-3K, which functions to modulate several aspects of cell physiology (Sweeney et al., 2000; Zhou and Carpenter, 2002).

It is evident from collected data, that the signaling diversity of the ErbB4 receptor is not only dictated by its protein expression patterns, but also by its structure due to the presence or absence of specific amino acids, as well as the large number of ligands and their varying affinities for the receptor.

1.6 The Behaviour of ErbB4 at the Cell Membrane

The regulation of growth factor receptors and various cell surface molecules generally follows a common trafficking pathway involving endocytosis. This process involves the rapid internalization of an activated receptor through clathrin-coated-pits, ultimately leading to either receptor degradation upon the fusion of the newly formed endosomes with lysosomes (Carpenter and Cohen, 1976) or inactivation of the receptor and subsequent return to the cell surface. These two pathways serve to down-regulate/desensitize or recycle the internalized receptor (Elenius et al., 1997b; Rio et al., 2000). Although EGFR (ErbB1)

activity is regulated in this manner, the remaining three members of the EGF receptor family are not subject to rapid internalization upon ligand binding (Sorkin et al., 1993; Baulida et al., 1996; Pinkas-Kramarski et al., 1996). ErbB2, ErbB3 and ErbB4 have been found to be inefficiently internalized and are therefore considered to be endocytosis impaired (Sorkin et al., 1993; Baulida et al., 1996; Pinkas-Kramarski et al., 1996; Peschon et al., 1998). The molecular basis for this difference is not clear, however speculation has lead to the discovery that only the activated EGF receptor is able to associate with the adaptin AP-2 coated-pitmolecule, a molecule observed to facilitate receptor internalization through coated pits (Sorkin and Carpenter, 1993; Baulida et al., 1996).

Alternative mechanisms for receptor down-regulation or desensitization are required in the absence of receptor cycling. Consequently, it has been discovered that ErbB4, but not ErbB2 or ErbB3 undergoes rapid sequential proteolytic cleavage upon heregulin binding (Vecchi et al., 1996; Vecchi and Carpenter, 1997). As seen in figure. 1-4, activation of ErbB4 due to ligand-binding or downstream activation of protein kinase C (PKC) has been shown to promote proteolytic cleavage to yield a ~120kDa ectodomain which is released into the extracellular space and an ~80kDa membrane-anchored molecule consisting of the transmembrane domain and cytoplasmic tail of the receptor (Vecchi et al., 1996; Vecchi and Carpenter, 1997). Inhibitor studies have shown that receptor cleavage is most likely dependent upon the action of a metalloprotease (Vecchi et al., 1998) which has since been identified as tumor necrosis factor-alpha-converting enzyme or TACE (Rio et al., 2000). Only the JM-a isoform undergoes TACE cleavage, indicating that the cut site is mostly likely located within the amino acid sequence unique to this receptor (Elenius et al., 1997b). The ~80kDa membrane-anchored molecule that remains following this initial cleavage is heavily



Figure 1-4. ErbB4 Activation and Cleavage. Schematic of erbB4 activation by the ligand neuregulin β 1 (NRG). Upon ligand binding, the erbB4 receptor dimerizes, autophosphorylates and is sequentially cleaved by TACE and γ -secretase to yield a cytoplasmic fragment that can translocate to the nucleus.

tyrosine phosphorylated and possesses catalytic tyrosine kinase activity toward an exogenous substrate *in vitro* (Vecchi and Carpenter, 1997). Recent studies have reported additional sequential cleaving of this molecule by γ -secretase to release the ErbB4 intracellular domain from the membrane (Ni et al., 2001; Lee et al., 2002). It has been hypothesized that release of the ~80kDa fragment into the cytosol upon secondary cleavage facilitates the translocation of the catalytically active molecule to the nucleus where is may be able to continue mediating receptor tyrosine kinase activity (Ni et al., 2001; Lee et al., 2002). Experiments using a heterologous system have shown that expression of the ~80kDa molecule does in fact translocate to the nucleus. Therefore γ -secretase cleavage of ErbB4 may represent an alternative mechanism for receptor tyrosine kinase-mediated signaling.

1.7 PC12 Cells as a System Representing Neuronal Development

Studies involving loss of function mutations in the gene encoding the ErbB4 receptor have revealed the importance of its intrinsic tyrosine kinase activity in neuronal development. The migration of neuronal precursors along radial glial fibers is a critical step in the formation of the nervous system and requires the signaling pathways attributed to ErbB4 activity (Aicardi, 1994). It was shown that cerebellar granule cells failed to migrate along radial glial fibers when ErbB4 activity was blocked and that the formation of radial glia was inhibited (Rio et al., 1997). Similar aberrant patterns in cell migration were observed in hindbrain-derived cranial neural crest cells. Microtransplantation experiments involving wild-type and mutant neural crest cells demonstrated that ErbB4 expression contributed important cues for neural migration to the surrounding environment (Golding et al., 2000).
When transplanted, both cells migrated in a pattern consistent with the host environment, deviating from the normal pathway only when transplanted into mutant embryos. Experimental data therefore demonstrates the importance of ErbB4 receptor activity in neuronal development.

The influence that ACPT may exert upon ErbB4 activity could possibly manifest itself as a modifier of neuronal development. Preliminary data was gathered through the use of PC12 cells. The PC12 cell line is derived from rat adrenal medullary pheochromocytoma tumour cells. Since these cells can be easily induced to differentiate into sympathetic neuronlike cells, they are ideally suited to serve as a paradigm for studies on the regulation of neural differentiation (Greene and Tischler, 1976). Addition of various neurotrophic factors that include nerve growth factor (NGF) and basic fibroblast growth factor can be added to the cells in order to bind and activate either tyrosine kinase trkA or fibroblast growth factor receptors, respectively, to initiate downstream signaling events, and subsequently induce differentiation of PC12 cells.

PC12 cells express EGFR, ErbB2 and ErbB3 receptors, but not ErbB4 receptors (Vaskovsky et al., 2000). Although each of these receptors are capable of inducing neurite extensions in PC12 cells, it is only when they are overexpressed that they are able to do so. It has been shown by Gamett and Cerione (Gamett and Cerione, 1994) that overexpression of transforming ErbB2 results in neurite outgrowth. Similarly, over expression of either ErbB2 or ErbB3 leads to neuregulin-stimulated neurite outgrowth and activation of phosphatidylinositol 3-kinase (Gamett et al., 1995). Overexpression of EGFR in PC12 cells can also lead to differentiation in response to EGF (Traverse et al., 1994). More significantly, it has been shown that overexpression of ErbB4, the more relevant EGF

receptor in neural development, can lead to neuregulin-induced early cell division that is followed by neuronal differentiation (Vaskovsky et al., 2000; Erlich et al., 2001). These effects of NRG on cell morphology have been shown to be mediated by the Erk and protein kinase C-dependent pathways (Vaskovsky et al., 2000).

Since these cells are capable of responding to several different growth factors by producing neurite extensions, a number of these factors can serve as positive controls. Also, activating receptors other than ErbB4 to induce differentiation can be used to aid in distinguishing the level at which the "activity-altering" proteins function.

1.8 Study Aims

The search for a novel member of the histidine acid phosphatase family was rooted in the desire to clone an enzyme which is only known to exist on the basis of histochemical staining. Based on its specific localization within a subset of small sensory neurons, this enzyme termed fluoride-resistent acid phosphatase or FRAP is believed to play an important role in nociceptive transmission and would therefore be an ideal target for analgesic therapy. However, the existence of this novel phosphatase has been a topic of debate. The immunohistochemical studies have shown that FRAP shares similar substrate specificity with PAP, and its fluoride resistance and tartrate sensitivity are mirrored by PAP, thereby leading to the proposal that the enzyme is PAP rather than a novel isoform.

The objective of this study was to further pain research by cloning and characterizing this novel histidine acid phosphatase. Although a novel acid phosphatase (ACPT) was cloned during the pursuit of FRAP, it was quickly determined that it was a phosphatase not previously proposed to exist. Due to its expression in the brain and its high degree of homology to PAP which functions as a tyrosine phosphatase regulator of ErbB2, it was hypothesized that ACPT may have tyrosine phosphatase activity towards the epidermal growth factor receptor, ErbB4.

CHAPTER II

Methods and Materials

2.1 Database Searches

A database search was conducted to identify genes encoding for novel, full-length proteins from the histidine acid phosphatase family. Using regions of homology amongst the members of the histidine acid phosphatase family, a search against human expressed sequence tags (ESTs) was conducted using the tBLASTN algorithm on the National Centre for Biotechnology Information server (<u>http://www.ncbi.nlm.nih.gov/BLAST/</u>). By piecing together overlapping ESTs, a sequence beginning with ATG and ending with a stop codon was assembled.

2.2 Cloning and Amplification of ACPT

Amplification of a gene representing the full-length sequence for a novel acid phosphatase was accomplished by running a polymerase chain reaction (PCR). Primers were designed based on the sequence derived from the alignment of overlapping EST sequences. Human testis cDNA (Clontech) was used as a template to perform a 25µl PCR reaction containing 1ug of cDNA, 60pmol of each primer (forward primer 5'-CGTCTGGGCTGGGT GGAAATG-3', reverse primer 5'-TGTCAGCTGGGGTAGGGAAG-3'), 1.5mM MgCl₂, 200µM dNTPs, 1.25 units Vent DNA Polymerase and 1X PCR reaction buffer. The mixture was subjected to 35 cycles of 94°C for 45 sec. for denaturing, 61°C for 1 min. for annealing and 72°C for 1.5 min. for primer extension. The resulting products were separated on an agarose gel, purified and subcloned into pCR2.1 vector (Invitrogen). To verify the gene product, the clone was sequenced (LoneStar Labs).

Additional amplifications of ACPT were performed using primers which incorporated the sequence for an HA-tag located at the C-terminus of the enzyme (forward primer 5'-GGCCGGTACCGCCACCATGGCCGGCCGGCCTGGGGT-3', reverse primer 5'-TAGCAAGCTTCGCATAATCCGGCACATCATACGGATACACGGGGCCCCCCAAGG C-3')

2.3 Restriction Digests

Restriction digests were performed to verify the integrity of plasmid clones and to subclone DNA into alternative vectors. The Gw1 and EGFP-N3 vectors encoded for a GFP tag priot to ACPT subcloning. Restriction enzymes were obtained from either Gibco-BRL or New England Biolabs. Each restriction digest was performed in the appropriate buffer using 1-5 units of enzyme per μ g DNA. The samples were incubated for a minimum of 1h at 37°C or at room temperature for overnight. The following constructs were created with full-length ACPT as the insert:

Plasmid	tag	tag location	Restriction Enzymes
pCR2.1	n/a	n/a	n/a
pCEP4	n/a	n/a	KpnI/HindIII
pCEP4	HA	C-terminus	KpnI/HindIII
pGw1	GFP	C-terminus	HindIII/KpnI
pEGFP-N3	GFP	N-terminus	EcoRI/BamHI

To verify the gene insert of the pcDNA₃/ErbB4 construct (JM-a isoform; a gift from Dr. L. Mei), digests using various combinations of restriction enzymes were conducted.

2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to determine the size of restriction fragments, PCR products and for estimating the DNA concentration of samples. Prior to loading, 1/5 volume of 5X loading buffer (10% v/v glycerol, 0.01% w/v xylene cyanol or bromophenol blue) was added to the samples. The samples were then loaded into the wells of a gel composed of 0.6-1.0% w/v agarose dissolved in 1X TAE buffer (50X TAE: 2M Tris, 5.7% w/v glacial acetic acid, 0.1M EDTA, pH~8.5) with 0.1 µg/ml ethidium bromide. Electrophoresis was conducted at 80-120V in 1X TAE buffer. An ultraviolet transilluminator ($\lambda = 300$ nm) was used to visualize the DNA. To determine the sizes of the fragments, a 1kB or 100bp molecular weight marker (New England Biolabs) was run simultaneously with the samples.

2.5 Gene Analysis

The nucleotide and amino acid sequence of the novel acid phosphatase was analyzed with the aid of the following programs: BLAST a basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST/); BCM Search Launcher provided multiple sequence alignment (ClustalW 1.8) and six-frame translation tools

(http://searchlauncher.bcm.tmc.edu/); Boxshade for outputting sequence alignment data with

identical and similar amino acid residues highlighted appropriately

(http://www.ch.embnet.org/software/BOX_form.html); SOSUI for the classification and secondary structure prediction of membrane proteins

(http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html); PSORT II for the prediction of protein sorting signals and localization sites (http://psort.ims.u-tokyo.ac.jp/) and Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html) for predicting the isoelectric point and molecular weight of the protein.

2.6 Expression in Human Tissue

To establish a distribution pattern of ACPT within human tissues a standardized RT-PCR panel was purchased from Clontech. Using primers specific for the amplification of a ~560 bp ACPT fragment (forward primer 5'-CTTTGGATATTGGAGCCCACG-3'; reverse primer 5'-TGTCAGCTGGGGGTAGGGAAG-3') 1 µg of cDNA from various human tissues was subjected to a PCR reaction using an annealing temperature of 59.3°C. The samples were subsequently run on an agarose gel for analysis. The specificity of the primers was verified through the purification and sequencing of the amplified fragment.

2.7 Cell Culture

2.7.1 COS7

COS7 cells were obtained from the American Type Cell Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100U/mL of both penicillin and streptomycin. During this period, the cells were incubated at 37°C in 5% CO₂. The cells were passaged every 3-4 days or when reaching 100% confluency. When passaged, the medium was aspirated and cells washed with pre-warmed (37°C) PBS. Trypsin-EDTA (0.25% trypsin, 1 mM EDTA·4Na in HBSS; GibcoBRL) was added to the COS7 cells and the plate incubated for 3-5 min. at 37°C in 5% CO_2 to completely detach the cells from the plate. Cells were then rinsed off of the plate, collected and plated onto fresh plates at the desired confluency.

2.7.2 Hippocampal Neuronal Cultures

Neuronal cultures were prepared from the hippocampi of E18/E19 rats as described by Craven et al. (Craven et al., 1999). Hippocampi were dissociated by enzyme digestion with papain followed by brief mechanical trituration and incubation at 37°C for 10 min. Samples were centrifuged at 3000 rpm and the supernatant removed. Addition of trypsin inhibitor solution (2.5 mg/ml trypsin inhibitor, 2.5 mg/ml BSA, 0.7 mg/mL DNase I in neurobasal media) to neutralize the papain was followed by brief trituration and

centrifugation. Cells were plated on poly-D-lysine (PDL)-treated glass coverslips and maintained in neurobasal media (Life Technologies, Inc.) supplemented with B27 (Gibco BRL), penicillin, streptomycin, and L-glutamine.

2.7.3 Pheochromocytoma Cells

Rat pheochromocytoma (PC12) cells were obtained from the American Type Culture Collection (Manassas, Virginia). The cells were grown on PDL-coated plates in F-12 nutrient mixture (Gibco) supplemented with antibiotics, 15% heat-inactivated horse serum and 2.5% FBS. Cells were incubated at 37°C in 5% CO₂ and the medium changed every 2-3 days. Upon reaching 80-100% confluency, the cells were passaged. The medium was aspirated and the cells gently washed with pre-warmed (37°C) PBS. The cells were resuspended in 4ml of media and triturated through a 20 and 22 gauge syringe, four times each. This was followed by replating of the cells onto freshly PDL-coated plates at the desired confluency.

2.7.3.1 Differentiation of PC12 Cells

Two methods were used to induce neurite sprouting in PC12 cells. The methods differ only in the ligand and receptor employed. For one method, it was necessary to transfect a construct expressing the full-length coding region for ErbB4 (pcDNA₃/ErbB4) into rat pheochromocytoma cells (PC12). To induce differentiation, cells were plated at a confluency of 5 x 10^5 cells/ml on PDL-coated plates in F-12 nutrient mixture supplemented

with antibiotics and 5% FBS (complete F-12 media) in the presence of 100 ng/ml neuregulin β 1. Medium was replaced with fresh medium containing neuregulin every 2-3 days.

The second ligand used to induce neurite sprouting of PC12 cells was nerve growth factor (NGF). Cells were cultured in the same manner as those treated with NRG, however, NGF was the ligand applied. It was added at a concentration of 50 ng/ml. Images were taken after 4 days of treatment and the neurites measured. Cells were considered elongated if they were at least twice as long as the original diameter of the rounded cells as determined by Northern Eclipse analysis.

2.8 Cell Transfection

2.8.1 COS7

Foreign DNA was introduced into COS7 cells with the aid of the lipid-mediated reagent LipofectAMINE in conjunction with Plus reagent (Invitrogen). Cells were plated 6-12 h prior to transfection. A total of 1 μ g DNA per 35 mm plate was incubated with 6 μ l of Plus reagent and 100 μ l of serum-free DMEM at room temperature for 15 min. This was followed by the addition of a mixture composed of 4 μ l of LipofectAMINE in 100 μ l of serum-free DMEM. The sample was mixed and incubated at room temperature for an additional 15 min. During the incubation, media was aspirated from the COS7 cells and replaced with 800 μ l of serum-free media. The LipofectAMINE/DNA cocktail was added directly to these cells. The cells were incubated in 5% CO₂ and 37°C for 6-12h. Following

the incubation, the transfection medium was replaced with DMEM + 10% FBS and the cells incubated in 5% CO₂ and 37°C for 24h.

2.8.2 PC12 Cells

Prior to transfection, PC12 cells were plated onto coverslips in 24-well, PDL-coated plates (1.25×10^4 cells per well) in growth medium. Plasmids were transfected with the aid of LipofectAMINE and Plus reagent (Invitrogen). A total of 0.25 µg DNA per well was incubated with 1.5 µl of Plus reagent and 25 µl of serum-free F-12 at room temperature for 15 min. This was followed by the addition of a mixture composed of 1 µl of lipofectAMINE in 25 µl of serum-free F-12. The sample was mixed and incubated at room temperature for an additional 15 min. During the incubation, media was aspirated from the PC12 cells and replaced with 200 µl of serum-free media. The lipofectAMINE/DNA cocktail was added directly to these cells. The cells were incubated in 5% CO₂ and 37°C for 6-12h. Following the incubation, the transfection medium was replaced with F-12 nutrient mixture supplemented with antibiotics, 15% heat-inactivated horse serum and 2.5% FBS.

2.9 Antibodies

Antibodies employed for immunoblotting included rabbit polyclonal to the carboxy terminus of ErbB4 (Santa Cruz), mouse monoclonal anti-green fluorescent protein (GFP) (Clontech), mouse monoclonal anti-phosphotyrosine (Upstate Biotechnology), rabbit

polyclonal anti-synaptophysin (Zymed), mouse monoclonal anti-NR1 (Synaptic Systems), rabbit polyclonal anti-actin and rabbit polyclonal antiserum generated to ACPT by the immunization with the peptide LSPEYRREEVYIRC (Affinity Bioreagents Inc.). Prior to use, anti-ACPT was affinity purified using a SulfoLink kit (Pierce).

Immunostaining required the use of the rabbit polyclonal ACPT antiserum, mouse monoclonal anti-GFP, mouse monoclonal HA (Sigma), mouse monoclonal anti-ErbB4 (Neomarkers), mouse monoclonal anti-PSD-95 (Affinity Bioreagents) and mouse polyclonal synaptophysin (Sigma).

2.10 ACPT Antibody Characterization

2.10.1 Immunocytochemistry

A custom made antibody was used to detect endogenous ACPT in Western blot assays and immunostaining. The antibody was generated against a peptide sequence located near the N-terminus of the enzyme by immunizing rabbits with a peptide of the following sequence: LSPEYRREEVYIRC. The peptide was chosen due to the high degree of specificity towards ACPT as well as the strong presence of hydrophilic residues. These residues are less likely to be contained within the hydrophobic cell membrane making this region more accessible to the antibody. This resulted in the production of a polyclonal antiserum to ACPT. The antibody was purified using an affinity purification column (Pierce Technology) to reduce non-specific binding of additional elements found within the crude serum. Specificity of the ACPT antibody was determined using immunocytochemistry.

COS7 cells were transfected with HA-tagged ACPT. The transfected cells were incubated with anti-HA or a pre-determined dilution of anti-ACPT or with preabsorbed ACPT antibody which served as a negative control. The ACPT antibody was preabsorbed with 100 μ g/ml of the peptide used to generate the antibody dissolved in PBS-T with 0.2% sodium azide and incubated at room temperature for 2 h. The immunocychemical staining procedure was conducted as described in section 2.11.

2.10.2 Western Blot

The specificity of the anti-ACPT antisera was analyzed by immunoblotting. COS7 cell lysate expressing either ACPT or GFP-tagged ACPT was run on an 8.5% SDS-PAGE and immunoblotted with the antisera, a GFP antibody or peptide blocked antisera. Analysis was accomplished using ECL. See section 2.13.5 for a detailed description of the immunoblotting protocol.

2.11 Immunocytochemistry

Coverslips were removed from culture wells and fixed in 4% paraformaldehyde or 100% ice-cold methanol for 10 min. The cells were washed with phosphate-buffered saline containing 0.1% Triton-X-100 (PBST) three times. The appropriate primary antibody was diluted in blocking solution (PBST + 3% normal goat serum), added to the cells and incubated with shaking for 1 hr at room temperature. This was followed by three 1 min.

washes in PBS-T and a 1h, room temperature incubation with either donkey anti-mouse or donkey anti-rabbit antibodies conjugated to Cy3 or Alexa680 fluorophores diluted in blocking solution. The cells were then washed three times for 1 min. in PBST. Coverslips were mounted on slides with Fluoromount-G (Southern Biotechnology Associates), and images were taken under fluorescence microscopy with a 40x or 63x objective affixed to a Zeiss microscope.

2.12 Immunohistochemistry

Tissue for immunohistochemical staining was prepared according to Vincent et al. (Vincent et al., 1982). Adult rats were anesthetized and perfused with 4% PFA in 0.1 M PBS, pH 7.4. The brains were removed and immersed in the same fixative at 4°C for 4-18 h and then transferred to 5% sucrose in 0.1 M phosphate buffer at 4°C for 24 h. Tissue was frozen with CO2 and serial sections of the brain were cut at a thickness of 20-25 μm on a cryostat.

Samples were immunohistochemically stained using an immunoperoxidase-based procedure as follows: sections were incubated for 24 h at 4°C in affinity purified ACPT antibody diluted to 5 μ g/ml in PBS-T (PBS containing 0.3% tween) and 2% normal goat serum. Following 3 x 15 min. rinsing in PBS-T, samples were incubated with biotinylated rabbit anti-sheep secondary antibody (Vector Laboratories) diluted 1:1000 in PBS-T containing 2% NGS for 1 h. Sections were rinsed a second time in PBS-T for 3 x 15 min. followed by a 1 h room temperature incubation in VECTASTAIN Elite ABC reagent (Vector Laboratories) diluted in PBS-T. This resulted in the

formation of an avidin-biotinylated horseradish peroxidase macromolecular complex. Following a series of rinses in PBS-T, the immunoreactivity was performed using a peroxidase substrate solution (50mM Tris-HCl, pH 7.6, 100mg/ml nickel ammonium sulfate, 50mM imidazole, 10mg/ml diaminobenzidine plus nickel ammonium sulfite as the chromogen, $1.2\% \text{ v/v H}_2\text{O}_2$). The samples were incubated for 10-15 min. in this solution to develop the stain. After staining, the sections were mounted and dehydrated in graded alcohols, cleared in xylene and coverslipped. Phase and brightfield optics were used to examine the samples.

2.13 Co-Immunoprecipitation and Immunoblotting

2.13.1 Preparation of Brain Homogenates

Rats were anaesthetized and decapitated and their brains dissected into various anatomical regions. The tissue was homogenized in 10w/v ice-cold homogenizing buffer (320 mM sucrose, 4 mM Hepes, pH 7.4, 1 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride). Centrifuging for 10 min. at 1000 x g cleared the supernatant of large cell fragments and nuclei. The protein concentration of the supernatant was determined using the BCA protein assay (Pierce) and 30 µg of each sample was analyzed by immunoblotting.

2.13.2 Preparation of Sepharose Beads

An appropriate amount of protein A-Sepharose CL4B or protein G sepharose 4 Fast Flow was weighed out and resuspended in 10 mL of 100 mM Tris-HCl (pH 7.4) and rotated for 15 min. at 4°C. Bovine serum albumin (2X crystallized) was added to 2 mg/ml and the beads rotated for 30 min. at 4°C. To pellet the beads, the samples were centrifuged at 1000 rpm for 5 min. The supernatant was removed and the pellet resuspended in 10 mL of 100 mM Tris-HCl (Ph 7.4). Rotation for 10 min. at 4°C was followed by centrifugation at 1000 rpm for 5 min. The supernatant was removed and the beads resuspended in the appropriate 1X immunoprecipitation buffer (refer to immunoprecipitation or co-immunoprecipitation protocols. An additional cycle of rotation for 10 min. at 4°C and centrifugation at 1000 rpm for 5 min. followed. After removing the supernatant, 1 volume of 1X immunoprecipitation buffer was added to the sepharose beads to make a final 1:1 suspension.

2.13.3 Cell Lysate and Immunoprecipitation

Cells were washed with ice-cold PBS and harvested in 0.5 mL of lysis buffer containing 20 mM Hepes, pH 7.0, 0.5% deoxycholic acid, 0.1% NP-40, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 0.1 mM ZnCl₂, 2mM sodium orthovanadate and 0.25 mM phenylmethylsulphonylfluoride. After extracting for 20 min at 4°C, insoluble material was removed by centrifugation at 10,000xg for 10 min. For immunoprecipitation experiments, the samples were then incubated with 2µg of ErbB4 antibody for 1 hr at 4°C. After addition of 30 µl of Protein A-Sepharose beads (Pharmacia), samples were incubated for 1 hr at 4°C. The beads were then washed three times with lysis buffer to removed non-binding material and boiled in 2X SDS-PAGE sample buffer with 1 mM DTT for 3 min. in preparation for Western blot analysis.

2.13.4 Co-Immunoprecipitation From Brain

Pregnant rats were anaesthetized with halothane, decapitated and embryos (E18/19) removed. The brains were removed and placed in ice-cold HBSS until they were weighed. Brains were homogenized in 10 w/v of IP buffer (20 mM Hepes, pH 7.0, 0.5% deoxycholic acid, 0.1% NP-40, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 0.1 mM ZnCl₂, 2mM sodium orthovanadate and 0.25 mM phenylmethylsulphonylfluoride) and incubated for 20 min at 4°C. Insoluble material and cellular debris was removed by centrifugation at 800 x g for 10 min. Using a BCA protein assay, the protein concentration of the supernatants was determined and 400 µg aliquoted into fresh microfuge tubes. These samples were diluted 4X in IP buffer and 25 µl of protein G sepharose 4 Fast Flow (Amersham Pharmacia Biotech) added. Incubation with gentle mixing at 4°C for 30 min. ensued. Microfuge tubes were centrifuged at 5000 rpm for 1 min and the supernatant removed to fresh microfuge tubes. ErbB4 mouse monoclonal antibody or IgG was added to the supernatant at 2 μ g antibody/mg protein. The samples were left on ice for 1 h. Addition of 30 µl of protein G sepharose 4 Fast Flow was followed by an incubation on the nutator at 4°C for 1 h. Three sequential washes with IP buffer removed non-binding material. The beads were resuspended in 70 µl

of 2 X sample buffer (1X Tris, pH 6.8, 20% glycerol, 140 mM SDS, 2% 2-mercaptoethanol, 1% w/v bromophenol blue) and boiled for 3 min. prior to SDS-PAGE analysis.

2.13.5 Immunoblotting

After boiling, the samples were electrophoretically separated on 8.0% SDS-PAGE gels and transferred at 150V for 1h to nitrocellulose membranes (Amersham) for western blotting. Membranes were blocked for 1h in TBS-T buffer (0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) containing 5% milk powder and blotted with the appropriate primary antibody for 1h and washed with TBS-T + 0.5% milk powder for 10 min., twice. A 1h incubation with a horseradish peroxidase-linked secondary antibody followed. The membranes were then washed three times in TBS-T + 0.5% milk powder for 10 min. and twice for 10 min. in TBS. The bound antibody was detected with enhanced chemiluminescence reagent ECL (Amersham) according to the manufacturer's protocol and Bio-Max film (Kodak).

2.13.6 Stripping Nitrocellulose Membranes

Nitrocellulose was submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl, pH 6.7) and incubated at 55°C for 30 min. with gentle agitation. The membrane was subsequently washed 2 x 10 min. in TBS-T at room temperature using generous amounts of the wash buffer. Blocking was accomplished by

incubating the membrane in 5% blocking solution (5% w/v milk in TBS-T) for 1h at room temperature and the western blotting protocol was followed for the remainder of the method.

2.14 Subcellular Fractionation

Subcellular fractions of 6 whole brains from 150-200 g rats were prepared as described by Huttner et al. (Huttner et al., 1983). All steps were performed on ice. Brains were homogenized with 9 strokes of a Dounce homogenizer at 900 rpm in 50 ml of homogenizing buffer (320 mM sucrose, 4 mM Hepes, pH 7.4, 1 mM EGTA, 1mg/ml pepstatin A, 200 mM pmsf). Centrifugation for 10 min. at 1000 x g removed large debris and nuclei (P1). The supernatant (S1) was centrifuged at 12 000 x g to obtain the S2 fraction containing small cell fragments. The pellet was resuspended in homogenizing buffer and centrifuged for 15 min. at 13 000 x g resulting in a supernatant (S2') of small compartments and a pellet of crude synaptosomal membranes. Homogenizing buffer was used to resuspend the pellet, followed by 9 volumes of ice-cold water. The sample was then homogenized at a very slow speed, followed by hypo-osmotic lysis of the pellet by the addition of 1 M Hepes-NaOH (pH 7.4). This sample was centrifuged for 20 min. at 33 000 x g to yield heavy membranes (LP1) and a supernatant which additionally centrifuged for 2 h at 251 000 x g. This resulted in a supernatant of presynaptic cytosol (LS2) and a pellet (LP2) of synaptic vesicles which was resuspended in 40 mM sucrose. The protein concentration of the sample was determined using the BCA protein assay and analyzed by SDS-PAGE and immunoblotting.

2.15 Topology and Orientation

The orientation of ACPT within the cell membrane was examined in a heterologous system. COS7 were transfected with full-length ACPT tagged with GFP at the C-terminus or a construct expressing the NR2B subunit of the NMDA receptor, tagged at its N-terminus with GFP (courtesy of Dr. L.A. Raymond). This construct was to serve as a positive control as its orientation within the cell membrane is known to result in the GFP-tag being extracellularly expressed. Following cell fixation on ice with 4% PFA for 10 min., the samples were washed with one of two solutions, PBS-T or PBS. To determine if the N-terminus of the enzyme was extracellular and the C-terminus intracellular, cells washed with PBS-T and PBS were incubated with a GFP or ACPT primary antibody.

2.16 Enzyme Deglycosylation

COS7 cells transiently expressing ACPT or the glutamate receptor subunit GluR6 (a gift from Dr. L.A. Raymond) were harvested in lysis buffer and denatured in 1X denaturing buffer (10X = 5% SDS, 10% β -mercaptoethanol) by boiling for 10 min. Addition of 10% volume of each 10X G7 buffer (0.5M sodium phosphate, pH 7.5) and 10% NP-40 as well as 1U of N-glycosidase F (PNGase F; New England Biolabs) was followed by a 3h incubation at 37°C. Samples were then analyzed by western blotting. GluR6 is a protein known to be N-linked glycosylated and served as the positive control.

2.17 ErbB4 Activation

To induce receptor activity, cells were treated with the ErbB4 ligand neuregulin β 1 (NRG β 1). Prior to ligand exposure, the cells were incubated in serum-free media (DMEM) for 90min. in 5% CO₂ at 37°C. This was followed by the direct addition of 100 ng/ml NRG β 1 to the media. A brief 5 min. incubation in 5% CO₂ at 37°C ensued. Cells were quickly washed in room temperature PBS and harvested in preparation for further analysis.

2.18 Inhibition of Tyrosine Phosphatase Activity

Inhibition of tyrosine phosphatase activity was accomplished by pervanadate treatment (Vecchi et al., 1998). Pervanadate, a tyrosine phosphatase inhibitor was prepared no more than 30 min. before use. Prior to exposure, cells were incubated in serum-free media (DMEM) for 90min. in 5% CO₂ at 37°C. This was followed by the direct addition of 100μ M pervanadate (1M vanadate, 1M H₂O₂ mixed to a final concentration of 0.5M) to the media. Incubation of the cells in 5% CO₂ at 37°C lasted for 30min. Cells were then quickly washed in room temperture PBS and harvested in preparation for further analysis.

2.19 Statistical Analysis

Statistical analysis was performed using Origin 6.1 software (OriginLab Corporation). Data was subjected to the ANOVA test for one-way analysis of variance, followed by determination of significant difference based on $P \le 0.05$. Data was presented as the mean \pm the standard error of mean of the trials.

CHAPTER III

Results

3.1 Cloning of a cDNA Encoding for Human ACPT

Searching human EST databases with regions of acid phosphatase homology, in particular the conserved catalytic domains, and aligning the overlapping EST sequences lead to the derivation of a novel family member. EST-based primers resulted in the PCR amplification of a 1278 bp full-length coding sequence of predicted molecular mass of 43.1 kDa from human testis cDNA. While this work was underway, the human form of a novel acid phosphatase, named testicular acid phosphatase (ACPT) was cloned by Yousef et al. (Yousef et al., 2001). The two sequences were identical. The classification of ACPT as a histidine acid phosphatase is based on the presence of the two catalytic motifs that are generally used to classify members of this family, RHGxRxP near the N-terminal and HD close to the C-terminal (Van Etten et al., 1991). ACPT displays ~44% identity and greater than 60% similarity to human prostatic acid phosphatase (PAP). Analytical programs predict that ACPT has a signal sequence at its N-terminus, a transmembrane domain near the Cterminus and four potential N-linked glycosylation sites (fig. 3-1). Although a transmembrane domain is not present in the original PAP clone, a more recently identified precursor protein of PAP possesses a similar putative transmembrane domain.

АСРТ рАР	1	MAGLGFWGHPAGPLLLLLLVLPPRALPEGPLVFVALVFRHGDRAPLASYPMDPHKE MRAAPLLLARAASLSLGFLFLLFFWLDRSVLAKELKFVTLVFRHGDRSPIDTFPTDPIKE
АСРТ	58	VASTLWPRGLGQLTTEGVRQQLELGRFLRSRYEAFLSPEYRREEVYIRSTDFDRTLESAQ
рАР	61	SSWPQGFGQLTQLGMEQHYELGEYIRKRYRKFLNESYKHEQVYIRSTDVDRTLMSAM
ACPT	118	ANLAGLFPEAAPGSPEARWRPIPVHTVPVAEDKLLRFPMRSCPRYHELLREATEAAEY
pAP	118	TNLAALFPPEGVSIWNPILLWQPIPVHTVPLSEDQLLYLPFRNCPRFQELESETLKSEEF
ACPT	176	QEALEGWTGFLSRLENFTGLSLVGEPLRRAW-KVLDTLMCQQAHGLPLPAWASPDVLRTL
pAP	178	QKRLHPYKDFIATLGKLSGLHGQDLFGIWSKVYDPLYCESVHNFTLPSWATEDTMTKL
АСРТ	235	AQISALDIGAHVGPPRAAEKAQLTGGILLNAILANFSRVQRLGLPLKMVMYSAHDSTLLA
рАР	236	RELSELSLLSLYGIHKQKEKSRLQGGVLVNEILNHMKRATQIPSYKKLIMYSAHDTTVSG
АСРТ	295	LQGALGLYDGHTPPYAACLGFEFRKHLGNPAKDGGNVTVSLFYRNDSAHLPLPLSLPGCP
рАР	296	LQMALDVYNGLLPPYASCHLTELYFEKGEYFVEMYYRNETQHEPYPLMLPGCS
АСРТ	355	APCPLGRFYQLTAPARPPAHGVSCHGPYEAAIPPAPWVPLLAGAWAVLVALSLGLGLF
рАР	349	PSCPLERFAELVGPVIPQDWSTECMTTNSHQVLKVIFAWAFCLISAWLMVLLFIHIRRGL
АСРТ	413	WRPGCLRALGGPV
рАР	409	CWQRESYGNI

Figure 3-1. Sequence Alignment. Nucleotide sequence alignment of the novel histidine acid phosphatase (ACPT) and the novel, transmembrane domain-containing prostatic acid phosphatase (pAP). Each enzyme has two catalytic domains (outlined in black), a signal sequence (yellow), putative transmembrane domain (green) and N-linked glycosylation sites (underline).

3.2 Antibody Characterization

3.2.1 Immunocytochemistry

Experiments designed to characterize and study the functional aspects of proteins often require tools such as antibodies. To aid with experiments involving ACPT, enzymespecific anti-sera was generated by immunizing rabbits with a peptide sequence corresponding to the amino acids LSPEYRREEVYIRC located near the N-terminus.

To determine the specificity of the antibody generated, COS7 cells were transfected with an ACPT construct that was HA-tagged at the C-terminus, fixed and immunostained using an antibody generated to an N-terminal amino acid sequence specific to the enzyme. Immunocytochemistry revealed a complete overlap in anti-HA and anti-ACPT staining in cells expressing the HA-tagged ACPT (fig. 3-2A). ACPT did not appear to be localized within the nucleus, rather its expression was concentrated within the cytoplasm in a pattern that could indicate localization within the Golgi apparatus and in some instances, the endoplasmic reticulum. Surrounding non-transfected cells which were defined by a lack of HA immunoreactivity, also failed to immunostain for the phosphatase. This suggests that the generated antibody is able to specifically detect ACPT to the exclusion of other proteins expressed by the COS7 cells, including lysosomal acid phosphatase.

COS7 cells transfected in parallel with the above experiments were fixed and immunostained with peptide-blocked antisera to serve as a negative control (Fig. 3-2B). Treatment of antisera with the immunizing peptide results in the saturation of all available antibody binding sites, preventing anti-ACPT immunoreactivity when subsequently applied to transiently expressing COS7 cells. These samples failed to detect any protein, indicating that the detection of ACPT was blocked by the peptide used to generate the antibody. These results support the initial antibody characterization data, indicating that the generated antibody selectively detects ACPT protein and that the enzyme is not endogenously expressed by COS7 cells.



Figure 3-2. Antisera Specifically Immunostains for ACPT. COS7 cells transfected with HA-ACPT and subsequently immunostained with A) anti-ACPT or anti-HA and B) anti-HA or pre-absorbed anti-ACPT. Photomicrographs are representations of results from n=3 different experiments. Scale bar = $10 \mu m$

3.2.2 Western Blotting

The specificity of the anti-ACPT antisera was additionally verified by immunoblotting (fig. 3-3). Cell lysates were harvested from COS7 cells transfected with ACPT and ACPT-GFP. Immunoblotting of these samples with the ACPT antibody revealed a band which corresponded to the expected size of the protein as well as multiple bands indicating the presence of proteins of higher molecular weights. Anti-ACPT revealed proteins of ~43 kDa, the predicted size for the full-length enzyme, and a larger protein of ~65 kDa, due to the addition of the GFP tag. Stripping of the nitrocellulose, followed by immunoblotting with anti-GFP detected the ~65 kDa protein only in the ACPT-GFP containing lane. Anti-GFP failed to detect any bands in the lane containing ACPT protein. Additionally, the detection of ACPT protein was blocked by the peptide used to generate the antibody. These results indicate that the generated ACPT antisera specifically detects ACPT protein and that this protein either undergoes post-translational modifications or has alternative isoforms.



Figure 3-3. Antisera Specifically Detects ACPT Protein. Cell lysate from ACPT or ACPT-GFP transfected cells (indicated above blot) were harvested and immunoblotted with anti-ACPT, +/- immunizing peptide (indicated below blots) (A). The blots were subsequently stripped and re-blotted with anti-GFP (B). Results were replicated in n=3 different experiments.

3.3 Tissue Distribution of Human ACPT cDNA

3.3.1 Human Multiple Tissue cDNA Panel

A human multiple tissue cDNA panel standardized for mRNA levels was screened with ACPT-specific primers. Screening of this panel revealed a restricted pattern of ACPT expression with varying levels amongst the different tissues (fig. 3-4). The enzyme was found to be highly expressed in brain, heart, liver, testis, ovary and small intestine. Lower levels of expression were also detected in skeletal muscle, spleen, prostate and leukocytes. It was not detected in lung, kidney, pancreas, thymus and colon tissues.



Figure 3-4. Distribution of ACPT cDNA in Human Tissue. RT-PCR of multiple cDNAs from human tissues using ACPT-specific primers. The source of the cDNA was a human multiple tissue panel (Clontech) which is standardized with several housekeeping genes, including G3PDH. Results replicated in n=3 different experiments.

3.3.2 Brain Region Distribution

Regional distribution of the ACPT protein was established by immunoblotting dissected rat brain tissue with anti-ACPT. Protein was loaded in equal quantity (40µg per lane) as determined by a BCA protein assay and confirmed by actin immunodetection. Western blot analysis revealed the presence of ACPT protein in the cerebellum, cortex, hippocampus, hypothalamus and striatum.

In the cortex, striatum and hippocampus, the ACPT antibody detected a doublet, with bands of ~43 kDa and ~46 kDa, with the lower band corresponding to the predicted molecular weight (fig. 3-5). In contrast, the cerebellum and hypothalamus extracts only contained a single band that corresponds to the higher molecular weight product. This product was observed previously when immunoblotting COS7 cells transfected with ACPT (refer to fig. 3-3). The shift in molecular weight may be the result of post-translational modifications or the expression of an alternative isoform.



Figure 3-5. ACPT Protein Expression Within the Brain. Adult rat brain was dissected into various gross regions, homogenized and analyzed by Western blot. The blot was probed with anti-ACPT. Even protein loading amongst the lanes was controlled by monitoring actin expression. Representative blot from n=4 different experiments.

3.3.3 Immunohistochemistry

Localization of ACPT within the brain was immunohistochemically determined using an ACPT antibody and an immunoperoxidase technique. Neuronal cells in various brain regions stained positively for ACPT. As seen in figure 3-6 (middle panel), immunostaining for ACPT was evident in pyramidal cells of the cerebral cortex. Staining was observed within their cell bodies as well as along their axons which can be observed to extend into the subcortical region. In the cerebellum (fig. 3-6, bottom panel), glutamatergic granule cells within the granule cell layer stained for ACPT protein. In the molecular layer, where interneurons are immunoreactive, staining appears less abundant than in the granule layer. A negative control using anti-ACPT preabsorbed with the peptide antigen displayed a lack of cellular staining (fig. 3-6, top panel).



Figure 3-6. ACPT Localization in Rat Brain Slices. Immunohistochemical staining of rat brain slices using the DAB technique. Preabsorbed ACPT staining of the cerebral cortex (top panel) and ACPT staining of the cerebral (middle panel) and cerebellar (bottom panel) cortices. Scale bar = 50μ m

3.4 Orientation of ACPT Within the Cell Membrane

The orientation in which ACPT is inserted into the cell membrane is of importance because the catalytic domain and putative transmembrane domain are located at opposite ends of the protein. If the catalytic domain is extracellularly located it is not in an orientation favourable for interaction with most cellular proteins. Alternatively, reversed orientation of ACPT, although making potential substrates more accessible, implies a lack of posttranslational modifications, such as glycosylation, which are displayed by both PAP and LAP (Pohlmann et al., 1988; Sharief and Li, 1992). Therefore, the topology of ACPT was explored.

To accomplish this task, an immunocytochemical approach with the mentioned modifications was used. It was necessary to have available a means by which to target or mark each end of the protein. Antibodies directed to the C-terminus (GFP tag) and the N-terminus (anti-ACPT) of the enzyme were used for the determination of the orientation of ACPT within the cell membrane (fig. 3-7). The presence of triton X-100 in PBS-T (blocking solution) permeabilized the cell membrane allowing the primary antibody to access any proteins expressed within the confines of the cell membrane. Alternatively, washing with PBS in the absence of triton X-100 left the cell membrane unpermeabilized thereby limiting the primary antibody to detecting proteins expressed on the cell surface.

When using the N-terminally directed antibody (anti-ACPT), immunostaining was only evident in permeabilized cells. Non-permeabilized cells failed to display any immunofluorescence. This suggests that the N-terminus of ACPT is located intracellularly (fig. 3-8). To test for a reversed orientation within the membrane, permeabilized and
nonpermeabilized cells were subjected to immunocytochemistry with anti-GFP to detect the C-terminal GFP tag of ACPT (fig. 3-9). GFP immunostaining was observed in permeabilized cells. The nonpermeabilized cells from these samples failed to immunofluoresce, suggesting that the C-terminus is also intracellularly expressed. The GFP control for both sample sets fluoresced green in the presence or absence of triton X-100 in the blocking solution, indicating that the transfection was not at fault.

However, some primary antibodies might not detect the antigen in the absence of mild detergent due to inaccessibility of the epitope when treated under non-detergent conditions. To control for this possibility, a NR2B construct (courtesy of the Raymond lab), known to express an extracellular GFP-tag was transfected into COS7 cells and immunostained in parallel with ACPT-GFP. The results from this experiment were similar to those of the previous by displaying a reduction in GFP immunodetection in the absence of detergent, thereby suggesting the need for triton X-100 in the blocking solution for epitope binding to be successful. Nevertheless, as can be seen in fig. 3-9, the lack of triton X-100 did not result in the complete blockage of antibody binding. Immunocytochemistry of neighbouring transfected cells revealed a mix of immunostained and non-immunostained, GFP-expressing cells. Since in the absence of detergent, some GFP-NR2B transfected cells were immunostained, whereas none of the ACPT-GFP transfected cells were immunostained, these results suggest that ACPT either has the conformation of a hairpin loop so that both termini are located intracellularly or that ACPT is not located at the surface of the cell membrane. However, due to the inconsistencies observed, the location of the phosphatase catalytic domain and ACPT's orientation within the membrane are still unclear.



Figure 3-7. Possible Orientations of ACPT at the Cell Surface. A schematic of the possible orientations of ACPT following insertion into the membrane. The N-terminus can be specifically targeted by anti-ACPT (yellow) and the GFP tag is located at the C-terminus (green).



Figure 3-8. Topological Arrangement of ACPT - Is the N-terminus Extracellular? COS7 cells expressing full-length, C-terminally GFP-tagged ACPT were immunocytochemically stained for the N-terminus in the presence (permeabilized) or absence (non-permeabilized) of triton X-100. Results are representative of n=4 different experiments. Scale bar = $10\mu m$



Figure 3-9. Topological Arrangement of ACPT - Is the C-terminus Extracellular? COS7 cells expressing full-length, C-terminally GFP-tagged ACPT or N-terminally GFP-tagged NR2B were immunocytochemically stained for the GFP tag in the presence (permeabilized) or absence (non-permeabilized) of triton X-100. Results are representative of n=4 different experiments. Scale bar = $10\mu m$

3.5 Post-Translation Modifications of ACPT

Following translation, many proteins are subjected to post-translational modifications such as phophorylation and glycosylation. Such alterations can often result in a protein being of higher molecular weight than that predicted. Earlier experiments involving immunoblotting for ACPT detected a protein doublet. Although this could indicate that there are multiple isoforms of ACPT arising from RNA splicing, it could also suggest that the enzyme undergoes post-translation modifications. Computer analysis of ACPT structure predicted four potential N-glycosylation sites and characterization of the known histidine acid phosphatases has revealed the presence of such post-translational modifications. Lysosomal acid phosphatase is heavily glycosylated with the presence of 9 N-linked glycosylation sites. In contrast, PAP only has 3 such sites. Therefore, the potential glycosylation of ACPT was examined.

Treating cell extracts from COS7 cells transfected with full-length ACPT with Nglycosidase F (PNGase F), which cleaves oligosaccharides from N-linked glycoproteins, resulted is the disappearance of the higher molecular weight band (fig. 3-10). In contrast, the lower molecular weight band did not shift following PNGase F treatment. The identical treatment was applied to cell lysate obtained from COS7 cells transfected with the glutamate receptor subunit GluR6, and the extract was run in parallel to serve as a positive control. The glutamate receptor subunit is known to be N-glycosylated (Roche et al., 1994) and treatment with PNGase F yielded the expected shift. These data therefore suggest that both glycosylated and non-glycosylated forms of ACPT exist, which may account for the appearance of two bands in the immunoblots as seen in figure. 3-5.



Figure 3-10. Post-translational Modifications of ACPT. Cell lysate from COS7 cells expressing the full-length ACPT protein or the glutamate receptor subunit GluR6 was treated with (+) or without (-) PNGaseF. GluR6 is known to be glycosylated served as a positive control. Samples were immunoblotted with anti-ACPT or anti-GluR6 as appropriate. Representative blot from n=4 different experiments.

3.6 Subcellular Distribution

3.6.1 Subcellular Fractionation

To determine the subcellular localization of ACPT, western blot analysis of a rat brain subcellular fractionation was conducted. Immunoblotting with NR1, a subunit of the postsynaptic NMDA receptor protein, and synaptophysin, a presynaptic vesicle-associated protein, confirmed the quality and success of the fractionation. Further analysis with anti-ACPT indicated that ACPT is mainly enriched in the postsynaptic density containing (PSD) fraction (fig. 3-11, LP1). In contrast, the enzyme was completely absent from the synaptic vesicle-enriched fraction (LP2). The enrichment of ACPT in the synaptic membranes corresponds well with ErbB4 expression. The ErbB4 receptor is a component of the postsynaptic density and has been shown to be associated with PSD-95 (Garcia et al., 2000; Huang et al., 2000). Even protein loading amongst the lanes was determined by blotting with anti-actin.



Figure 3-11. Subcellular Localization of ACPT. Subcellular fractination was performed on rat brains. Synaptophysin and NR1 distribution patterns confirm the success of the fractionation. P1 = large cell fragments and nuclei; S2 = supernatant; S2' = small compartments; LP1 = synaptosomal membranes; LS2 = presynaptic cytosol; LP2 = synaptic vesicle enrichment. Even protein loading amongst the lanes was controlled by monitoring actin levels. Results replicated in n=3 different experiments.

3.6.2 Hippocampal Neuron Immunocytochemistry

To localize ACPT expression within neurons, cultured 15 *div* hippocampal neurons were immunostained for endogenous ACPT, PSD-95, synaptophysin and ErbB4. Examination by fluorescence microscopy revealed a pattern of punctate staining for ACPT within the axons and dendrites of the neurons. ACPT was observed to co-localize with the presynaptic marker protein synaptophysin and the post-synaptic marker protein PSD-95 (fig. 3-12). Overlap in expression patterns with the ErbB4 receptor was also observed (fig. 3-13). This data supports the results of the subcellular fractionation and suggests that ACPT subcellular localization within neurons is favourable for supporting a possible interaction with the ErbB4 receptor to allow for the regulation of its phosphorylation state at the synapse.



Figure 3-12. Co-localization of ACPT with Synaptic Proteins. Immunocytochemical staining of 15 div hippocampal cultures. The boxed region in each panel is enlarged in the small panels to the right of the originals. Arrows indicate where ACPT and a synaptic protein co-localize (puncta appear yellow) and the arrowhead indicates where there is no co-localization of the proteins. ACPT (red) appears to have punctate staining that can co-localize with synaptophysin (green, upper panel) or PSD-95 (green, lower panel). The merged images with ACPT and synaptophysin or PSD-95 are in the far right panel. Photomicrographs are representative of results from n=3 different experiments. Scale bar = $20\mu m$



Figure 3-13. ACPT Co-localizes with ErbB4 in Neurons. Immunocytochemical staining of 15 div hippocampal cultures. The boxed region in each panel is enlarged in the small panels to the right of the originals. Arrows indicate where ACPT and erbB4 co-localize (puncta appear yellow) and the arrowhead indicates where there is no co-localization of the two proteins. ACPT (red) appears to have punctate staining that can co-localize with erbB4 (green). The merged images with ACPT and erbB4 are in the right panel. Photomicrographs are representative of results from n=4 different experiments. Scale bar = $20\mu m$

3.7 ACPT and ErbB4 Interact

Since ACPT and PAP share a high degree of homology, it is likely that they not only function using similar mechanisms, but may also utilize similar substrates. It has been shown that the EGF receptor ErbB2 is an endogenous substrate for PAP in human prostatic epithelial cells (Meng and Lin, 1998; Zhang et al., 2001). Another member of the same family of receptor kinases, ErbB4, shares high homology with the ErbB2 kinase domain (Plowman et al., 1993), is expressed in the brain, and unlike ErbB3, has an active tyrosine kinase domain (Kraus et al., 1989; Guy et al., 1994). Moreover, we have shown by immunocytochemistry that ErbB4 co-localizes with ACPT in dendrites of cultured hippocampal neurons (see section 3.6). Together, these data suggested ErbB4 as a potential ACPT substrate.

To determine whether ACPT may serve as a regulator of ErbB4, a direct association of the enzyme and receptor was investigated in a heterologous system. COS7 cells expressing ErbB4, ACPT-GFP or both ErbB4 and ACPT-GFP were subjected to immunoprecipitation with an ErbB4 antibody. Subsequent immunoblotting revealed that ErbB4 could only be precipitated from samples transfected with the receptor (fig. 3-14). Cells overexpressing only ACPT-GFP failed to immunoprecipitate ErbB4 indicating that no non-specific binding was occurring during the procedure. Immunoprobing of the ErbB4 immunoprecipitate from cells transiently expressing ErbB4 and ACPT-GFP with anti-GFP revealed a ~65 kDa band which corresponds to the predicted molecular weight of GFP-tagged acid phosphatase. Co-immunoprecipitation of ACPT with ErbB4 was alternately tested by receptor immunoprecipitation from COS7 cells transfected with ErbB4, ACPT-HA or ErbB4

and ACPT-HA, followed by immunoblotting for a C-terminal HA-tagged ACPT (data not shown). Results mirrored those obtained using ACPT-GFP. This indicates that the enzyme can directly interact with ErbB4 in a heterologous system.

Discovering an interaction between two or more proteins in a heterologous system is not always representative of what occurs in an *in vivo* model. To ascertain the plausibility of a direct interaction of ACPT with ErbB4 in an *in vivo* system, co-immunoprecipitation experiments were conducted on embryonic rat brain homogenate. As seen in figure 3-15, one isoform of endogenous ACPT is pulled down with the receptor when ErbB4 is immunoprecipitated. This interaction is specific since immunoprecipitation with the control IgG did not result in the immunodectection of ACPT.

It can therefore be concluded that ErbB4 and ACPT interact in both a heterologous system and *in vivo*.



Figure 3-14. ACPT Interacts with ErbB4. Prior to harvesting, COS7 cells were transfected with erbB4 only, ACPT-GFP only, or both (as indicated by the label above the blots), incubated with serum-free media for 90 min. and then treated either with (+) or without (-) 100 ng/ml NRG β -1 for 5 min. Lysates were immunoprecipitated with an erbB4 antibody and subsequently analyzed by Western blotting. Immunoprecipitates and crude lysates were probed with anti-GFP to detect GFP-tagged ACPT. Results were replicated in n=5 different experiments.



Figure 3-15. ACPT Interacts with ErbB4 in vivo. Rat brain homogenate (H) was immunoprecipitated using anti-erbB4 (IP) or IgG (IgG). The samples were subsequently Western blot analyzed with anti-erbB4 and anti-ACPT. Results are representative of n=2 different experiments.

3.8 ACPT Acts as a Tyrosine Phosphatase

The interaction demonstrated to occur between the ErbB4 receptor and ACPT suggests that ACPT could act to regulate receptor activity. The means by which this alteration would most likely be achieved would require direct action on the receptor itself. To demonstrate this type of functional interaction between these two proteins, the dephosphorylation of tyrosine residues on ErbB4 by ACPT was explored.

Tyrosine phosphatase activity is the hypothesized function of ACPT since PAP, the acid phosphatase to which it is highly homologous, acts as a tyrosine phosphatase towards its endogenous substrate, ErbB2, to regulate its kinase activity (Lin and Meng, 1996; Meng and Lin, 1998; Meng et al., 2000; Zhang et al., 2001). To explore this possibility, COS7 cells were transfected with ErbB4, ACPT-GFP alone, or ErbB4 together with ACPT-GFP. Prior to cell lysis, half the cells were treated with 100 ng/ml of NRGB-1 for 5 min. In the presence of this ligand, ErbB4 is activated, resulting in increased phosphorylation of tyrosine residues on the cytoplasmic tail of the receptor (Plowman et al., 1993). The cell lysate was then harvested from these cells and subjected to immunoprecipitation with anti-ErbB4. ErbB4 immunodetection indicated that receptor protein only immunoprecipitated in the expected samples, confirming previous findings of no endogenous ErbB4 in COS7 cells. It also served to indicate the relative proteins levels of the receptor in each lane. Additional immunoblotting of the precipitate with an anti-phosphotyrosine antibody confirmed the results of earlier studies that revealed an increase in tyrosine phosphorylation of the receptor upon ligand exposure. When co-expressed with ACPT, this increase in tyrosine

phosphorylation of ErbB4 was blocked (fig. 3-16). A reduction of basal tyrosine phosphorylation was also observed in the presence of ACPT (fig. 3-16).

In summary, the interaction of ACPT with the receptor results in the reduction of both basal and neuregulin-induced levels of tyrosine phosphorylation of ErbB4. These data therefore suggest that ACPT can act as a tyrosine phosphatase to regulate the activity of ErbB4.



Figure 3-16. ACPT Acts as a Tyrosine Phosphatase Towards ErbB4. Prior to harvesting, COS7 cells were incubated with serum-free media for 90 min. and then treated either with (+) or without (-) 100 ng/ml NRG β -1 for 5 min. Lysates were immunoprecipitated with an erbB4 antibody and subsequently analyzed by Western blotting. Immunoprecipitates were probed with antiphosphotyrosine to detect phosphorylated erbB4. Representative blot from n=4 different experiments.

3.9 ErbB4 Cleavage is Regulated by ACPT

The lack of receptor regulation via endocytosis due to the slow internalization of ErbB4 from the cell surface (Baulida et al., 1996; Pinkas-Kramarski et al., 1996) has led researchers to search for other compensatory mechanisms. Recently, it has been shown that ErbB4 is cleaved by the metalloprotease TACE into an ectodomain and a membrane-associated fragment (Vecchi et al., 1998; Rio et al., 2000). This fragment can be further cleaved by γ -secretase to yield a soluble fragment which can translocate to the nucleus to regulate transcription (Ni et al., 2001). Evidence indicates that these cleavage processes are regulated by the phosphorylation state of the receptor (Vecchi et al., 1998). We therefore investigated the role of ACPT in the regulation of ErbB4 receptor cleavage by expressing ErbB4 alone or together with ACPT-GFP in COS7 cells. Using an antibody directed towards the C-terminal region of ErbB4, cell lysates or immunoprecipitates, as indicated in the figure, were analyzed.

Stimulation of the tyrosine kinase receptor by neuregulin increased the amount of the cleaved C-terminal ~80 kDa ErbB4 fragment as shown previously (Vecchi and Carpenter, 1997; Zhou and Carpenter, 2000). This response to activation was prevented or reduced when the receptor was co-expressed with ACPT (fig. 3-17).

If ACPT is exerting its actions via tyrosine phosphatase activity, than pre-treatment of the transfected cells with a tyrosine phosphatase inhibitor should result in a reversal of the blocked cleavage. To investigate this possibility, the cells were treated with the tyrosine phosphatase inhibitor, pervanadate, for 30 min. prior to harvesting. This resulted in a dramatic increase in cleavage in cells overexpressing ErbB4, thereby confirming earlier

reports (Vecchi et al., 1998; Zhou and Carpenter, 2000). However, in the presence of the tyrosine phosphatase inhibitor, ACPT expression was unable to prevent ErbB4 cleavage when the two proteins were expressed together.

It should be noted that pervanadate treatment of ErbB4, with or without the coexpression of ACPT, resulted in a mobility shift of the cleaved fragment from the expected ~80 kDa fragment to ~89 kDa. This is consistent with previously published data (Vecchi et al., 1998; Zhou and Carpenter, 2000), but the observation was not commented on in those studies.



Figure 3-17. Regulation of ErbB4 Cleavage is Phosphotyrosine Dependent. COS7 cells were incubated with serum-free media for 90 min. followed by a 30 min. exposure to PBS or 100 uM pervanadate. Cells were harvested, immunoprecipitated and immunoblotted with anti-erbB4 and/or anti-phosphotyrosine. Representative blot from n=4 different experiments.

3.10 ACPT Dephosphorylation of ErbB4 Regulates PC12 Cell Differentiation

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The crucial role that ErbB4 plays in neuronal development was evident from studies involving loss of function mutations in the gene encoding for the receptor tyrosine kinase (Gassmann et al., 1995; Rio et al., 1997; Golding et al., 2000). Particularly evident were the erroneous migration patterns of hindbrain-derived neural crest cells. To investigate the role that ACPT may play in neuronal development as a result of ErbB4 regulation, differentiation of PC12 cells was examined.

Members of the ErbB receptor family can regulate the differentiation of PC12 cells into "neuronal-like" cells upon activation by their respective ligands. PC12 cells transfected with ErbB4 have been shown to undergo neurite outgrowth in response to neuregulin treatment that is indistinguishable from that mediated by nerve growth factor activation of endogenous Trk A receptors (Vaskovsky et al., 2000). We therefore used this model to examine the functional significance of ACPT regulation of ErbB4. PC12 cells transfected with the ErbB4 receptor were identified immunocytochemically with an ErbB4 specific antibody. As can be seen in figure 3-18A, activation of ErbB4 with neuregulin, promoted dramatic neurite outgrowth in PC12 cells, as previously described (Vaskovsky et al., 2000). Co-transfection with ACPT-GFP completely prevented this neuregulin-induced neurite extension even after 4 days of treatment (fig. 3-18B). In contrast, when PC12 cells were transfected with ACPT-GFP alone and then treated with nerve growth factor, which induces neurite extension via TrkA, the cells were able to differentiate in an identical manner as those cells which were untransfected (fig. 3-18A). This indicates that although ACPT has the

ability to block ErbB4-dependent differentiation, it is not able to block TrkA-induced differentiation.

Quantitative analysis of the cells included measurement of the length of the neurite extensions and counting of the number of neurites per cell. Comparing the average number of neurites grown per cell amongst the sample groups indicated that there is no significant difference in the number of extensions sprouted by NGF treated cells versus ErbB4 + NRG cells (fig. 3-19). The remaining two sample groups failed to grow neurites upon treatment. Measurement of the length of the neurite extensions did not reveal any significant difference in neurite length when comparing neuregulin versus nerve growth factor-induced differentiation (fig. 3-20). This indicates that ErbB4-induced differentiation of PC12 cells emulates TrkA-induced differentiation and that ACPT is not able to block TrkA-induced signaling, but rather selectively regulates the ErbB4 tyrosine kinase pathway. overlayImage: state state

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Figure 3-18. Effect of ACPT Expression on ErbB4-induced Differentiation of PC12 Cells. A. PC12 cells were transfected with erbB4 and treated with 100 ng/ml NRG or transfected with ACPT-GFP and cultured in the presence of 50 ng/ml NGF for 4 days prior to analysis. B. Cells co-transfected with erbB4 and ACPT-GFP and subsequently grown in the presence of 100 ng/ml NRG for 4 days. Immunofluorescent staining with an erbB4 specific antibody (red) or GFP visualization identified the transfected cells. Cells expressing both erbB4 and ACPT appear yellow. Red, green and yellow fluorescent images were overlayed with the bright-field view. Images are representative of results replicated in n=3 different experiments. Scale bar = $20\mu m$



Figure 3-19. ErbB4-induced Differentiation of PC12 Cells is Prevented by ACPT. The average number of neurite extensions per differentiated PC12 cell was quantitated. ErbB4 + 100ng/ml NRG (n=15), ACPT + 50ng/ml NGF (n=13), erbB4 + ACPT + 100ng/ml NRG (n=30), ACPT + 100ng/ml NRG (n=28). Expression of ACPT significantly blocked NRG-mediated neurite outgrowth. ***, P<0.001, treatment vs. control; bars, SD. Results from n=3 different experiments of cultured PC12 cells.



Figure 3-20. ErbB4-induced Differentiation of PC12 Cells is Not TrkA-activity Dependent. The average neurite length of the differentiated PC12 cells was measured. ErbB4 + 100ng/ml NRG (n=15), ACPT + 50ng/ml NGF (n=13), erbB4 + ACPT + 100ng/ml NRG (n=30), ACPT + 100ng/ml NRG (n=28). Expression of ACPT significantly blocked NRGmediated, but not NGF-mediated neurite outgrowth. ***, P<0.001, treatment vs. control; bars, SD. Results from n=3 different experiments of cultured PC12 cells.

CHAPTER IV

Discussion

4.1 Structural Features and Characteristics of ACPT

Amino acid sequence analysis of the newly cloned enzyme from human testis cDNA revealed the presence of a string of amino acids, RHGxRxP, that are used for the classification of histidine acid phosphatases. The presence of an additional catalytic motif, HD, near the C-terminus, indicates that the novel acid phosphatase can be further subclassified as a high molecular weight histidine acid phosphatase (Van Etten et al., 1991). Other members of this family, with which ACPT shares a high degree of homology include prostatic, lysosomal and lysophosphatidic acid phosphatases. The ACPT sequence also indicates the presence of additional structural features that are shared amongst histidine acid phosphatase family members. These features include a signal sequence, conserved cysteine and proline residues, a putative transmembrane domain and multiple potential N-linked glycosylation sites.

The N-terminally located signal sequence is a common feature of all of the histidine acid phosphatases (Pohlmann et al., 1988; Vihko et al., 1988; Sharief et al., 1989; Hiroyama and Takenawa, 1998). This relatively short sequence of mostly hydrophobic amino acids is responsible for directing the protein from the cytosol and importing it into the endoplasmic reticulum, mitochondria, chloroplasts or nucleus. In some instances, this sequence can be used to retain rather than import certain proteins into the endoplasmic reticulum (ER). Each destination is specified by a different type of signal peptide which is defined by its amino

acid composition and its location within the protein.

Proteins destined for initial transfer to the ER usually have amino-terminal signal peptides with a central part of the sequence composed of 5-10 hydrophobic amino acid residues. Most of these proteins pass from the ER to the Golgi apparatus; those with a specific sequence of 4 amino acids (KDEL) at their carboxyl terminus are permanently retained within the lumen of the ER. Mitochondria-destined proteins have signal peptides with positively charged amino acid residues that alternate with hydrophobic ones. Nucleus bound proteins carry signal peptides which are generally internally located and formed from a cluster of 5 basic amino acids or 2 smaller clusters of basic residues separated by ~10 amino acids. This sequence directs the proteins to the membranes without insertion into the ER. In most cases, the signal peptide is cleaved once a protein reaches its final destination. In the case of ACPT, the signal sequence may serve to localize the phosphatase to the endoplasmic reticulum or at synaptic membranes as seen in ACPT-overexpressing cells or subcellular fraction and immunocytochemistry studies.

The conserved cysteine and proline residues within ACPT, implies marked tertiary structural similarities to the three other known histidine acid phosphatases (Peters et al., 1989). The helices, strands and random coils that represent the secondary structure of a protein are arranged in a compact internal scaffold dictated by the hydrophobic interactions between the nonpolar side chains. In histidine acid phosphatases, there are six cysteine and twenty proline residues contributing to these hydrophobic interactions to aid in the formation of the tertiary structure by influencing the arrangement of the secondary structures. The cysteine residues which are positionally conserved can form three intramolecular disulphide bridges essential for stabilizing the tertiary structure of the protein and the highly conserved

proline residues influence tertiary structure by their strong helix breaking potential (Peters et al., 1989). Conservation of these particular residues indicates that the tertiary structure of PAP, LAP, LyAP and ACPT is similar.

In contrast to some of the common characteristic features, not all of the members of the acid phosphatase family have a transmembrane domain at the C-terminus. Although it has been long known that lysosomal acid phosphatase has a transmembrane domain (Pohlmann et al., 1988), it was not until recently that such a domain has been suggested to exist in PAP. No studies regarding this putative transmembrane domain have been conducted and any previous suggestions of the existence of this isoform have been largely ignored. In contrast, the presence of a transmembrane domain in lysosomal acid phosphatase has been well established. It has been demonstrated in baby hamster and canine kidney cells, that lysosomal acid phosphatase (LAP) is synthesized and transported to lysosomes as a transmembrane protein (Waheed et al., 1988; Prill et al., 1993).

The transmembrane domain is the structure that forces LAP to insert into the lysosomal membrane in a manner that orients the N-terminus into the lumen (Waheed et al., 1988). This results in the catalytic domains and glycosylated regions to be oriented within the acidic luminal environment. Once LAP is transported to the lysosomal membrane, it is rendered soluble when it is released into the lumen by proteolytic cleavage, thereby removing the C-terminal of the peptide, including the transmembrane domain and the cytosolic tail of 18 amino acids (Waheed et al., 1988).

If ACPT was to follow a similar orientation due to the presence of the putative Cterminal transmembrane domain, the catalytic sites of the acid phosphatase would not be located within the cytoplasmic space. This suggested orientation is supported by the presence

of potential N-linked glycosylation sites. However, such an orientation would make it impossible for ACPT to interact with the ErbB4 receptor, as its tyrosine kinase domain is within the intracellular space and therefore on the opposite side of the cell membrane from the enzyme. Additionally, if ACPT was to undergo proteolytic cleavage in this orientation, the catalytic domain would be released into the extracellular space, to float away from other potential membrane-anchored substrates if it were inserted into the cell surface membrane. If the phosphatase were inserted into an organelle membrane like LAP, cleavage of the transmembrane domain would release the amino terminal into the lumen of the organelle.

Immunohistochemical studies using antibodies directed towards the N- and C-termini were conducted to discern the orientation of ACPT at the cell membrane and did little to clarify the question at hand. Although it was shown that anti-ACPT (raised against an Nterminal epitope) could not bind to the epitope in the absence of detergent, it cannot be conclusively stated that this is the result of the sequence being cytoplasmically located and the membrane unpermeabilized, since identical results were obtained when immunostaining for the C-terminal with anti-GFP. The binding of an antibody to the appropriate epitope can occasionally be hindered in the absence of various chemicals such as detergents. This appeared to be a contributing factor to the lack of immunostaining with anti-GFP, as demonstrated by the NR1 positive control. Immunostaining of the NR1 subunit of the glutamate recpetor known to have an extracellularly expressed GFP tag was greatly attenuated in the absence of detergent. A few cells stained for anti-GFP, whereas the majority of the cells that expressed the tag failed to immunostain for it. These sparse patches of staining amidst the majority of the non-GFP immunodetecting cells was not observed in either set of ACPT-GFP immunostained cells.

In contrast to the GFP tag, the peptide sequence to which the ACPT antibody is targeted is at the distal N-terminus, separated from the cell membrane by 261 amino acids. Therefore the antibody should have easy access to its binding epitope. However, without a means of controlling for the ability of anti-ACPT to bind to the epitope in the absence of detergent, the results must be viewed with considerable caution. The lack of immunostaining of unpermeabilized cells would suggest that ACPT is inserted into the membrane in such a way as to orient the N-terminus into the cytoplasm. This is only possible if the enzyme were not post-translationally modified. However, members of the histidine acid phosphatase family are known to be heavily glycosylated proteins. Lysosomal acid phosphatase has nine N-glycosylation sites, consistent with its localization in the lysosomal membrane and PAP contains three such sites. Treatment with N-glycosidase F removed sugar residues from ACPT, resulting in a shift of the molecular weight of one of the two bands respresenting the protein. However, the lower number of N-linked glycosylation sites, as well as the possible presence of an endogenous, non-glycosylated form, as indicated by a lack in shift of one of the proteins bands upon N-glycosidase F treatment, suggests that ACPT, as with PAP, exists as both a glycosylated and non-glycosylated enzyme.

If a non-glycosylated form of ACPT exists *in vivo*, there are two possible topological explanations for the data gathered. One hypothesis would suggest that the N-terminus is located within the cytoplasm with the C-terminus facing the extracellular space. These circumstances would result in N-terminal immunostaining only when the cells were permeabilized and GFP tag immunodectection under permeabilized or non-permeabilized conditions. A second possible orientation would be the presence of a hairpin turn within the cell membrane by the transmembrane domain, so that both the N- and C-termini are

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cytoplasmically oriented. This situation would result in immunodetection of either termini only under cell membrane permeabilizing conditions.

Neither of these suggested orientations is possible for the glycosylated, insoluble form of ACPT. The inability to reliably immunostain for GFP in the absence of detergent in the blocking solution prevented us from deciphering whether or not ACPT is even located at the cell membrane. As already discussed, if the glycosylated form of ACPT is located at the cell membrane, then its N-terminus would be within the extracellular space, away from most potential substrates such as ErbB4. Such an unfavourable orientation would imply that the glycosylated form of ACPT is located at the cell surface, but unable to accommodate prospective physical interactions with its substrate(s).

Another possible solution, which not only suits a potentially glycosylated protein, but also supports data suggesting that the acid phosphatase is not located at the surface of the outer cell membrane is one involving an alternative version of receptor cycling. This hypothesis revolves around the formation of multivesicular bodies which would provide ACPT with the opportunity to form a direct physical interaction with the ErbB4 receptor.

Multivesicular bodies (MVBs) are a form of late endosomes that play a crucial role in several processes, including receptor downregulation (Katzmann et al., 2002). As a key regulatory step in ensuring the correct duration and magnitude of cell signaling, downregulation involves endocytosis of a stimulated receptor into a clathrin-coated pit. From this endosomal compartment, membrane proteins generally follow one of two pathways: some proteins are rapidly recycled to the plasma membrane where they can be activated once more, while others are destined for lysosomal processing where they are degraded. This is a common constitutive process, but there are proteins that undergo a slightly modified

endocytic pathway. It has been observed for some proteins that during the process of endocytosis, the endosomal membrane invaginates towards the compartmental lumen (Hirsch et al., 1968), forming a vesicle within a vesicle (fig. 4-1).

The epidermal growth factor receptor is one receptor known to be internalized into endosomes upon ligand binding (Felder et al., 1990; Hopkins et al., 1990). It is believed that the endocytosed ligand-receptor complex may maintain its ability to generate cell signaling from this compartment, and recent data have confirmed the interaction between EGFR and various signaling proteins in endosomes (Cohen and Fava, 1985; Lai et al., 1989; Burke et al., 2001; Sorkin, 2001). If the receptor continues to signal from this locale, regulatory proteins such as phosphatases would be located within the vicinity in order to maintain a balance of cell signaling by continued receptor activity or to prepare the protein for degradation within the merging vacuole.

Eventually, this late endosome/MVB fuses with a vacuole, delivering MVB vesicles to the lumen of the vacuole where the process of degradation begins (Futter et al., 1996; Dupre et al., 2001; Katzmann et al., 2002). Initially, the membrane of the MVB vesicle breaks down and exposes the catalytic domain of the EGF receptor to the lumen of the vacuole, where it is possible that a phosphatase such as ACPT may reside. Whether ACPT is membrane bound or not, whether it is glycosylated or non-glycosylated, or whether it is not expressed at the outer membrane of the cell, this sequence of events accommodates each potential scenario, while positioning the enzyme in a favourable orientation in regards to the catalytic domains of potential substrates such as the ErbB4 receptor.



Dupre et al., Curr. Biol. (2001)

Figure 25. Receptor Cycling via the Formation of Multivesicular Bodies. A schematic of the downregulation of receptor (pink) from the cell membrane. The receptor is delivered to the early endosomes (EE) and then to the late endosomes (LE). Invagination of the late endosome results in the formation of a vesicle within the late endosome to produce a multivesicular body. Fusion of the multivesicular body with the vacuole leads to degradation of the receptor.

In summary, members of the high molecular weight histidine acid phosphatase family which includes PAP, LAP, LyAP and most recently ACPT, share two catalytic motifs, a signal sequence at the N-terminus and conserved cysteine and proline residues. As with PAP and LAP, ACPT is heavily glycosylated at its N-terminus and can also exist as a non-glycosylated form. At its C-terminus is a putative transmembrane domain. A similar domain, which is cleaved, resulting in a soluble enzyme has been known to exist in LAP for sometime, however the discovery of a novel isoform of PAP, whose only modification from the previous known form is the prescence of a putative Cterminal transmembrane domain has recently been cloned.

4.2 Tissue Distribution of Human ACPT cDNA

Discovering the function of a novel protein is often guided by its tissue distribution pattern. Limited expression to specific tissues or higher levels of mRNA and protein in various regions can aid in deducing hypothetical interacting proteins that display identical or similar patterns of distribution and expression. In the case of PAP, this approach led to the discovery of an endogenous substrate expressed by prostatic epithelial cells. The epidermal growth factor receptor, ErbB2 can be regulated by PAP activity (Lin and Meng, 1996; Meng and Lin, 1998). The tyrosine phosphorylation of ErbB2 inversely correlates with the cellular activity of PAP (Lin et al., 1994; Lin and Meng, 1996; Meng et al., 2000).

It could be hypothesized that PAP can act in a similar manner towards the other members of the EGF receptor family, however, the expression of PAP is limited to prostatic epithelium while the EGF receptors are widely expressed in epithelial, mesenchymal and

neuronal tissue (Vihko, 1979; Morris et al., 1989; Prigent and Lemoine, 1992). In contrast to PAP, ACPT has a more favourable expression pattern when compared to EGF receptors, in particular to ErbB3 and ErbB4. High levels of ACPT mRNA expression were found in brain, heart, liver, testis, ovary and small intestine. Immunoblotting of specific brain regions revealed the presence of ACPT protein in the cortex, striatum, hippocampus, cerebellum and hypothalamus. A doublet, mostly likely representing the glycosylated and non-glycosylated forms of ACPT appeared in all but the cerebellum and hypothalamus, which appear to only express the glycosylated form of the acid phosphatase.

The overlapping pattern of expression of EGF receptors with an acid phosphatase that shares ~50% homology and 46% identity with PAP suggested that ACPT may be responsible for EGF receptor regulation in these other tissues. With the exception of ErbB1, the remaining three receptors are expressed to varying degrees within the brain. However, ErbB2 does not have high affinity for any ligand and its expression is limited to Schwann cells within the central nervous system, with its expression in the brain a debated topic at present (Press et al., 1990; Gerecke et al., 2001). In contrast ErbB3 is tyrosine kinase dead (Guy et al., 1994). Therefore, ErbB4, a receptor with high brain levels of expression that has been shown to be crucial in neuronal development is an ideal candidate substrate (Gassmann et al., 1995; Srinivasan et al., 1998; Golding et al., 2000).

In conclusion, ACPT has a restricted expression pattern that includes brain, making it an ideal candidate for regulating the tyrosine kinase activity of the EGF receptors expressed there.
4.3 ACPT Interaction with a Potential Endogenous Substrate

The expression of ACPT in the brain is of interest because two members of the EGF receptor family, ErbB3 and ErbB4 are thought to be important in early neuronal development and differentiation. Although expression of ErbB2 has been found in Schwann cells of the central nervous system, its presence in the brain is currently being debated within the field (Press et al., 1990; Gerecke et al., 2001). In addition to it being the substrate for PAP, the tendency or preference of this receptor to dimerize due to the low binding affinity of this receptor towards ligands does not make ErbB2 an ideal potential substrate for ACPT.

The function of ErbB3 as suggested by ErbB3(-/-) embryos revealed its crucial role in neuronal development. These mice exhibit striking developmental defects in the midbrain/hindbrain region by displaying little differentiation of the cerebellar plate (Erickson et al., 1997). However, the lack of an active tyrosine kinase domain suggested that any regulation of ErbB3 activity must be indirect (Guy et al., 1994).

ErbB4 which has conclusively been shown to be expressed in the brain (Srinivasan et al., 1998) and has tyrosine kinase activity is therefore suggested to be a potential substrate for ACPT. In both developing and adult neurons, ErbB4 is enriched at glutamatergic synapses where it can associate with the post-synaptic density scaffolding protein PSD-95, a protein implicated in the regulation of glutamate receptor trafficking and synaptic plasticity (Garcia et al., 2000; Huang et al., 2000). ACPT is expressed in CNS neurons in a punctuate pattern and its localization overlaps with several synaptic markers, including PSD-95. In hippocampal interneurons that highly express ErbB4, ACPT displayed a synaptic distribution pattern with considerable overlap with ErbB4. In addition, it was also shown that ACPT and

ErbB4 are both present in the synaptic membrane fraction that is enriched for postsynaptic proteins. Since these two proteins are co-localized at synaptic sites, it is possible that ErbB4 can serve as a substrate for ACPT *in vivo*. ACPT-dependent regulation of the ErbB4 receptor could therefore directly influence the protein interactions of this tyrosine kinase receptor, and its role in synaptic development and plasticity.

Immunoprecipitation studies indicated that ErbB4 and ACPT can physically interact in a heterologous system with additional functional consequences. Detection of tyrosine phosphorylated residues on the ErbB4 receptor demonstrated the tyrosine phosphatase activity of ACPT. The acid phosphatase was shown to dephosphorylate the ErbB4 receptor under basal conditions, and antagonized the phosphorylation induced by neuregulinstimulated activation of the receptor.

The discovery that ACPT possesses tyrosine phosphatase activity like PAP, supports the idea that there is a distinct subtype of protein tyrosine phosphatase. Classically, protein tyrosine phosphatases have been viewed as a family of structurally and functionally unrelated tyrosine phosphatases. What little sequence homology exists amongst the numerous members of this family is limited to the catalytic domain which is defined by the signature motif [CXXXXR(S/T)] (Hiraga and Tzuiki, 1991; Zhang et al., 1994). The cysteine and arginine residues within this motif are essential for enzymatic activity and as a result, each enzyme possessing this string of amino acids functions using the same catalytic mechanism (Ramponi and Stefani, 1997).

The low molecular weight (~20 kDa) and high molecular weight phosphatases (~50 kDa) are a sub-classification of the protein tyrosine phosphatase family. They share a high degree of sequence homology amongst the members within each sub-group. Members of the

low molecular weight acid phosphatases have the [CXXXXR(S/T)] signature motif and therefore are capable of phosphotyrosine phosphatase activity. However, these enzymes are not limited to this function. Research has revealed that the low molecular phosphatases also possess dual specificity, indicating that in addition to dephosphorylating tyrosine residues, they are capable of hydrolyzing phosphorylated serine/threonine resides (Ramponi and Stefani, 1997). Initial kinetic assays indicated that these phosphatases were optimally catalytic under acidic conditions and therefore were termed low molecular weight acid phosphatases (Saini et al., 1981; Taga and Van Etten, 1982; Zhang and VanEtten, 1991).

The high molecular weight phosphatases also achieve optimal catalytic activity at an acidic pH, hence they can also be referred to as acid phosphatases. In addition to possessing a higher molecular weight, this family of phosphatases differs from the low molecular weight phosphatases in that it does not possess the typical catalytic motif. By virtue of their distinct signature motif of RHGxRxP which confers tyrosine phosphatase activity to members of this family, they form a distinct class of tyrosine phosphatases (Van Etten, 1982; Vincent et al., 1992). It is to this family that PAP and ACPT belong.

For additional proof of the existence of this newly emerging subtype of tyrosine phosphatase, evidence of a direct interaction of ACPT with ErbB4 and regulation of activity in a natural system would be required. Co-immunoprecipitating ACPT with ErbB4 from brain homogenate as seen in figure 3-15 demonstrates a physical interaction between these two proteins *in vivo*.

In summary, ACPT and ErbB4 have a similar distribution pattern which supports the physical interaction detected in an *in vivo* system. The co-

immunoprecipitation of ACPT with ErbB4 indicates an interaction between these two proteins. The neuregulin-induced phosphotyrosine activity of ErbB4 was shown to be regulated by ACPT.

4.4 Downstream Signaling of ErbB4

Downregulation and desensitization of receptors commonly occurs via a rapid internalization trafficking pathway involving endocytosis. However, ErbB4 receptors have been found to be inefficiently internalized and are therefore endocytosis impaired (Baulida et al., 1996). Instead, it has been discovered that ErbB4 undergoes sequential proteolytic cleavage upon agonist binding (Vecchi and Carpenter, 1997; Zhou and Carpenter, 2000), thus providing a possible alternative mechanism for receptor downregulation. Metalloprotease cleavage by TACE results in a ~120 kDa ectodomain (Rio et al., 2000) which is shed into the extracellular space and an ~80 kDa membrane-associated fragment (Vecchi and Carpenter, 1997; Vecchi et al., 1998) which is then cleaved by γ -secretase activity. This results in the release of a heavily phosphorylated ~80 kDa molecule which retains catalytic activity (Vecchi and Carpenter, 1997). Recent data shows that the cleaved fragment translocates to the nucleus where it may mediate protein phosphorylation important for the regulation of gene expression (Ni et al., 2001).

These cleavage processes are regulated by the phosphorylation state of the receptor (Vecchi et al., 1998). Thus, regulation of ErbB4 phosphorylation within a cell could lead to the regulation of gene expression important for the development or differentiation of a cell. In the presence of ACPT, it was shown that the basal and neuregulin-induced cleavage of

ErbB4 was reduced. Treatment with the tyrosine phosphatase inhibitor pervanadate reversed the effect of ACPT. It was noted that in the presence of pervanadate, the cleaved fragment ran at a higher molecular weight, consistent with previous studies (Vecchi et al., 1998; Zhou and Carpenter, 2000). It is possible that pervanadate, by inhibiting other tyrosine phosphatases is indirectly responsible for this mobility shift.

Because pervanadate is not a specific inhibitor of ACPT, it inhibits the activity of all tyrosine phosphatases present in the lysate. The activity attributed to these other tyrosine phosphatases cannot be accounted for even though their actions can result in significant changes in the sequence of usual signaling events. Changes in phosphorylation can result in alterations of receptor conformation and interactions with recruited adaptor proteins. This consequently affects any signaling cascade dependent on these intermediate molecules. It is known that the ErbB4 receptor is doubly cleaved (Vecchi and Carpenter, 1997; Vecchi et al., 1998; Ni et al., 2001; Lee et al., 2002). If the use of pervanadate were to indirectly inhibit the activity of γ -secretase, while leaving TACE unaffected, then the receptor will have only been cleaved once, resulting in a larger than predicted or expected fragment. Alternatively, another protease may be recruited to cleave and release the cytoplasmic tail of ErbB4. It is unlikely that this "backup" protease would recognize and cleave the receptor in the identical fashion of γ -secretase.

It appears that ACPT activity can influence proteolytic cleavage of the ErbB4 receptor. This implies that the phosphatase interacts with the receptor prior to the disassociation of the ErbB4 catalytic tail. *In vivo* co-immunoprecipitation studies support this hypothesis. When using an ErbB4 antibody directed to the N-terminus for immunoprecipitation, the C-terminus can only be isolated prior to its cleavage from the

remainder of the receptor. Since the phosphorylated tyrosine residues on ErbB4 are located on the C-terminal fragment, this indicates that this is the interaction site for ACPT. Therefore co-immunoprecipitating ACPT with this antibody was only possible when the receptor was not cleaved.

These data suggest that ACPT can regulate neuregulin-induced cleavage of ErbB4 by altering the tyrosine phosphorylation state of the receptor, thereby indirectly influencing ErbB4 activity-dependent downstream regulation of gene expression.

4.5 Possible Role in Differentiation

If ACPT alters the tyrosine kinase activity of ErbB4, the signaling cascades activated by this receptor should also be affected. The importance of ErbB4 during development would suggest that regulation of the receptor by ACPT could modulate neuronal differentiation or development. When the ErbB4 receptor is transfected into PC12 cells, the cells are able to differentiate in response to neuregulin (Vaskovsky et al., 2000).

The sustained activity of the Ras dependent MAPK/Erk pathway by NGF-stimulated TrkA receptors is the traditional method of inducing neurite sprouting in PC 12 cells (Gotoh et al., 1990; Gomez and Cohen, 1991). NGF binding to the TrkA receptor induces dimerization, autophosphorylation and subsequent recruitment of molecules such as the guanine nucleotide exchange protein son of sevenless (SOS) (Nakamura et al., 1996). Recruitment of SOS leads to the exchange of bound GDP for GTP thereby activating the small G protein ras. This triggers a cascade or series of phosphorylation events beginning with the serine/threonine kinase Raf, involving the tyrosine/threonine kinase MAP/Erk and

ending with the serine/threonine kinase erk (fig. 4-2). NGF is both necessary and sufficient for differentiation of the cells into sympathetic neuron-like cells (Tischler and Greene, 1975; Greene and Tischler, 1976).

This same signaling cascade can be initiated by neuregulin-induced activation of the ErbB4 receptor(Chao, 1992; Schlessinger and Ullrich, 1992). However, activation of the ras-Erk/MAP pathway can only result in neurite sprouting when the activity is sustained and only cells overexpressing EGF receptors are capable of this type of activity (Traverse et al., 1992; Traverse et al., 1994; Marshall, 1995). PC12 cells which endogenously express ErbB1, ErbB2 and ErbB3 promote cell survival when stimulated, even though the same pathway is activated (Marshall, 1995; Vaskovsky et al., 2000). To account for the distinct cellular responses to the activation of one signaling cascade, it has been suggested that signaling molecules such as suc-associated neurotrophic factor-induced tyrosine phosphorylated target are only activated in response to specific growth factors. It has also been suggested that the difference in duration of tyrosine phosphorylation between TrkA and the EGF receptors may be due to the presence of a protein tyrosine phosphatase which dephosphorylates the activated EGF receptor, but not TrkA (Yamada et al., 1997).



Figure 26. TrkA and ErbB4 Signaling Cascade in PC12 Cells. The intracellular signaling intermediates involved in the differentiation of PC12 cells upon TrkA and ErbB4 activation.

It was determined that regulating the phosphorylation of ErbB4 by co-expressing the receptor with tyrosine phosphatase ACPT blocked neuregulin-induced neurite outgrowth. To determine if the regulation of neurite growth was specific to dephosphorylation of the ErbB4 receptor, we examined the effect of ACPT on neurite outgrowth upon treatment with nerve growth factor, which induces PC12 differentiation via the TrkA receptor (Greene and Tischler, 1976). Overexpression of ACPT did not prevent nerved growth factor-induced outgrowth. Since neurite outgrowth in response to either neuregulin or nerve growth factor is dependent on the activation of the MAP kinase pathway (Vaskovsky et al., 2000), these results indicate that ACPT is working upstream of this cascade, most likely directly on the activated ErbB4 receptor, but not on the TrkA receptor.

It is therefore evident that the regulation of tyrosine phosphorylation of the ErbB4 receptor by ACPT influences neuregulin-induced neurite outgrowth of PC12 cells. This data suggests that ACPT plays an important role in neuronal differentiation and development.

4.6 General Overview

For many years, the endogenous substrates for the various members of the histidine acid phosphatase family have been unknown. The discovery that ErbB2 can be dephosphorylated by PAP in prostatic epithelial cells (Meng and Lin, 1998; Zhang et al., 2001) suggests that other members of this family may be capable of regulating tyrosine kinase receptors. We have now identified a novel histidine acid phosphatase, ACPT, which can dephosphorylate the ErbB4 receptor and inhibit the ligand-induced proteolytic cleavage of this receptor. ACPT physically associates with ErbB4 in a heterologous system and *in vivo*, and in neurons the two proteins are co-localized at synaptic sites. When co-expressed with ErbB4 in PC12 cells, ACPT prevents the neuregulin-induced differentiation indicating a possible role for the phosphatase in regulating ErbB4 function.

4.7 Future Directions

There are several experiments which could be performed to address issues raised by the results in this present study and to further uncover the signaling mechanism that utilizes ErbB4 and ACPT in concert.

A) Is ACPT membrane expressed?

The presence of a putative transmembrane domain at the C-terminus of ACPT raised questions regarding its ability to access the phosphorylation site of the ErbB4 receptor. The presence of glycosylation sites at the N-terminus typically suggests that the enzyme would be inserted into the membrane with its catalytic domain facing the extracellular space surrounding the cell or within an organelle membrane with the catalytic region facing the luminal domain and therefore physically incapable of associating with the catalytic domain of the receptor kinase. Experiments attempting to resolve the question of ACPT membrane orientation were not only inconclusive, but fueled the hypothesis that the enzyme is not located at the cell surface, but rather inserted into a membrane of an organelle.

An alternative approach to this question could involve treating ACPT-GFP expressing cells with trypsin prior to harvesting. A mild preparation of trypsin would cleave and remove

cell surface expressed proteins upon incubation with cells. To serve as a positive control, one sample set would be incubated with PBS in the absence of trypsin. Harvesting these cells and immunoblotting with anti-GFP would reveal a decrease in full-length ACPT in the trypsin treated cells versus the control sample if the protein is located on the cell surface with the C-terminus extracellularly expressed. Stripping and reprobing the membranes with anti-ACPT would therefore reveal only the cleaved product in the trypsin-treated cells and the full-length enzyme in PBS-treated cells. Alternatively, if the N-terminus is extracellularly located if ACPT is cell surface expressed, immunoblotting with anti-ACPT would result in a decrease in full-length protein in the trypsin treated cells and probing with anti-GFP would reveal the presence of a cleaved fragment.

If ACPT is not located on the cell surface membrane, trypsin treatment would not result in enzyme cleavage. Therefore immunoblotting with anti-GFP or anti-ACPT would not reveal any notable changes, suggesting that ACPT is located within the membrane of an internal organelle.

B) Does ACPT Affect the Translocation of the Cleaved ErbB4 Fragment

Experiments conducted on a fragment of the cytoplasmic tail of the ErbB4 receptor in COS7 cells has shown that this fragment can translocate to the nucleus (Ni et al., 2001) where it may mediate protein phosphorylation important for the regulation of gene expression. Since ACPT activity appears to affect ErbB4 cleavage as shown in this study, it would be of interest to explore its possible connection to gene regulation in the proposed pathway.

To begin, it would be necessary to determine if the interaction of ACPT with ErbB4 could alter the amount of cleaved fragment located within the cell nucleus. Immunocytochemical examination of COS7 cells tranfected with an ErbB4 receptor containing an internal HA-tag located on the C-terminal end, slightly downstream of the transmembrane domain (erb-int) compared to ACPT and erb-int co-transfected cells would give insight into the effect of ACPT activity. Should this experiment prove successful, a decrease in nuclear localized HA would be observed in the cells co-expressing ACPT.

In addition to conducting this experiment in COS7 cells, it would be necessary to repeat the protocol in neurons in order to verify that this regulation occurs in neurons as well.

C) Is ErbB4 Regulated by ACPT Along the Endocytic Pathway?

Most research concerning the involvement of multivesicular bodies in the endocytic cycling pathway has been focused on ErbB1. As a member of the same receptor tyrosine kinase family, it can be hypothesized that ErbB4 participates in this pathway as well. This would provide ACPT with ample opportunity to interact with ErbB4.

To determine if ErbB4 is incorporated into and continues to signal from multivesicular bodies it is necessary to be able to differentiate between signals generated from the MVB from plasma membrane-derived signals. Following the protocol of Wang et al. (Wang et al., 2002), the specific activation of endosome-associated receptor without activation of plasma membrane-associated receptor and without disruption of the overall endocytic pathway can be detected. This method involves a series of treatments of various agonists and antagonists. Freshly plated ErbB4 transfected cells in serum-free medium, prior to treatment express the non-phosphorylated receptor at the plasma membrane. Application

of a tyrosine kinase inhibitor followed by treatment with a solution containing the ErbB4 agonist neuregulin and a receptor internalization inducer such as AG1478 causes activation of the cycling pathway and therefore the internalization of the receptor or its cytoplasmic tail to be internalized into endosomes, while remaining nonphosphorylated. Subsequent washes and incubation of the cells in serum-free medium leads to the specific activation of the endosome-associated kinase (i.e. the receptor is located in endosomes and is heavily phosphorylated).

At this stage, the cells can be fixed and immunostained for the cytoplasmic tail of the ErbB4 receptor and phosphorylated tyrosine residues. Immunofluorescent analysis of cells will reveal co-localization in staining of the two antibodies if there is specific activation of endosome-associated receptor.

During the execution of this protocol, samples co-expressing ErbB4 and ACPT should be run in parallel. If ACPT activity is localized within the multivesicular bodies of the endocytic pathway, then a striking difference in ErbB4 endosomal signaling should be observed.

Abbreviations

ACPT – testicular acid phosphatase

ATP – adenosine triphosphate

CNS – central nervous system

div – days *in vivo*

DMEM – Dulbecco's modified Eagle's medium

ECL – enhanced chemiluminescence

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ER – endoplasmic reticulum

ESTs – expressed sequence tags

FRAP - fluoride resistant acid phosphatase

GFP – green fluorescent protein

GTP – guanosine triphosphate

IB – immunoblot

Ins(1,4,5)P₃ – inositol-1,4,5-triphosphate

InsP – inositol phosphate

IP – immunoprecipitation

LAP – lysosomal acid phosphatase

LPA – lysophosphatidic acid

LyAP – lysophosphatidic acid phosphatase

MAPK - mitogen-activated kinase

MVB - multivesicular body

NGF - nerve growth factor

NRG – neuregulin

PA – phosphatidic acid

PAP – prostatic acid phosphatase

PBS – phosphate buffered saline

PC12 cells – pheochromocytoma cells

PCR – polymerase chain reaction

PDL – poly-D-lysine

PFA – paraformaldehyde

PI-3K - PI-3 kinase

PKC – protein kinase C

PNGase F - N-glycosidase F

PSD – post-synaptic density

S1P – sphingosine 1-phosphate

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SOS – son of sevenless

TACE - tumour necrosis factor-alpha-converting enzyme

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