

CHAPTER 1

INTRODUCTION

SUMMARY

Rhythmic cellular behaviour is a well known characteristic of nature. Fine control of cellular phosphorylation allows for better regulation of dynamic processes such as proliferation, differentiation and neoplastic transformation. Protein phosphatases catalyse the dephosphorylation of phosphoproteins, thereby antagonizing the action of protein kinases. Initially, protein phosphatases were thought of as enzymes that served merely to counteract the action of the kinases, whether this resulted in the activation or inhibition of target enzymes. A good example of this is phosphorylase phosphatase, which causes the inactivation of phosphorylase and activation of glycogen synthase. This reciprocal effect was thought to prevent a futile cycle resulting in ineffectual utilization of ATP. However, it soon became apparent that these interconvertible enzymes do not exist in fully active or inactive forms; that is, the kinases and phosphatases do not simply serve as on-off switches.

It has been shown that the cyclic interconversion of regulatory enzymes represents a dynamic process in which the steady-state equilibrium between active and inactive forms varies gradually depending on many parameters

determined by the metabolic state of the system (Stadtman and Chock, 1977). It is widely accepted that the steady state activity of an enzyme would be dependent on the ratio of its phosphorylated to dephosphorylated forms. It has been shown that the mechanisms used by tyrosine residues as opposed to those of serine-threonine may be different. Tyrosine phosphorylation is transient and substoichiometric, yet, normal physiological responses can be elicited.

Proteins phosphorylated on tyrosine residues are often phosphorylated on seryl and threonyl residues as well, with the implication that both of these modifications could act cooperatively with one another. Because most protein tyrosine kinases are associated with the cell membranes, tyrosine phosphorylation has been regarded as a signal capable of affecting the state of activity of second messengers (Ray and Sturgill, 1987).

Protein phosphatases found in eukaryotic cells represent a diverse class of enzymes that exhibit considerable variation with regard to their three-dimensional structure, substrate specificity, and catalytic function. Three distinct gene families have been discovered that code for the protein phosphatases. Two of these, the so-called PPP and PPM families, code for protein serine and threonine phosphatases respectively. A third family, the recently discovered protein

tyrosine phosphatases (PTPs) include structures that are both tyrosine-specific and dual-specific in their phosphatase activity.

This study focuses on three phosphoproteins; namely, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) which dephosphorylate serine and threonine residues and protein tyrosine phosphatase-1B (PTP-1B) which dephosphorylate tyrosine residues. The human acute promyelocytic cell line, HL-60 was used in this study. These cells were induced to differentiate either along the macrophage pathway using phorbol-12-myristate-13-acetate (PMA) or along the granulocytic pathway using, either, dimethyl sulfoxide, all-trans retinoic acid (ATRA) or 9-cis retinoic acid (9-cis RA). This cell line provided a model for the study of the reversal of transformation for all three enzymes at the transcriptional and translational level.

This chapter provides background information to the study and discusses cellular oscillations, the phosphorylation and dephosphorylation of phosphoproteins, the cell line and the inducing agents used.

CELLULAR OSCILLATORY DYNAMICS

This century has witnessed incredible developments in scientific endeavours. Notable among these has been the rediscovery of time and the realization that the rhythmic behaviour is characteristic of life itself. A large number of oscillatory systems are now well recognized in chemical, biochemical and cellular events. Rhythms occur at every level of biological organization, their periods ranging from fractions of a second, in the case of neuronal oscillations, to years, when considering epidemiological and ecological variations. For every second, throughout our lives, cardiac rhythms generate a heart beat. Liver cells produce regular pulses of calcium in response to hormones; intracellular calcium oscillations have periods varying from seconds to minutes. Many biochemical oscillations, the mitotic cycle, hormonal and circadian rhythms have higher periods of the order minutes to hours (Goodwin, 1963).

All living cells are dynamic since their functions, reproduction, behaviour and characteristics are determined by oscillatory variations influenced by internal and external control (Parrington et al., 1996). Until a few decades ago, the concept of equilibrium dominated almost all scientific research, cellular oscillatory behaviour was first described in the 1960's (Gilbert, 1968). Today, however, the concept has been demonstrated in many systems such as

fertilization, secretion, proliferation, neural signaling and cellular division. It has also been demonstrated that these oscillatory behaviour are controlled by reversible protein phosphorylation (Hammond et al., 1998).

Cellular dynamics have been best demonstrated by the existence of oscillatory variations in the levels of cellular constituents (Gilbert, 1968; 1974; 1980), as they represent the only kind of characteristics which can be self generated and can persist indefinitely under suitable conditions. Such behaviour is also a reflection of the fact that living cells are far from equilibrium as there is a constant flux of components in and out of each cell. For a system to be in equilibrium it must therefore by definition be isolated.

In the past two decades, much interest has been shown in the subject of irregular, deterministic oscillations, or chaos, in various fields, including cell biology (Goldbeter and Decroly, 1983; Markus et al., 1984; Olsen and Degn, 1985; Lloyd and Lloyd, 1995; Goldbeter, 1996). Evidence continues to accumulate which supports the long-standing contention that living cells are multioscillators and therefore chaotic behaviour is likely to be met, if only on occasions. However, irregularity is not a sufficient indication of chaos since it

can arise through interactions between the many distinct periodicities that have been observed in mammalian cells in culture (Gilbert et al., 2000).

Cells exist far from equilibrium and depend on fluxes of materials and kinetics of many reactions. The driving force for all life processes is the non-equilibrium state of living cells, not gene expression which is a dependent variable of systems. Differentiation, ageing and cancer all involve prolonged temporal effects but indefinite continuous monotonic changes are not possible and cannot account for these various remaining enigmas. On the basis of that observation and the above argument, it seems reasonable to suggest that, under defined conditions, cells have characteristic patterns of temporal organization reflecting disparate metabolic states and the distinct patterns of interactions between dynamic control reactions (Gilbert, 1968; 1969; 1984). It was also pointed out that the existence of oscillations permits a wider range of cell characteristics. It follows that changes in cell properties and behaviour (Gilbert, 1968; 1984; Gilbert and MacKinnon, 1992) could be expected to result from altered patterns of temporal organization. Oscillations thus allow explanations for the major dynamic enigmas presented by cells. It can therefore be postulated that if oscillations are associated with life then death must be associated with disruption of cellular oscillatory behaviour (Gilbert, 1995).

It has been suggested that each species has its own fundamental pattern of temporal organization which may be altered or modified by changes in the concentrations of cellular constituents, which in turn depend on the concentrations of the oscillating components. The course that a cell may follow is determined by the actual state of the cell, either static or dynamic, and by the particular set of rhythmic parameters to which the cell is subjected (Gilbert, 1968).

As indicated (Edmunds, 1988), oscillatory variations occur in the active and apparent isozyme patterns of a number of glycolytic enzymes and distinct phasing relationships have been reported in untransformed and polyoma transformed BHK cells (Gilbert, 1968; 1974; 1984; Gilbert and Tsilimigras 1981). Tsilimigras and Gilbert, 1977, have shown that the extractable protein oscillations cannot entirely account for enzyme periodicities though it probably contributes toward them.

In earlier experimental studies using several cell species, Gilbert and co-workers showed high frequency oscillations in the level of NADH in the activities and effective isoenzyme patterns of a number of enzymes associated with glycolysis

(Gilbert and Tsimiligras, 1981; Gilbert and MacKinnon, 1992) and in the activity and amount of active isoenzyme of LDH (Ferreira et al., 1996). Temporal variations and oscillatory behaviour were also evident in the activities of phosphoamino protein phosphatases in murine erythroleukaemic (MEL) cells (Hammond et al., 1998); in the phosphorylation potential of certain specific proteins and in the expression of the protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein tyrosine phosphatase 1B (PTP1B) proteins (Hammond et al., 1985, 1989, 1998; Bhoola and Hammond, 2000).

Earlier studies in our lab also showed that phosphorylation is highly dynamic in resting HL-60 cells (Hammond et al., 1988; Hammond, 1998; Calvert-Evers and Hammond, 2000). When these resting dynamics were disturbed by differentiating agents such as hexamethylene bisacetamide (HMBA) and ATRA, four distinct tyrosine kinase and phosphatase enzyme patterns were identified (Calvert-Evers and Hammond, 2002). Similar oscillatory behaviour was also shown to occur in *H-ras* and *N-ras* mRNA expression in MEL cells (Hammond et al., 2000).

At present several different chemical-kinetic models to predict cellular behaviour have been devised to simplify the complex network of biochemical reactions in a mathematical formula. These studies try to explain the dynamic cellular behaviour through the use of graphical models (Norel and Agur, 1991; Tyson, 1991; Goldbeter, 1991). Recently Tyson and coworkers (1996) translated the cell cycle into a set of differential equations and by assigning parameters to specify the rates of various steps in the mechanism, the oscillatory behavioural dynamics could be predicted. Although biochemical models often ignore important mechanisms such as feedback control and secondary phosphorylation, mathematical modeling could serve as a powerful tool for understanding complex regulatory systems.

Numerous simulations of complex, multivariable models analyzed by computer result in the following projections:

- (a) the behaviour of the control system is dependent on its parameters;
- (b) parameter values can be changed by altering the concentrations of key regulatory enzymes;
- (c) two behavioural patterns, namely, stable steady states and stable oscillations, were evident;
- (d) metabolic state of cells determines the shape of the curve.

When a non-linear system was subjected to non-equilibrium constraints, limited cycles, steady states, chaotic behaviour and complex oscillations as predicted by mathematical modeling, were observed (Tyson, 1991).

PROTEIN PHOSPHORYLATION AND PHOSPHATASES

The precise and rapid propagation of cellular signaling events demands strict and flexible regulatory processes. Protein phosphorylation is one of the major post-translational modification mechanisms that cells utilize to control various cellular regulatory processes. The addition or removal of a phosphoryl moiety from a protein can generate a recognition motif for protein-protein interactions, control protein stability, and most importantly, modulate enzyme activity (Cohen, 1989).

The reversible phosphorylation of proteins is now recognized to be a major mechanism for the control of intracellular events in eukaryotic cells. Processes as diverse as metabolism, contractility, membrane transport and secretion, the transcription and translation of genes, cell division, fertilization and even memory are all regulated by this post-translational modification (figure.1, Barford et.al., 1998). The phosphorylation or dephosphorylation of serine, threonine and tyrosine residues on regulatory proteins triggers conformational changes that alter the biological properties and level of phosphorylation at any instant, reflecting the relative activity of the protein kinase and phosphatase (Cohen, 1989).

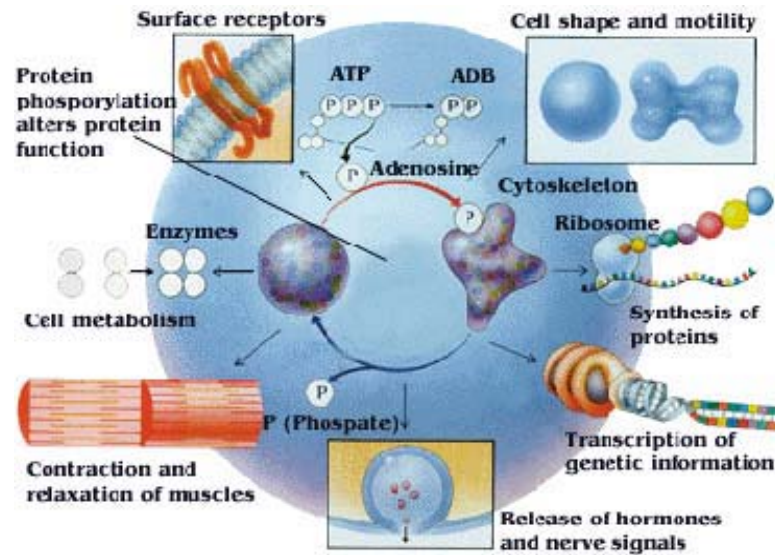


Figure 1: The role of protein phosphorylation and dephosphorylation in cellular function

Hundreds of different protein kinases and phosphatases in eukaryotic cells are organized into complex networks of signaling pathways that help coordinate the cell's activities, drive the cell cycle, and relay signals into the cell from the cell's environment. Individual protein kinases serve as "processing" devices in the integration process. An important part of the input to these proteins comes from the control that is exerted by phosphatases. Specific phosphate groups serve to activate a protein, while others function to inactivate (adapted from Barford et al., 1998)

Eukaryotic protein phosphatases are structurally and functionally diverse enzymes that are represented by three distinct gene families. Two of these, the PPP and PPM families, dephosphorylate phosphoserine and phosphothreonine residues, respectively, whereas the protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine. The most abundant protein serine/threonine phosphatases of the eukaryotes, PP1, PP2A and PP2B belong to the PPP family, whereas PP2C and the related mitochondrial pyruvate dehydrogenase phosphatase are members of the PPM family (Cohen, 1989).

Within each family, although the catalytic domains are highly conserved, similarities in tertiary structure and catalytic mechanisms, considerable structural and functional diversity of individual protein phosphatases is created as a result of a combination of associated regulatory domains and subunits (Wera and Hemmings, 1995).

THE PPP FAMILY

Protein serine/threonine phosphatases of the PPP family play numerous roles in mediating intracellular signaling processes. In addition to PP1, PP2A and PP2B, novel protein phosphatases have recently been characterized that occur at low abundance in a tissue- and developmental-specific manner (table 1, Hubbard and Cohen, 1993).

PP1 and PP2A are specifically and potently inhibited by a variety of naturally occurring toxins such as okadaic acid and microcystin (MacKintosh and MacKintosh, 1994). Whereas PP2B is only poorly inhibited by the toxins that affect PP1 and PP2A, it was recently defined as the immunosuppressive target of FK506 and cyclosporine in association with their major cellular binding proteins, the cis-trans peptidyl prolyl isomerases FKBP12 and cyclophilin, respectively (Liu J., 1991).

Table 1: Nomenclature of protein Ser/Thr phosphatase catalytic and regulatory subunits (adapted from Hubbard and Cohen, 1993)

CATALYTIC SUBUNIT	REGULATORY SUBUNITS
PPP family	
PP1	GM, GL, M110 + M21, NIPP-1, RIPP-1, R110, P53BP2, L5, sds22, RB gene product, Inhibitor-1, DARPP-32, inhibitor-2, Splicing factor, kinesin-like
PP2A	A-subunit (PR65) B-subunit (PR55, PR72, PR61), eRF1, PTPA, SET, polyoma middle and small T-antigens, SV40 small T antigen
PP2B	B-subunit, calmodulin, AKAP-79
PPM family	
PP2C	Arabidopsis AB11 Arabidopsis KAPP-1 Pyruvate dehydrogenase phosphatase Bacillus subtilis SpolIE phosphatase

The structural complexity of PP1 and PP2A holoenzymes in vivo resolves the seemingly paradoxical situation that a relatively small number of protein phosphatase catalytic subunits are responsible for the specific dephosphorylation of a variety of cellular proteins, and that both PP1 and PP2A have been implicated in regulating many diverse cellular functions, including glycogen metabolism, muscle contraction, control of the cell cycle and RNA splicing.

The individual catalytic subunits of PP1 and PP2A catalyse the dephosphorylation of a broad range of substrates by altering the selectivity of the enzyme towards a particular substrate or by targeting the phosphatase to the sub-cellular location of its substrates. This is achieved by regulatory or targeting subunits that bind to the phosphatase catalytic subunits. In addition, the regulatory subunits allow the activity of the phosphoprotein phosphatases to be modulated by protein phosphorylation and second messengers (Faux and Scott, 1996).

Phosphoprotein phosphatase 1 (PP1)

PP1 is a major eukaryotic protein serine/ threonine phosphatase that regulates an enormous variety of cellular functions through the interaction of its catalytic subunit (PP1c) with over fifty different established regulatory subunits. Multiple genes encode PP1c isoforms in most eukaryotes. Four mammalian PP1c gene products initially identified were designated PP1 α , PP1 β , PP1 γ 1 and PP1 γ 2, the latter two arising through alternative splicing (Sasaki et al., 1990). Mammalian PP1c isoforms possess distinct tissue distributions and subcellular localizations (Andreassen et al., 1998). Although a small fraction of PP1c molecules may be inhibited by phosphorylation during the cell cycle (Berndt et al., 1997; Kwon et al., 1997), most forms of regulation are achieved through the regulatory subunits.

A classification of 45 established regulatory subunits of PP1c is discussed by Cohen (2002). An additional nine proteins have been identified from the human genome sequence (Ceulemans et al., 2001) and there are likely to be more PP1-interacting proteins (Campos et al., 1996). The regulatory subunits have been implicated in many physiological processes including glycogen metabolism, smooth muscle relaxation, actin cytoskeleton, pre-mRNA splicing, cell cycle, transcription, protein synthesis and centrosomal function (Cohen, 2002). The regulatory subunits target PP1c to specific subcellular locations and interact with a small hydrophobic groove on the surface of PP1c through a short

conserved binding motif – the RVxF motif (Egloff et al., 1997). Several ‘modulator’ proteins bind to PP1c but do not possess a domain targeting them to a specific location. Most are potent inhibitors of PP1c and possess at least two sites for interaction with PP1c, one of which is identical or similar to the RVxF motif (Conner et al., 2000).

Regulation of PP1c in response to extracellular and intracellular signals occurs mostly through changes in the levels, conformation or phosphorylation status of targeting subunits. Understanding of the mode of action of PP1c complexes may facilitate development of drugs that target particular PP1c complexes and thereby modulate the phosphorylation state of a very limited subset of proteins (Liu et al., 2000).

Phosphoprotein phosphatase 2A (PP2A)

Protein phosphatase 2A describes an extended family of intracellular protein serine/threonine phosphatases sharing a common subunit that regulates a variety of processes by means of diverse regulatory subunits. The characterization of at least 15 distinct regulatory B-subunits that individually assemble with a core heterodimer consisting of PP2Ac and a 65 kDa A-subunit suggests a similar level of diversity of PP2A holoenzymes as compared with PP1 (table 1). Insight into the role of PP2A regulatory subunits has been obtained from genetic analysis of a 55 kDa B-subunit in *Drosophila* and yeast (Mayer-Jaekel et al., 1993) where mutations in this gene lead to defects in cell division.

The PP2A holoenzyme in brain extracts from *Drosophila* display specific decreased activity towards the CDK-1 phosphorylated histones H1 and caldesmon. The core heterodimer of PP2A is a target of tumour antigens SV40 and polyoma viruses. In vivo, small T antigens inhibit PP2A dephosphorylation of mitogen-activated protein kinase (MEK) and extracellular-signal-regulated kinase (ERK) resulting in receptor mediated activation of these kinases and cell proliferation (Sontag et al., 1993).

Protein phosphatase 2A was first implicated in the control of DNA replication when PP2Ac was purified as replication protein C on the basis of its ability to

stimulate the efficient initiation of simian virus 40 (SV40) DNA replication (Virshup and Kelly, 1989). PP2Ac restored SV40 DNA replication activity in G1 phase cells, suggesting that specific PP2A activity was regulated in the cell cycle (Virshup et al., 1989).

It has been shown by Kleinberger and Shenk (1994) that adenovirus E4orf4 protein, which induces apoptosis in transformed cell lines, binds to the PR55 B-subunit of PP2A. They have also shown that mutations in E4orf4 interfere with binding to the B-subunit and interfere with induction of apoptosis (Shtrichman et al., 1999). New studies have implicated PP2A to be the phosphatase that dephosphorylates Bcl-2 and promotes apoptosis and phosphorylation to block cell death (Ruvolo et al., 1999).

Phosphoprotein phosphatase 2B (PP2B)

PP2B is characterized by its dependence on Ca^{2+} for activity. The enzyme consists of an A-subunit with an amino-terminal catalytic domain and a carboxy-terminal regulatory region containing binding sites for the PP2B B-subunit and calmodulin, and at the extreme carboxyl terminus, an auto-inhibitory sequence. Maximal Ca^{2+} stimulation of PP2B requires association of Ca^{2+} to both the B-subunit and to calmodulin. PP2B plays a crucial role in the Ca^{2+} -signalling cascade of activated T cells. Increases in Ca^{2+} concentrations in T cells, promoted by antigen presentation to the T-cell receptor, stimulate PP2B to dephosphorylate the cytosolic subunit of the transcription factor NFATp (Jain et al., 1993).

Dephosphorylated NFAT translocates into the nucleus where, in concert with other transcription factors, it induces expression of the IL-2 gene, one of the early genes in the T-cell activation pathway. Inhibition of this Ca^{2+} -signalling cascade by immunosuppressant drugs suppresses T-cell activation. Similarly to PP1 and PP2A, PP2B is ubiquitously produced with pleiotropic roles including regulation of long-term depression in neurons and recycling of integrins to the front of migrating neutrophils (Lawson and Maxfield, 1995).

THE PPM FAMILY

This is a large and varied family of protein phosphatases found in both eukaryotes and prokaryotes whose defining member is PP2C. Within the PP2C domain are numerous structural contexts that reflect functional diversity. For example, the PP2C domain of the Arabidopsis AB11 gene is fused with EF-hand motifs (Leung et al., 1994), whereas in KAPP-1, a kinase-interaction domain associates with a phosphorylated receptor which precedes the phosphatase domain. Other less-closely related examples include the Ca^{2+} -stimulated mitochondrial pyruvate dehydrogenase phosphatase, which contains a catalytic subunit sharing 22% sequence identity with mammalian PP2C and the SpoIIE phosphatase of *Bacillus subtilis*, which has ten membrane spanning regions preceding the PP2C-like catalytic domain (Duncan et al., 1995).

In eukaryotes, one of the roles of PP2C is to reverse protein kinase cascades that become activated as a result of stress. The role of PP2C-like protein phosphatase in regulating stress-response pathways is also conserved in prokaryotes. The protein phosphatase SpoIIE controls the sporulation of *Bacillus subtilis* by dephosphorylating the anti-transcription factor SpoIIAA, reversing the actions of the SpoIIAB protein kinase in a process that is governed by the ADP-ATP ratio (Duncan et al., 1995).

PROTEIN TYROSINE PHOSPHATASES

Protein tyrosine phosphatases (PTPases) are signaling enzymes that control a diverse array of cellular processes, they account for only 0.01% to 0.05% of total phosphorylated proteins. However, upon oncogenic transformation or growth factor stimulation, the level of tyrosine phosphorylation increases to 1%-2% of the total cellular protein (Hunter and Sefton, 1980). Although tyrosine phosphorylation occurs to only a small extent, it has become clear that it is still vital for controlling normal cellular growth, differentiation, metabolism, cell cycle, cell-cell communications, cell migration, gene transcription, ion channels, the immune response and cellular survival (Hunter, 2000). Abnormal tyrosine phosphorylation is associated with many human diseases including cancers, diabetes, rheumatoid arthritis and hypertension (Zhang, 2002).

In vivo, tyrosine phosphorylation is reversible and dynamic; the phosphorylation states are governed by the opposing activities of protein tyrosine kinases (PTKases) and protein tyrosine phosphatases (PTPases). Like PTKases, PTPases constitute a large family of enzymes (Li and Dixon, 2000) which exert both positive and negative effects on a signaling pathway and play crucial physiological roles in a variety of mammalian tissues and cells (Van Vactor et al., 1998).

Protein Tyrosine Structure and Function

The PTPases are a family of enzymes whose structural diversity and complexity rival those of the PTKases. Unlike PTKases, which share sequence identity with protein serine/ threonine kinases, the PTPases show no sequence similarity with the protein serine/ threonine phosphatases. Protein tyrosine phosphatases are characterized by the presence of the active site signature motif (H/V)C(X)₅R(S/T) in the conserved catalytic domain (Zhang, 1998). In addition to a catalytic domain, protein tyrosine phosphatases also have a wide array of structural elements including SH2 domains, PDZ domains, extracellular ligand binding domains, and many others (Li and Dixon, 2000).

The recently completed human genome project suggests that there are approximately 112 protein tyrosine phosphatases (International Human Genome Sequence Consortium, 2001), which contain tyrosine specific and dual-specific phosphatases. The tyrosine specific phosphatases, such as PTP1B, hydrolyse phosphotyrosine containing proteins, while the dual-specific phosphatases, such as Cdc25, utilize protein substrates that contain phosphotyrosine (pTyr), as well as phosphoserine (pSer) and phosphothreonine (pThr). The tyrosine specific PTPases can be further categorized into two groups: receptor-like and intracellular. The receptor-like PTPases, like CD45, have an extracellular ligand binding domain, a signal transmembrane region, and one or many cytoplasmic PTP domains. The intracellular PTPases, like PTP1B, contain a single catalytic

domain and various amino or carboxyl terminal extensions, including SH2 domains that have targeting or regulatory functions. Examples of dual-specificity phosphatases include the MAP kinase phosphatase, the cell cycle regulators cdc25 phosphatases and the tumour suppressor PTEN (figure 2). A low molecular weight phosphatase family has been identified in mammals, yeast and bacteria, but no function is known yet for this group. All PTPases are characterized by their sensitivity to vanadate, ability to hydrolyze p-nitrophenyl phosphate, insensitivity to okadaic acid, and lack of metal ion requirement for catalysis (Fauman and Saper, 1996).

The three dimensional structures of over a dozen PTPases have been determined. These structures have helped us understand PTPase function at the molecular level by defining the interactions that stabilize the active site structure and substrate complex formation. The PTPases are α/β proteins with tertiary folds composed of a highly twisted mixed β -sheet flanked by α helices on both sides. Despite the variation in amino acid sequences and differences in substrate specificity, the crystal structures of the dual-specific phosphatases display general folds that resemble the tyrosine-specific PTPase structure. In addition, key structural features [e.g., the active site PTPase loop formed by the (H/V)C(X)₅R(S/T) sequence motif and the general acid-containing surface loop] important for catalysis are conserved among all PTPases (Barford, 1999).

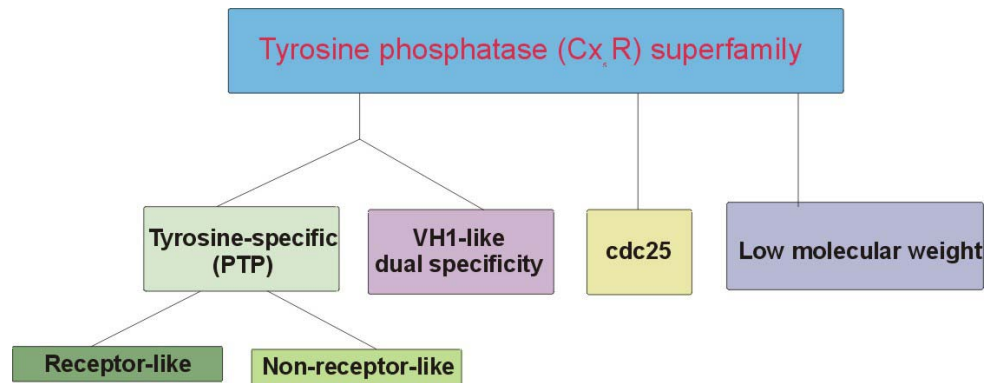


Figure 2: Protein tyrosine phosphatase family

Hierarchy based on amino acid sequence comparisons, function and three-dimensional structure (adapted from Fauman and Saper, 1996)

The active site is located within a crevice (~ 9 Å deep for the tyrosine-specific PTPases and 6 Å deep for the dual-specificity phosphatases) on the surface. The much deeper active site pocket specifically selects phosphotyrosine-containing substrates (Jia et al., 1995), while the shallower active site cleft for the dual-specific phosphatases may accommodate either phosphotyrosine (pTyr) or phosphoserine/ phosphothreonine (pSer/pThr) (Yuvaniyama et al., 1996).

At the bottom of the active site is the phosphate binding loop or P-loop formed by the PTPase signature motif. The backbone of the P-loop creates a positive electrostatic potential owing to the outward arrangement of the side chains and the inward orientation of the partially positive-charged amide hydrogens. The bound phosphate is further stabilized by a closely positioned α -helix dipole, located near the amino-terminal of the P-loop, and the active site arginine. The design creates a complementary electrostatic binding pocket for the phosphoryl moiety of incoming substrates. The active site is surrounded by several surface loops, which are important for catalysis and substrate recognition. In PTP1B, one of these loops, called the substrate recognition loop ($\alpha 1$ - $\beta 1$ loop), contains Tyr46. A second loop contains the essential general acid/base Asp 181. One important structural feature in PTPases is that ligand binding promotes a major conformational change of the general acid/base-containing loop, which brings Asp181 into the catalytic site and forms a hydrogen bond to the phenolic oxygen of phosphotyrosine to provide the general acid function (Jai et al., 1995).

In the ligand bound form, the aryl side chain of Tyr46 and Phe182 sandwiches the phosphotyrosine ring and delineates the boundaries of the tyrosine-binding pocket in PTPases. Protein tyrosine phosphatases employ covalent catalysis, utilizing the thiol group of the active site cysteine residue (Cys215 in PTP1B) as the attacking nucleophile, to form a thiophosphoryl enzyme intermediate (E-P) (Guan and Dixon, 1991). Substitutions of Cys residue stops PTPase activity (Streuli et al., 1990). The nucleophilic cysteine is housed within the active site architecture specifically designed to bind a negatively-charged substrate. The E-P formation is assisted by a conserved aspartic acid (Asp 181 in PTP1B) functioning as a general acid to neutralize the build-up of a negative charge on the leaving group (Zhang et al., 1994). The PTPases further accelerate the formation and hydrolysis of E-P by preferentially binding the pentacoordinated transition states with the guanidinium side chain of the active site arginine residue (Arg221 in PTP1B) (Zhang et al., 1999).

There is rich literature describing the structure and function of PTPases based on kinetic analysis of various protein tyrosine phosphatase mutations. The structure and function of PTPase amino acid residues have been carefully examined and the effects of mutations on the structure are summarized in Table 2 (Zhang, 2002).

Table 2: Correlation between effect of mutagenesis and structure of PTPases (adapted from Zhang Z-Y, 2002)

RESIDUE	CONSERVATION	EFFECT OF MUTATION	STRUCTURE/FUNCTION
Tyr46	Conserved	800-fold decrease in E-P formation	Hydrophobic packing with pTyr in the substrate
Arg47	Variable	10-fold decrease in E-P formation, no effect on E-P hydrolysis and pNPP reaction	Flexible, adopts two conformations for peptide recognition
Asp48	Conserved	20-fold decrease in E-P formation, no effect on E-P hydrolysis and pNPP reaction	Forms two H-bonds with the main chain N atoms of pTyr and the +1 position of peptide substrates
Glu115	Invariant	100-fold decrease in k_{cat}	Coordinates Arg221 with two H-bonds
Trp179	Invariant	Similar effects to those observed for the Asp181 mutants	Located at one of the hinges of the general acid loop, important for precise positioning of Asp181
Asp181	Invariant	10^3 - 10^4 -fold decrease in E-P formation, 10^1 - 10^2 -fold decrease in E-P hydrolysis	General acid in E-P formation, general base in E-P hydrolysis
Phe182	Variable	10-20 fold decrease in both E-P formation and E-P hydrolysis	Hydrophobic stacking with pTyr in the substrate, important for precise positioning of Asp181
His214	Invariant	Increase in the pKa of the active site thiol group, 100-fold decrease in k_{cat}	The side chain of His214 interacts with the carbonyl of Cys215
Cys215	Invariant	Destroys phosphatase activity, retains substrate binding ability	Active site nucleophile for phosphate ester hydrolysis
Arg221	Invariant	10^4 -fold decrease in k_{cat} , 10-fold increase in K_m when replaced with residues other than lysine.	Forms bidentate hydrogen bonds with the nonbridge phosphoryl oxygens in the substrate.
Ser222	Conserved	<10-fold decrease in E-P formation.	H-bond to stabilize the active site thiolate in E-P hydrolysis
Arg254	Invariant	50-fold decrease in k_{cat} , can be replaced by a Lys	Surface exposed, participates in a H-bond array to stabilize the P-loop
Arg257	Invariant	10^3 -fold decrease in k_{cat}	Buried, H-bonds with carbonyls of residues in the P-loop, structural role
Gln262	Invariant	10-fold decrease in E-P formation, 100-fold decrease in E-P hydrolysis, confers phosphotransferase activity	Coordinates scissile oxygen in the substrate and the attacking water
Gln266	Invariant	10^2 -fold decrease in E-P formation, 10-fold decrease in E-P hydrolysis	Makes H-bonds with the phosphoryl group and active site waters

Involvement of protein tyrosine phosphatases in the control of the cell cycle

In contrast to the full-length enzyme, expression of the truncated 37kDa form of PTPase in BHK cells produced striking changes in morphology in some cases; the cells became multinucleated, apparently due to a defect in cytokinesis. Even more unexpected was the fact that nuclear division was asynchronous. While cases of multinucleation are well documented, to our knowledge, nuclear division is always synchronous. The truncated PTP therefore seems to interfere with signal synchronization. One candidate substrate for PTPases is p32^{cdc2}, a protein serine threonine kinase that functions in a cell cycle control mechanism ubiquitous in eukaryotes (Lewin, 1990).

The activity of p34^{cdc2} varies dramatically during the cell cycle, a phenomenon associated with various regulatory molecules. Phosphotyrosine is first detected in p34^{cdc2} at the onset of DNA synthesis and rises to a maximum in G₂ phase. Activation of p34^{cdc2} in *S. pombe* requires dephosphorylation of a tyrosyl residue in the adenosine triphosphate binding site, driving the cell from G₂ to the M phase (Draetta and Beach, 1988).

Protein tyrosine phosphatase substrate specificity

Extensive biochemical and structural studies on PTPases have led to a detailed understanding of the mechanism by which PTPases catalyze phosphate monoester hydrolysis. In spite of the remarkable progress in the identification and characterization of new PTPases, the understanding of PTPase catalysis and the molecular basis by which PTPases distinguish and recognize the diverse substrates in the cell remains largely undefined. This is partly because the physiological substrates for most PTPases are unknown. Furthermore, since the physiological substrates for PTPases are phosphoproteins, even if the identity of the substrate is known, it remains a major technical challenge to obtain quantities of specifically and stoichiometrically phosphorylated proteins required for detailed enzymological studies. Based on the available data, it appears that members of the PTPase family display a spectrum of substrate specificity. In general, the tyrosine specific phosphatases show only moderate specificity in the context of peptide substrates, while the dual-specificity phosphatases exhibit more stringent substrate specificity. For example, at one end of the spectrum, PTP-1B possesses extraordinary plasticity in accommodating various phosphotyrosine containing sequences, while at the other end of the spectrum, MAP kinase phosphatase 3 (MKP3) displays exquisite substrate specificity, which requires extensive protein-protein interactions away from the active site for substrate recognition (Zhang, 2002)..

Regulation of protein tyrosine phosphatase function by reversible oxidation

Recently emphasis has been placed on the importance of production of reactive oxygen species (ROS) as a mechanism for fine-tuning phosphorylation-dependent signaling pathways through transient oxidation and inactivation of members of the PTPase family. Due to the unique environment of the PTPase active site, the essential Cys residue of the signature motif displays an unusually low pK_a and is present predominantly as the thiolate anion at neutral pH. This enhances its nucleophilic property and also renders the PTPases susceptible to oxidation with concomitant inhibition of activity. Pervanadate inhibits PTP-1B by oxidation of the active site Cys to sulfonic acid, the triply oxidized ($-SO_3H$) form; even though this oxidation to sulfinic acid ($-SO_2H$), is an irreversible modification (Huyer et al., 1997).

In contrast, H_2O_2 , a ROS produced in response to a variety of physiological stimuli, leads to oxidation of PTPases to a stable, singly oxidized sulfenic acid ($-SOH$) (Sundaresan et al., 1995). This inhibits activity because the Cys can no longer function as a nucleophile, but this modification is reversible and therefore can form the basis for a mechanism for transient inhibition of PTPase activity. Interestingly, PTP-1B has now been shown to be oxidized reversibly under physiological conditions, for example in response to epithelial growth factor (EGF) (Lee et al., 1998) and insulin (Mahadev et al., 2001).

One function of ROS generated in response to a physiological stimulus is to inactivate the critical PTPases that provide an inhibitory constraint upon the signal transduction pathway induced by that stimulus, thereby enhancing tyrosine phosphorylation and facilitating the signaling response (Tonks, 2003).

PROTEIN TYROSINE PHOSPHATASE-1B

Protein tyrosine phosphatase-1B is a prototype protein tyrosine phosphatase which was first isolated from human placenta. PTP-1B was shown to comprise a single N^α-acetylated polypeptide chain of 321 residues (Charbonneau et al., 1989). Placental PTP-1B is not structurally related to the serine-threonine-specific phosphatases, indicating that the phosphatases have evolved as separate families in contrast to the protein kinases, which all appear to be derived from a common progenitor (Hunter, 1987).

Further studies revealed that the protein tyrosine phosphatase family can be subdivided into two broad categories, the classical, protein tyrosine specific enzymes typified by PTP-1B and CD45, and the dual-specific phosphatases, which dephosphorylate Ser/Thr as well as tyrosine residues in proteins (Tonks, 2003). These latter enzymes, which largely maintain the same catalytic mechanism as the classical protein tyrosine phosphatases but display differences in the architecture of the active site, have been implicated in fundamentally important signaling events from control of MAP kinases in cell proliferation to the regulation of cyclin-dependent kinases in the cell cycle. These observations helped to shift the emphasis in research into the signaling function of tyrosine phosphorylation to include both protein tyrosine kinases and phosphatases (Tonks, 2003). Since the discovery of PTP-1B about 15 years ago continued investigation has revealed important insight into the structure, regulation and

function of this enzyme, culminating into a major target for a novel therapeutic strategy for the treatment of diabetes, obesity and cancer.

However, PTP-1B was found to be homologous to each of two tandem domains in the cytoplasmic portion of the leukocyte common antigen (CD45) (figure 3, Charbonneau et al., 1988). CD45 is a major transmembrane protein expressed on the surface of hematopoietic cells which has been shown to possess intrinsic tyrosine phosphatase activity (Tonks et.al., 1988). Subsequently, other transmembrane PTPases have been identified, indicating that the PTPases represent a diverse family of enzymes with both intracellular and integral membrane forms. The structural organization of CD45 and its transmembrane homologs resembles that of several integral membrane tyrosine kinases such as the epidermal growth factor receptor. Thus, these receptor-linked PTPases may be capable of initiating transmembrane signaling in response to external stimuli. Sequence analysis revealed a segment of approximately 230 residues conserved among two nonreceptor and four transmembrane PTPases. This segment was later identified in all PTPases and comprised a catalytic domain containing a common cysteinyl residue that is required for activity (Streuli et al., 1990).

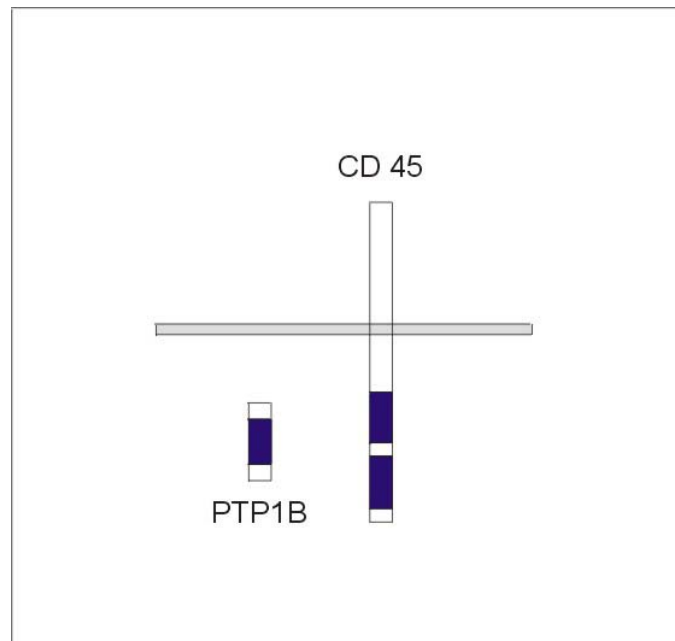


Figure 3: Structural relationship between transmembrane CD45 and PTP-1B (adapted from Charbonneau et al., 1988)

These findings account for the known sensitivity of PTP-1B to thio-directed reagents and suggest that this cysteinyl residue participates in the catalytic mechanism. This essential cysteine is located within a highly conserved segment of 11 residues with the consensus sequence, [I/V]HCXAGXXR[S/T]G, which is a hallmark of the PTP domain (figure 4, Fischer et al., 1991).

Isolation of cDNAs for the nonreceptor PTPases from human T-cells (Cool et al., 1989), rat brain (Guan et al., 1990), and human placenta (Brown-Shimer et.al., 1990) has been reported. The latter two cDNAs encode PTP-1B, but the T cell gene product represents a distinct enzyme displaying 65% sequence identity with PTP-1B (Cool et al., 1989). In all cases, the cDNAs encode proteins with an extension of about 11kDa at their COOH-terminal relative to the enzyme purified from placenta, which is a truncated product of the PTP-1B gene. The exon organization of the human PTP-1B gene indicates that truncation cannot be ascribed to RNA splicing (Brown-Shimer et al., 1990). The sequence of the COOH-terminal extensions of all three nonreceptor PTPases are related; however the sequence similarity is far lower than that among the corresponding catalytic domain. The sequence identity between the rat and human PTP-1B COOH-terminal extensions is 49%, whereas that among the T cell PTP and PTP-1B enzymes is only 18%. The last 19 residues of the COOH-terminal extension are hydrophobic and may be responsible for localization of the full length enzymes to the particulate fraction (Cool et al., 1990).

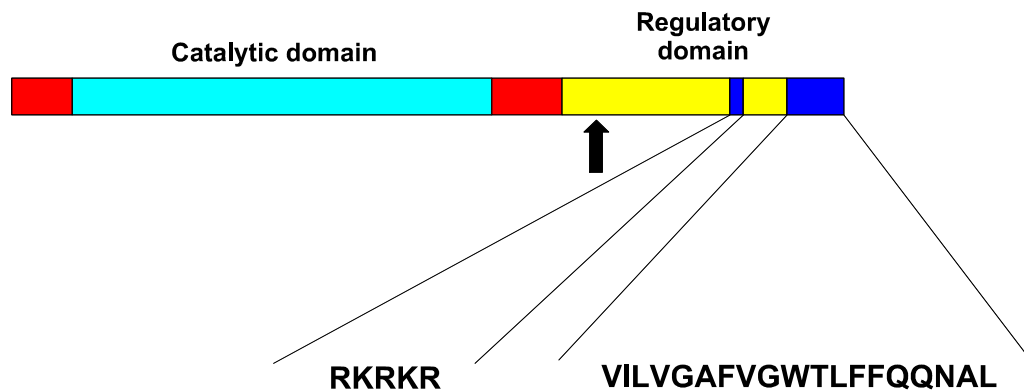


Figure 4: Diagram illustrating the structural organization of T cell PTP

The position of the conserved catalytic domain and a putative regulatory domain is indicated. Sequences for a potential nuclear localization signal and hydrophobic COOH-terminal segment are given in single letter codes (adapted from Fischer et al., 1991)

The PTP-1B protein: distinct catalytic and regulatory domains

Initially purified as a catalytic domain of 37kDa, the isolation of cDNA-encoding PTP-1B revealed a full length form of the protein that also contains an extension of 114 residues on the C-terminal side of the catalytic domain (Brown-Shimer et al., 1990). The C-terminal 35 residues are predominantly hydrophobic in nature and function in targeting the enzyme to the cytoplasmic face of membranes of the endoplasmic reticulum (Frangioni et al., 1992). The targeting of PTPases to defined subcellular locations contributes to the regulation of their function by restricting the spectrum of substrates to which they can gain access. Alternative mRNA splicing generates additional structural and functional diversity among several PTPase gene products. For example, TC-PTP, the closest relative of PTP-1B, exists in alternative spliced forms of either 48kDa, which like PTP-1B contains a hydrophobic C-terminus and is targeted to the endoplasmic reticulum, or 45kDa, which lacks the hydrophobic segment and shuttles in and out of the nucleus under the influence of a bipartite nuclear localization signal (Tiganis et al., 1997).

Two spliced variants of PTP-1B have also been reported, the relative levels of which are regulated by growth factors or insulin. The mRNA splicing event, which leads to retention of the last intron in the PTP-1B gene, results in only minor differences in the sequence at the extreme C-terminus of the protein, but major differences in the 3'-UTR of the mRNA, the function of which remains to

be established. It is important to realize that subcellular targeting is not the only aspect of the regulation of PTPase specificity (Tonks, 2003).

The C-terminal segment of PTP-1B not only controls activity indirectly by regulating subcellular location, but may also exert a direct effect on catalysis. PTP-1B is sensitive to proteolytic cleavage by calpain, for example following engagement of the gpIIb-IIIa integrin on the surface of platelets, which generate a truncated, soluble 42kDa form of the enzyme (Frangioni et al., 1993). Generation of this truncated form of PTP-1B, which lacks 75 residues from the C-terminal segment of the protein, including the endoplasmic reticulum targeting motif, is associated with enhanced PTPase activity, suggesting a role for the C-terminal segment in suppressing catalytic function. Such an inhibitory role has been demonstrated for the C-terminal segment of the closely related enzyme TC-PTP (Hao et al., 1997).

PTP-1B was one of the first PTPases shown to exist as a phosphoprotein in vivo. Several Ser/ Thr phosphorylation sites were identified in the regulatory C-terminal segment, including the phosphorylation of Ser 352 and 386, whose phosphorylation was regulated in a cell cycle-dependent manner (Flint et al., 1993). Although changes in activity were associated with changes in phosphorylation, the precise mechanism by which PTP-1B function may be regulated by phosphorylation is unclear. Nevertheless, phosphorylation of

members of the PTPase family by protein Ser/Thr kinases is now recognized as an important regulatory mechanism, with examples of both activation and inhibition of PTPase function. Interestingly, this represents a mechanism of potential cross-talk between signaling pathways, whereby activation of protein Ser/Thr kinases may lead to changes in tyrosine phosphorylation through effects on PTPase function. This represents a mechanism of potential cross-talk between signaling pathways, whereby activation of protein Ser/Thr kinases may lead to changes in tyrosine phosphorylation through effects on protein tyrosine phosphatase function. There have been reports of phosphorylation of tyrosyl residues in protein tyrosine phosphatases, including PTP-1B (Tao et al., 2001), although the functional significance of these modifications largely remains to be established.

The architecture of PTP-1B

Protein tyrosine phosphatase-1B is a 37-kD protein comprising a single domain topologically organized into 8 alpha helices and 12 beta sheets. Spanning the length of the molecule is a highly twisted, 10-stranded, mixed beta sheet. Beta-12, the final beta strand of this structure's primary sequence, is located proximally to a central region containing parallel strands with the order beta-3, beta-12, beta-4, and beta-11 (figure 5). This motif, in turn, is flanked by a series of anti-parallel beta strands. The center of the sheet is bounded on either side by alpha helix 2 on one side and helices alpha-3 and alpha-4 on the adjacent side. Following alpha-4, the chain is observed to form a four-helix bundle consisting of helices alpha-5, alpha-6, alpha-3, and alpha-4 (Walton and Dixon, 1993).

A short alpha-1 helix is found at the top of a beta-sheet neighboring beta-1. This segment denotes the beginning of the conserved PTP domain. Above the central beta-sheet is an additional anti-parallel beta-sheet containing beta-5 and beta-6. The carboxyl terminus of the chain is situated at the terminus of alpha-6. The non-conserved amino-terminal of the chain folds into two alpha-helices labelled alpha-1' and alpha-2'. These helices are found to wrap about the NH2-terminus of alpha-6 (Barford et al., 1998).

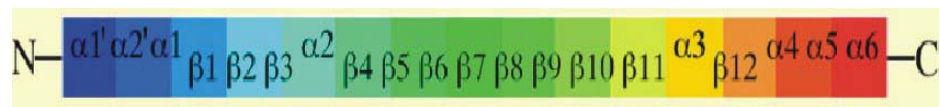
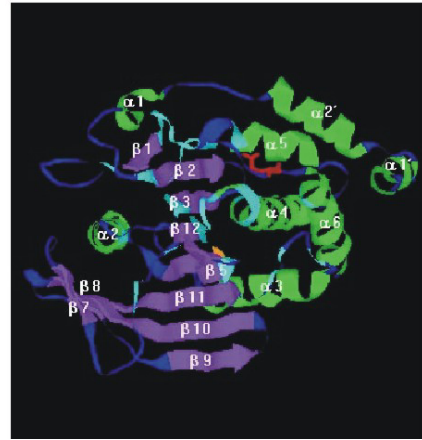
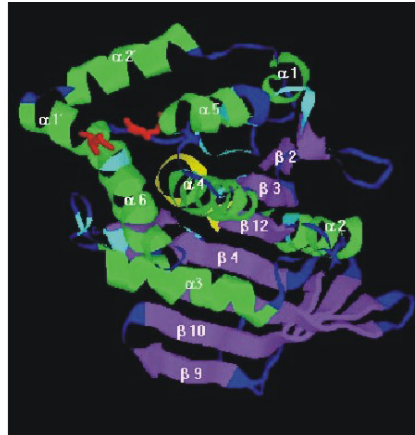


Figure 5: Secondary structure of PTP1B (from Walton and Dixon, 1993).

Situated on loop 15, a segment which connects beta-12 and alpha-4, is the cysteine residue (Cys215) critical to the catalytic function of this enzyme. Intervening loops connecting secondary structural elements are observed to converge about this region. Most of the 27 invariant residues in the sequences of PTP domains for which activity toward phosphotyrosine-containing proteins or peptides has been reported are found on these loops. Invariant residues more distally located from the catalytic site are buried and likely stabilize the protein's tertiary fold (Barford et al., 1998).

In both representations, both the N- and C-termini are highlighted in red (figure 6 and 7). Diversity within the PTP family of phosphatases is generated by the nature of the non-catalytic segments attached to these termini, which provide regulatory and sub-cellular targeting functions (Pannifer et al., 1998).



Figures 6 and 7: Ribbon representations of PTP 1B highlighting secondary structural elements

Green (alpha helices), purple (beta strands), loop sequences connecting these sequences (blue), invariant residues (cyan) and the catalytic site (yellow). The catalytic site is situated at the carboxyl-terminus of the central parallel region of the sheet (from Pannifer et al., 1998).

Molecular basis of phospho-peptides recognition by PTP-1B

Early studies show that PTP-1B displays limited selectivity towards peptides derived from *in vivo* phosphorylation sites (Zhang et al., 1993). The k_{cat}/K_m (substrate specificity constant) values for some peptide substrates approach the diffusion limit and are 3-4 orders of magnitude higher than those of phosphotyrosine (pTyr), which suggest that residues flanking the pTyr moiety make important contributions to the PTP-1B substrate recognition. It has been shown that DADEpYLIPQQG, which corresponds to residues 988-998 in EGF receptor, are the best substrate for PTP-1B. This peptide was subjected to a classical alanine scanning in order to identify amino acids important for PTP-1B recognition (Zhang et al., 1993). The kinetic analysis revealed that PTP-1B displays moderate preference for acidic residues at positions N-terminal to pTyr, particularly in the -1 position. It was also found that in EGF receptor peptides, the hexapeptide DADEpYL-NH₂ is an excellent substrate for PTP-1B with a k_{cat}/K_m of $2.24 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Zhang et al., 1994).

The crystal structure of DADEpYL-NH₂ bound to the catalytically inactive PTP-1B/C215S revealed key features for peptide binding (Jia et al., 1995). Besides vital hydrogen bonds and hydrophobic interactions between the pTyr residue and PTP-1B active site, several hydrogen bonds involve the peptide backbone that stabilizes the binding interface. This complex provides a structural interpretation for PTP-1B's preference for N-terminal acidic residues

and for the superiority of DADEpYL-NH₂ over pTyr. It appears that efficient substrate recognition by PTP-1B requires the presence of amino acids on both sides of phosphotyrosine (Pannifer et al., 1998).

An affinity-selection strategy was utilized to identify high-affinity peptides for PTP-1B, which contained the nonhydrolyzable phosphotyrosine mimic, phosphonodifluoromethyl phenylalanine. The -1 position was shown to accept aromatic residues such as tyrosine and phenylalanine with affinity comparable to substitutions by glutamine and aspartate. The position immediately C-terminal to pTyr (+1) did not show a strong preference, and residues glycine or methionine appeared to be somewhat selected, and the basic residue arginine was disliked in any position (Huyer et al., 1998).

The structural basis for the plasticity of PTP-1B substrate recognition was revealed by a comparison of the structure of PTP-1B-DADEpYL-NH₂ with two novel structures of PTP-1B with the peptides ELEFpYMDYE and DAD(Bpa)pYLIPQQG, respectively containing aromatic amino acids [Phe and p-benzoylphenylalanine (Bpa)] in position -1. In all three structures, the bound peptide is similarly orientated and maintains the same N- to C-terminal direction relative to PTP-1B surface. The dominant interaction involves phosphotyrosine itself with PTP-1B. All three structures maintain two conserved hydrogen bonds between the Asp48 carboxylate side chain and the main chain nitrogens of the

pTyr and the +1 residue, and a third between the main chain nitrogen of Arg47 and the main chain carbonyl of the -2 residue. The peptide backbone of residues from the -1 to the +1 position in all three structures adopts a twisted β -strand conformation, which ensures that the phosphotyrosine is bound to PTP-1B in an identical manner, regardless of the specific sequence adjacent to it (Sarmiento et al., 2000).

The ability to accommodate both acidic and hydrophobic residues appears to be conferred upon PTP-1B by a single residue, Arg47. Depending on the nature of the N-terminal amino acids, the side chain of Arg47 can adopt two different conformations, generating two sets of distinct peptide binding surfaces. When the acidic residue is positioned at the -1 position, a preference for a second acidic residue is observed at the -2 position. When a large hydrophobic group occupies the -1 position, Arg47 adopts a new conformation so that it can participate in hydrophobic interactions with both the -1 and -3 positions. Thus the conserved mode of interaction between the peptides and PTP-1B provides a structural basis for the observed plasticity in PTP-1B substrate recognition (Sarmiento et al., 2000).

Functional significance of PTP-1B active site plasticity

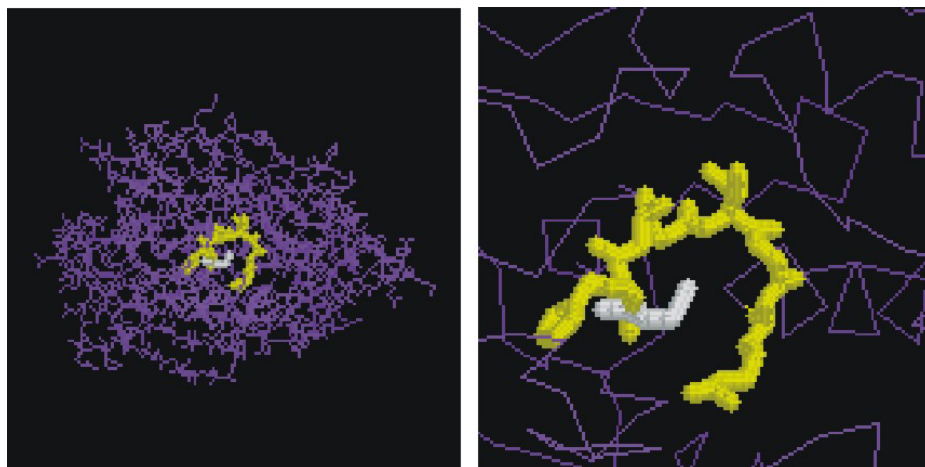
The ability of PTP-1B to recognize common substrate features and to generate altered binding surfaces to accommodate diverse amino acid sequences may be essential for its function as a broad specificity PTPase. Numerous studies have implicated PTP-1B in various signaling pathways. For example, PTP-1B has been suggested as a negative regulator of insulin signaling (Kenner et al., 1996). In addition to a role in insulin signaling, PTP-1B is over expressed in association with the expression of p185^{c-erbB-2} in human breast and ovarian cancers (Wiener et al., 1994). PTP-1B is capable of suppressing transformation by Neu (Brown-Shimer et al., 1992), v-Crk (Liu et al., 1998), v-Src (Woodford-Thomas et al., 1992), and v-Ras (Liu et al., 1998).

Recently, PTP-1B has been identified as the major PTPase that dephosphorylates and activates c-Src in several human breast cancer-cell lines (Bjorge et al., 2000). PTP-1B is also capable of antagonizing signaling by the EGF receptor (Liu and Chernoff J, 1997) and the oncoprotein p210^{bcr-abl} (LaMontagne K.R Jr et al., 1998) by dephosphorylating directly the EGF receptor and the p210^{bcr-abl} tyrosine kinase. Protein tyrosine phosphatase-1B can negatively regulate integrin-mediated adhesion and signaling by binding and dephosphorylating β -catenin (Balsamo et al., 1998) and p130^{cas} (Crk associated substrate) (Liu et al., 1996). These results suggest that PTP-1B may be a participant in several pathways.

Many of the suggested PTP-1B substrates are receptor tyrosine kinases (e.g. insulin receptor and EGF receptor) and adapter proteins that contain multiple tyrosine phosphorylation sites. A choice that a cell has to make is whether to enlist multiple PTPs that are highly specific for each phosphorylation site or to rely on a broad specificity PTP, such as PTP-1B, that is strategically positioned in the cell for efficient substrate dephosphorylation. Protein phosphorylation sites are generally located on surface loops, which may be mimicked by peptides. Because the k_{cat}/K_m values of peptide substrates for PTP-1B already approach the efficiency limited by diffusional events, it is unlikely that protein substrates for PTP-1B will exhibit kinetic properties that are any better than the peptide substrates. Thus it is possible that cellular localization may dictate the substrate accessibility for PTP-1B. Once the substrate is exposed to PTP-1B complete dephosphorylation occurs. Considering the large number of protein tyrosine kinases and their substrates, many of which have multiple phosphorylation sites, it is unrealistic to expect every protein tyrosine phosphatase to be specific for a single phosphorylation site (Zhang, 2002).

Protein tyrosine phosphatase-1B catalytic site organization

The catalytic site is located within a crevice on the molecules surface (figures 8 and 9). The base of the catalytic site is formed by the residues His214 through Arg221. Loops surrounding the site contain conserved amino acids at positions 37 through 55, 86 through 90, 177 through 187, and 255 through 263. The small beta sheets beta-5 and beta-6 are located above the site. Polar groups on neighboring conserved residues aid in the orientation of the phosphate binding loop by forming H-bonds to main-chain carbonyl groups on the loops (Pannifer et al., 1998).



Figures 8 and 9: Surface views of the catalytic cleft of PTP-1B

The catalytic cleft is emphasized on critical residue Cys-215 in white. The highly conserved residues His-214 to Arg-221, believed to be essential to the catalytic activity of PTP-1B is highlighted in yellow. The remainder of the protein is represented in purple (from Pannifer et al., 1998).

The phosphate binding active site is positioned at the amino terminus of alpha-4 (figure 9). It is created by main-chain atoms from residues located on the loop joining beta-12 with alpha-4 (Ser216-Gly218), and the amino-terminal residues of alpha-4 (Ile219-Arg221). In addition, the side chains of the invariant residues Cys215, Arg221, and Gln262 have also been observed to participate. Somewhat unusual in this region is the presence of a buried charged residue, Arg257. The ability of this residue to remain deep within the protein's core has been explained by noting that its guanidinium side chain can participate in a hydrogen bond network formed from a cluster of invariant amino acids, including Asn44, Tyr66, and Asn68 (Figure 10). In addition, the position of Arg257 adjacent to the phosphate binding site contributes to substrate affinity as a result of an increase in electrostatic potential at the catalytic site (Zhong-Yin et al., 1996).

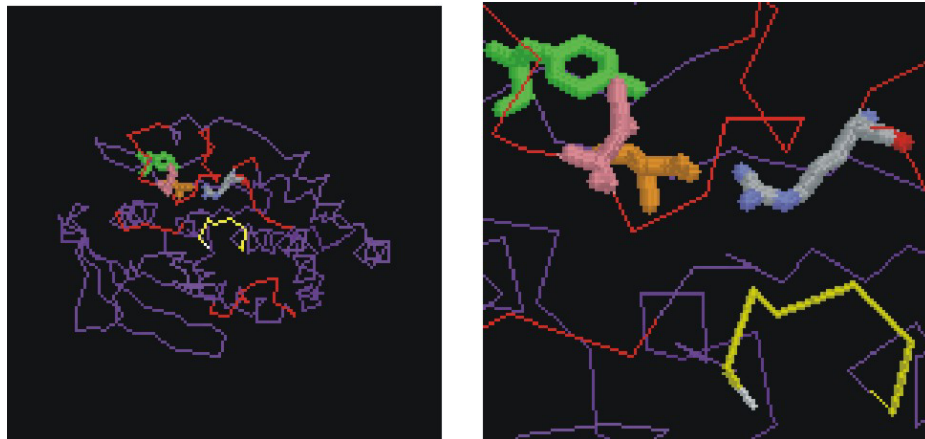


Figure 10: The unusual buried environment of Arg-257 in PTP-1B

Arg-257, participates in a hydrogen bond network with surrounding residues Asn-68 (orange), Tyr-66 (green), and Asn-44 (pink), stabilizing its presence within the protein's hydrophobic core. The close positioning of Arg-257 to PTP-1B's phosphate binding site suggests that this residue aids in substrate binding by increasing the electrostatic potential at the catalytic site (from Zhong-Yin et al., 1996).

Structural elements leading to binding interaction and substrate specificity

Protein tyrosine phosphatase-1B has been shown to preferentially dephosphorylate phosphotyrosine proteins. Kinetic studies (Zhang et al., 1993; Hippen et al., 1993) have shown that phosphotyrosine proteins are markedly less reactive toward free phosphorylated tyrosine residues. These findings suggest that substrate specificity arises from interactions between the enzyme and residues following tyrosine phosphorylation on the peptide ligand. It has also been shown that selectivity is strongly influenced by the nature of the residues following phosphotyrosine.

The structure of PTB-1B in complex with the tetra-peptide AC-DEpYL-NH₂ (Barford et al., 1998) reveals the molecular mechanisms underlying substrate recognition. The recognition pocket for phosphotyrosine containing peptides is the dominant feature observed in the binding site of PTP-1B. A region defined at its base by Arg-221 and Asp-48 correlates exactly with the length of the phosphotyrosine residue. Barford and co-workers (1998) have also shown that the depth of the binding pocket contributes to the phosphotyrosine specificity of the enzyme. In contrast to phosphotyrosine, both phosphoserine and phosphothreonine are too short to reach the catalytic residues situated at the base of the catalytic cleft.

The O-gamma atom of Ser215 (figure 11), situated within the center of the cradle, is positioned 3.2Å from the phosphorus atom of phosphotyrosine. This places Ser215, O-gamma, the phosphotyrosine phosphorus, and the phenolic oxygen in a roughly colinear relationship. As a result, it appears likely that a Cys residue at position 215 would be placed ideally to undergo a nucleophilic attack of the substrate phosphorus (Figure 12, Barford et al., 1998).

A primary determinant of the peptide conformation at the phosphotyrosine site is the formation of two hydrogen bonds between the *p+1* residue of the ligand with the side-chain of Asp48. These hydrogen bonds stabilize the α -helical main-chain conformation at the phosphotyrosine site of the peptide and, in part, define the peptide's orientation in the active site. Because the Asp48 residue is observed to be highly conserved in other PTPs, it is believed that this residue has a role to play in peptide recognition.

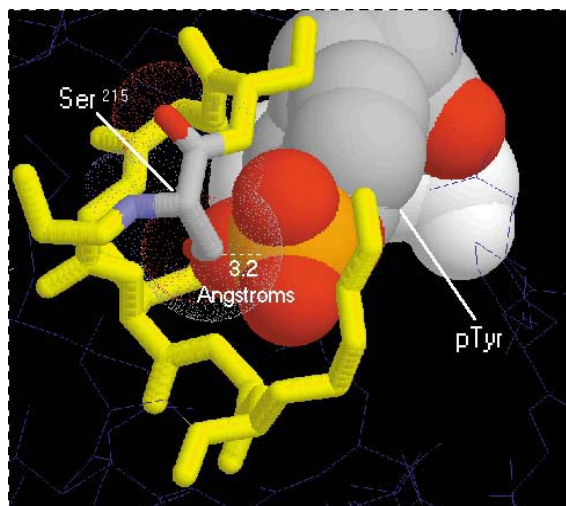


Figure 11: The O-gamma atom of Ser-215 in PTP-1B

Located within the center of the cradle, 3.2 angstroms from the phosphorus atom of phosphotyrosine is the Ser-215 O-gamma, phosphotyrosine phosphorus and phenolic oxygen atoms. This suggests that that the Cys residue in the catalytically active form of the enzyme is ideally positioned for nucleophilic attack of the substrate phosphorus (adapted from Barford et al., 1998).

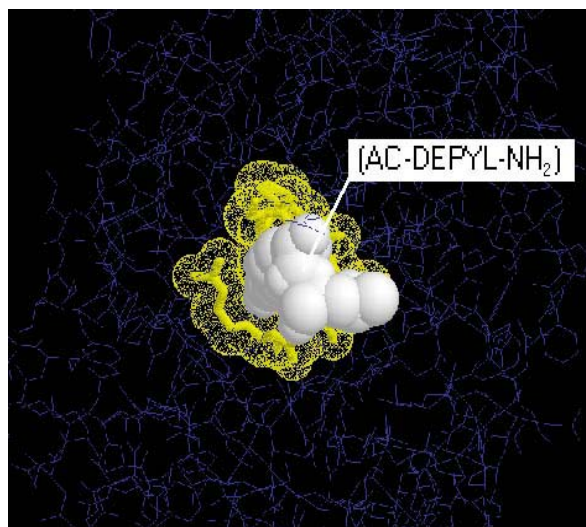


Figure 12: Structure of the catalytically inactive form of PTP-1B

The predominant feature of peptide binding is the deep penetration of the phosphotyrosine side chain into a recognition pocket whose base corresponds to the phosphate binding site situated on a loop (adapted from Barford et al., 1998).

The final factor that influences PTP-1B's ability to bind with specificity to its substrates is the number of non-polar side chains lining the catalytic cleft. Residues capable of hydrophobic interactions with the phenyl ring of the phosphotyrosine residue are found on the side chains of Ala217 and Ile219, Tyr 46, Val 49, Asp181, Phe 182, and the hydrophobic portion of the side chain of Gln262. Engagement of a phosphopeptide by PTP-1B promotes a major conformational change in the loop comprising residues 179-187. Individual atoms are observed to shift by more than 8 angstroms to close over the phenyl ring of phosphotyrosine, allowing the side chain Asp181 to come into close enough contact with the ligand to function as a general acid in the catalytic reaction. In addition a close association between phosphotyrosine and Phe182 provides for the hydrophobic packing of the phenyl ring. The sum of these movements is the formation of the stabilized, catalytically competent conformation of the loop (Barford et al., 1998).

Catalytic mechanism of protein tyrosine phosphatases

An understanding of the chemical steps involved in the catalytic mechanism of protein tyrosine phosphatases includes:

Cleavage of the P-O bond of the tyrosine residue: (figure 13)

The critical role of the Cys215 residue has been demonstrated both through kinetic and, more recently, crystallographic studies. Site-directed mutagenesis of the active site cysteine has been shown to result in the complete loss of enzymatic activity. These findings are explained by additional studies using rat PTP-1B which demonstrate that the cysteine forms a thio-phosphate intermediate during the reaction mechanism. The pKa of the active site Cys residue is 4.7, indicating that at physiological pH, the species is predominantly in its anionic form (Tonks, 2003).

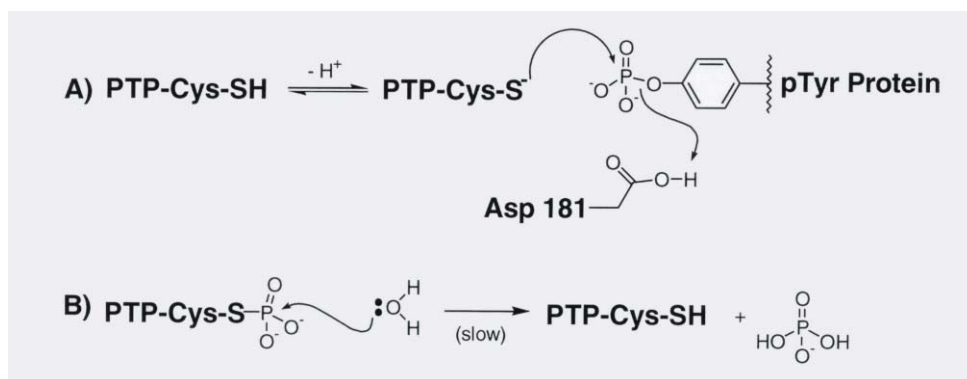


Figure 13: Proposed mechanism for the hydrolysis reaction catalyzed by PTP-1B

(A) Deprotonation of the catalytically essential residue Cys-215 allows a thiol anion, the reactive form of the enzyme. Nucleophilic cysteine may next attack the phosphate group of a phosphorylated protein. (B) Subsequent hydrolysis of the cysteinyl-phosphate intermediate by addition of a nucleophilic water molecule completes the catalytic cycle by regenerating the native enzyme. The cleaved inorganic phosphate diffuses into solution (adapted from Tonks, 2003).

Once the thiolate anion has formed, and the peptide ligand has been correctly positioned within the active site through the structural elements, dephosphorylation may commence. The reaction is initiated through a nucleophilic attack by the sulphur atom of the catalytic cysteine residue on the phosphotyrosine phosphorus atom. Cleavage of the scissile P-O bond is assisted through the protonation of the phenolic oxygen by the proximal acidic residue Asp181. This results in the subsequent formation of a phosphocysteine intermediate and the diffusion of the HO-tyrosine residue out of the active site. The anionic phosphate ion, now covalently attached to S-gamma Cys215, is stabilized through hydrogen bond interactions with main-chain nitrogens lining the catalytic cleft (Figure 14; Tonks, 2003).

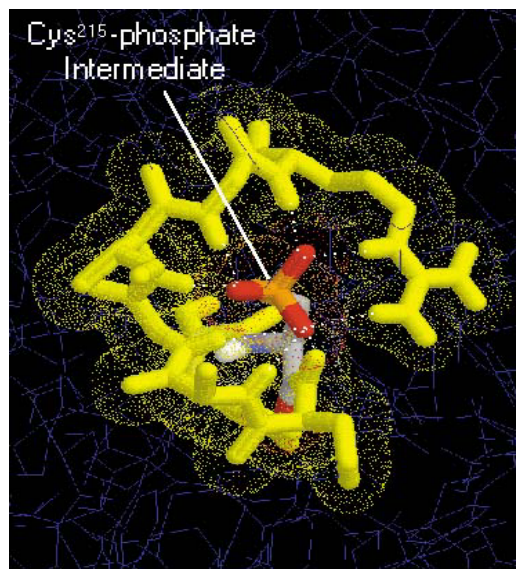


Figure 14: Hydrogen bonded cysteinyl-phosphate intermediate of PTP-1B

Once the tyrosine peptide has diffused out of the active site, a conformationally flexible loop is closed over the active site, preventing non-specific phosphoryl transfer reactions to extraneous phosphoryl acceptors. The subsequent step in the reaction sequence would be the hydrolysis of this transient intermediate by an activated water molecule (adapted from Tonks, 2003).

Stabilization of the cysteinyl-phosphate structure

A water molecule, coordinated by hydrogen bonds to the side chains of Gln262 and Asp181, is situated directly above and in line with the P-S bond of the phosphocysteine. The relative pKa values of the Asp and Gln residues suggest that Gln262 functions more to correctly position the water molecule, while Asp181 serves as a general base. Abstraction of a proton from the sequestered water molecule by this second residue increases the water's nucleophilicity, facilitating attack on the phosphorus atom of the intermediate (figure 15, Pannifer et al., 1998).

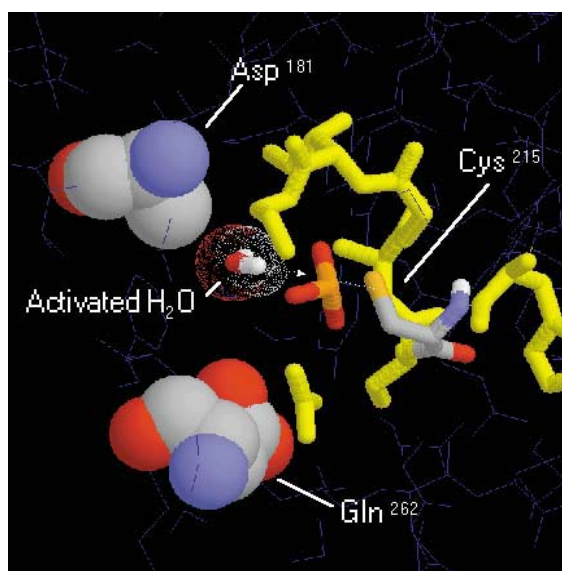


Figure 15: A sequestered water molecule of PTP-1B

Sequestration is coordinated by bifurcated hydrogen bonds to the side chains of Gln-262 and Asp-181. Abstraction of a proton by from the water molecule by Asp-181 increases the nucleophilicity of the water molecule, facilitating attack onto the phosphorus atom of the intermediate (adapted from Pannifer et al., 1998).

Protein tyrosine phosphatase inhibitor development

Protein tyrosine phosphatase function is critical for the maintenance of cellular homeostasis. Malfunction of PTPases has been linked to many human diseases (Zhang, 2001). When PTPase activity is inappropriately high, PTPase inhibitors may constitute as a valuable therapeutic tool. Drug development targeting protein phosphatases has yielded much success. For example, cyclosporine and FK506, inhibitors of the protein Ser/Thr phosphatase calcineurin, are among the most effective immunosuppressive drugs used after organ transplantation. Treating diseases with PTPase inhibitors was not seriously considered until recently.

Two major issues, both of which relate to specificity, are responsible for the hesitation in developing PTP-based therapy. There is concern that because the PTPase active site is highly conserved, it would be impossible to obtain selective PTPase inhibitors. There is also concern that a PTPase may regulate multiple signaling pathways while at the same time, a single pathway may be controlled by several PTPases. Protein tyrosine phosphatase-1B is ubiquitously expressed and is implicated in a variety of signaling processes, not making it an ideal target for drug development (Zhang, 2002).

Protein tyrosine phosphatase-1B inhibitors

Although phosphotyrosine by itself binds weakly to PTP-1B, phosphotyrosine (pTyr) is essential for binding of peptide/ protein-based substrates by PTP-1B. In fact, the recognition pocket for phosphotyrosine represents the dominant driving force for peptide binding since it contributes about 53% of the peptide solvent-accessible surface area (Jia et al., 1995). Since high-affinity substrate binding requires amino acids flanking the phosphotyrosine, one approach towards the design of potent and selective PTP-1B inhibitors relies on the incorporation of a nonhydrolyzable analog into specific optimal peptide templates. Several nonhydrolyzable analogs have been prepared and inserted into PTP-1B targeted peptides (Burke and Zhang, 1998).

The most commonly used phosphorus-based phosphotyrosine analog is phosphonodifluoromethyl phenylalanine (F₂Pmp) (Burke et al., 1993). When F₂Pmp replaces the phosphotyrosine in the hexapeptide DADEpYL-NH₂, the K_i for the resulting peptide bearing F₂Pmp is over 1000 times more potent than the same peptide containing phosphonomethyl phenylalanine (Zhang et al., 2000). This has been attributed to a direct interaction between the fluorine atoms and PTP-1B active site residues (Chen et al., 1995). The enhanced affinity of F₂Pmp over Pmp likely arises from the ability of the fluorine atoms in F₂Pmp to interact with the PTP-1B active site residues in a fashion analogous to that

involving the phenolic oxygen and side chains in the active site of PTP-1B (Burke et al., 1996).

Small molecule phosphotyrosine mimetics that exhibit higher or comparable binding to phosphotyrosine may not yield a potent PTP-1B inhibitor when introduced into a peptide template, owing to the need to maintain peptide binding outside of the pTyr pocket and the less than ideal interactions between the analog and the active site. It is known that phosphotyrosine alone is not sufficient for high-affinity binding by PTPases and residues. The binding site in PTPases is smaller than the ATP site in protein kinases, therefore inhibitor design, is even more critical to consider in adjacent peripheral sites than the active site in order to gain potency and selectivity (Gao et al., 2000).

Protein tyrosine phosphatase-1B and oncogenesis

The existence of oncoprotein protein tyrosine kinases, has led to suggestions of the potential role of PTPases as tumour suppressors and regulators of oncogenic transformation. Expression of PTP-1B in NIH3T3 cells has shown to suppress transformation by the PTKases Neu (Brown-Shimer et al., 1992) and v-Src (Woodford-Thomas et al., 1992). It has been shown that PTPase dephosphorylates and activates c-Src in human breast cancer cell lines (Bjorge et al., 2000), control cell adhesion, via effects on integrin signaling (Liu et al., 1998) and cadherin-catenin complexes (Xu et al., 2002).

Changes in the level of expression of PTP-1B have been noted in several human diseases, particularly those associated with disruption of the normal patterns of tyrosine phosphorylation. The expression of PTP-1B is induced by p210 Bcr-Abl oncoprotein, a PTK that is directly responsible for the initial manifestation of chronic myelogenous leukemia. These effects coincide with the ability of PTP-1B to antagonize p210 Bcr-Abl-induced transformation. Interestingly, expression of TC-PTP was not induced and, despite its structural similarity to PTP-1B, TC-PTP did not antagonize p210 Bcr-Abl function (LaMontagne Jr et al., 1998).

The induction of PTP-1B expression by p210 Bcr-Abl occurs at the level of transcription. A p210 Bcr-Abl responsive sequence (PRS) which is required for stimulation of activity in response to the protein tyrosine kinases has been identified (Fukada and Tonks, 2001). The PRS is contained in a sequence that displays features of a stress response element and functions as a binding site for three mammalian C₂H₂ zinc finger proteins; Egr-1, Sp1 and Sp3. Of these, both Sp1 and Sp3 function as positive regulators, whereas Egr-1 represses Sp3-mediated transactivation of the PTP-1B gene. The expression of p210 Bcr-Abl results in the down regulation of the levels of Egr-1. The reciprocal actions of Sp1/Sp3 and Egr-1 are an important aspect of the regulation of PTP-1B expression in response to the p210 Bcr-Abl oncoprotein.

In addition to the PRS, a transcription factor Y-box binding protein-1 (YB-1) has been identified which acts as a PTP-1B enhancer binding protein (Fukada and Tonks, 2001). The nucleic acid binding domain of YB proteins is highly conserved throughout evolution from bacteria to humans, displaying 43% identity with the cold shock response protein cs7.4 from *Escherichia coli*, suggestive of a fundamentally important function. YB proteins bind single- and double-stranded DNA and RNA and have been implicated in both transcriptional activation and repression, functioning directly as transcription factors and indirectly by altering promoter conformation. Although originally identified in terms of their ability to recognize a DNA sequence termed a Y box, the YB proteins are now known to recognize motifs with little similarity to the

classical Y box. There is no recognizable Y-box motif in the PTP-1B enhancer and so this interaction could not have been predicted from analysis of the promoter sequence. Using Rat1 fibroblasts, it has been demonstrated that over expression of YB-1 leads to an increase in the level of PTP-1B. Also depletion of YB-1 by expression of a specific antisense construct, led to a 70% decrease in expression of PTP-1B, but no change in the level of TC-PTP. Expression of antisense YB-1 resulted in increased sensitivity to insulin and enhanced signaling through the cytokine receptor gp130, which was suppressed by re-expression of PTP-1B. By targeting YB-1, the levels of PTP-1B and signaling through insulin and cytokine receptors can be manipulated (Fukada and Tonks, 2003).

INHIBITORS OF REVERSIBLE PHOSPHORYLATION

Effect of genistein on reversible phosphorylation

It has been known that diets rich in legumes and fiber decrease cancer risk relative to those diets which consists mainly of animal products (Saio, 1990). Recently the consumption of soy bean products has been associated with low rates of hormone-dependent and hormone-independent cancers (Barnes et al., 1990). Asians consume 20-50 times more soy per capita than Americans and have shown to have a lower incidence of breast and prostate cancer (Lee et al., 1991). Since soy contains the isoflavones genistein and daidzein at mg/g concentrations, it has been suggested that isoflavones might be acting as natural chemopreventive agents.

It has subsequently been shown that genistein inhibits the growth of a wide variety of tumour cells in culture. It has also been shown that growth inhibition is more effective with genistein in leukaemic cell lines than cell lines derived from solid tumours. Genistein inhibits cell growth stimulated by a variety of agents including steroid hormones, serum and those factors which act through receptors with protein tyrosine kinase activity, such as EGF and those which act through G-proteins such as thrombin (Fotsis et al., 1993).

Mechanism of action of genistein

Initial interest in genistein showed that genistein displays estrogenic properties in several animal species. This is not surprising since the structure of genistein closely resembles that of the estrogenic steroids. This led several investigators to hypothesize that genistein may act as an anti-estrogen and thus may have a role to play in cancer prevention. However it has subsequently been shown by Paterson and Barnes (1993) in both cells that express and lack estrogen receptors that growth is equally inhibited in both, disproving genistein to act through an estrogen receptor.

Genistein inhibits the activity of topoisomerase II in vitro by stabilizing the DNA-topoisomerase II complex, called the cleavable complex. Stabilization of this complex in tumour cells leads to double and single strand breaks in cellular DNA, leading to growth inhibition or cell death (Gewirtz, 1991). However at IC_{50} values for growth inhibition, little DNA damage is observed, suggesting that genistein does not inhibit cell growth through inhibition of topoisomerase II activity (Constantinou et al., 1990).

Production of reactive oxygen species has been postulated to play a role in carcinogenesis, particularly in tumour promotion. Although genistein has been used for several decades as a chemical antioxidant in the food industry, only

recently it has been shown to decrease production of reactive superoxides (ROS) by tumour cells. Genistein can inhibit both the priming events necessary for high level ROS production or can directly inhibit agonist-stimulated ROS production, suggesting that it may exert some of its anti-cancer effect through antioxidant mechanisms.

In 1987, it was discovered that genistein was a specific and potent inhibitor of PTK activity, with little inhibitory effect on serine/ threonine kinases (Akiyama et al., 1987). Genistein inhibited the activity of several tyrosine kinases in vitro, such as c-src and v-abl. Genistein is a potent inhibitor of epidermal growth factor receptor (EGF-R) PTK activity. Interestingly, genistein does not inhibit all PTK activities equally, and some such as p94 PTK are not inhibited at all, suggesting specificity to PTK inhibition by genistein (Akiyama et al., 1987).

Effect of okadaic acid on reversible phosphorylation

Okadaic acid is a polyether fatty acid first isolated from the marine sponges *H.okadaei* and *H.melanodocia* (Tachibana et al., 1981). Its structure is very similar to acanthifolicin isolated from the sponge *Pandoras acanthafolium*, which was the first polyether carboxylic acid to be reported from marine sources (Schmitz et al., 1981).

Okadaic acid was subsequently shown to be produced by several types of dinoflagellates and to concentrate in marine sponges by filter feeding. Okadaic acid is a member of the okadaic acid class of non-12-O-tetradecanoylphorbol-13-acetate (TPA)-type tumour promoter, which does not bind to the phorbol ester receptors in cell membranes or activate protein kinase C in vitro. It has potent tumour promoting activity on mouse skin, as strong as TPA-type tumour promoters, such as TPA, teleocidin and aplysiatoxin. DNA samples isolated from tumours induced with okadaic acid have a mutation at the second nucleotide of codon 61 (CAA to CTA) in the c-H-ras gene. Treatment of primary human fibroblasts and human keratinocytes with okadaic acid induced the hyperphosphorylation of a 60kDa protein in nuclear and cytosolic fractions, due to the inhibition of protein phosphatases (figure 16).

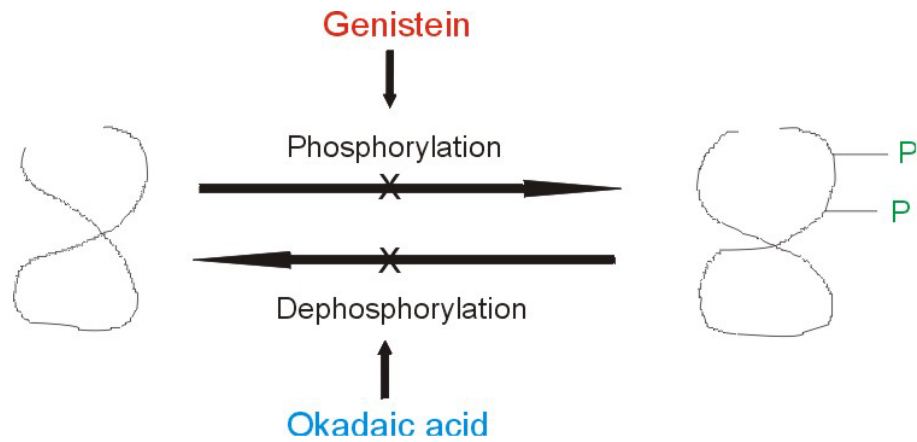


Figure 16: Effect of okadaic acid and genistein on protein phosphorylation

Genistein inhibits phosphorylation by affecting the activity of protein kinases and okadaic acid inhibits protein phosphatase activity and hyperphosphorylates proteins.

Mechanism of action of okadaic acid

The first clue to the mechanism of action of okadaic acid came with the discovery that okadaic acid caused a long-lasting contraction of isolated vascular smooth muscles from human umbilical arteries and rabbit aorta. Contraction was not mediated by neuronal mechanisms releasing neurotransmitters, because antagonists and 5-hydroxytryptamine (5HT) did not affect the response to okadaic acid. Smooth muscle contraction is triggered by the phosphorylation of one of the small subunits of myosin, the P-light chain, suggesting that okadaic acid might activate myosin P-light chain phosphatase.

It has also been shown that okadaic acid is a potent inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), two of the four major protein phosphatases in the cytosol of mammalian cells that dephosphorylate serine and threonine residues (Cohen and Cohen, 1989). Of the other two major protein-serine/ threonine phosphatases, the Ca^{2+} / calmodulin-dependent protein phosphatase 2B (PP2B) is far less sensitive, while the Mg^{2+} -dependent protein phosphatase 2C (PP2C) is unaffected (Bialojan and Takai, 1988). The mitochondrial pyruvate dehydrogenase phosphatase, protein phosphatases that dephosphorylate tyrosine residues, acid and alkaline phosphatases and inositol triphosphatase are all insensitive to the toxin. There is also no inhibition of the eight different protein kinases that have been tested, such as cyclic AMP or

Ca^{2+} / calmodulin-dependent protein kinases or protein kinase C (Haystead et al., 1989, figure 18).

Identification and quantitation of protein phosphatases using okadaic acid

Okadaic acid has improved the procedures for measuring protein phosphatase activity. Three of the major protein-serine/ threonine phosphatases in eukaryotic cells (PP1, PP2A and PP2C) have broad and overlapping substrate specificities in vitro, and their identification and quantitation in cell extracts presented problems before the advent of okadaic acid. Under normal assay conditions PP2A is completely inhibited by 1nM okadaic acid, while PP1 is unaffected at this concentration, its IC_{50} being 10-15 nM. PP2A can therefore be taken as the proportion of activity inhibited by 1nM okadaic acid, PP1 as the activity resistant to 1nM okadaic acid, and PP2C as the Mg^{2+} -dependent activity resistant to 1 μM okadaic acid.

The sensitivities of both PP1 and PP2A to okadaic acid have been remarkably conserved and are virtually identical in organisms as diverse as mammals, yeast and even higher plants (MacKintosh and Cohen, 1989). These observations are consistent with the extreme structural conservation of these enzymes that has recently emerged from cDNA cloning studies. The only exception so far is the

protozoan *Paramecium* where all PP2A-like activity is resistant to okadaic acid (Klumpp et al., 1990).

The effect of okadaic acid in cell-free extracts

The effect of okadaic acid on cell free extracts has led to the identification of several proteins whose activities are controlled by phosphorylation. In reticulocyte lysates, protein synthesis is known to become inhibited when phosphorylation of initiation factor eIF2 reaches a certain threshold level. However addition of low levels of okadaic acid (50nM) to reticulocyte lysates was found to block protein synthesis, without increasing eIF2 phosphorylation. Using ^{32}P -labelling revealed that okadaic acid caused a striking increase in the phosphorylation of a 100kDa protein that co-migrated with elongation factor-2 (EF2), a protein identified as the target for a specific Ca^{2+} / calmodulin-dependent protein kinase (Nairn and Palfrey, 1987). Addition of a Ca^{2+} chelator EGTA prevented okadaic acid from stimulating phosphorylation of the 100 kDa protein and inhibiting protein synthesis.

Maturation promoting factor (MPF) is a protein kinase whose catalytic subunit (p34cdc2) is activated just prior to M-phase and plays a central role in driving cell cycle events (e.g. nuclear-envelope breakdown, chromosome condensation and mitotic-spindle reorganization) in eukaryotic cells. Since MPF activation is

known to involve dephosphorylation of threonine and tyrosine residues on the p32cdc2 component, it was surprising when okadaic acid was found to increase the rate and extent of MPF activation in frog egg extracts (Felix et al., 1990), a cell free system which reproduces certain aspects of the mitotic cycle.

Large T-antigen is a multifunctional protein encoded by the SV40 viral genome, which regulates viral gene expression, initiates viral DNA replication and stimulates host cell replication. Okadaic acid has been shown to inhibit T-antigen stimulated DNA replication, while preincubation of T-antigen with PP2A stimulated the rate of replication. These observations indicated that the T-antigen is most active in stimulating DNA synthesis when a serine or threonine residue on the protein has been dephosphorylated by PP2A.

Effect of okadaic acid on intact cells

Okadaic acid is hydrophobic and can therefore enter cells easily. It has been shown in ³²P-labelled hepatocytes (Haystead et al., 1989), adipocytes (Haystead et al., 1989) and chromaffin cells (Haavik et al., 1989) that the addition of okadaic acid to the incubation medium increases the phosphorylation states of many proteins within minutes, and stimulates overall levels of protein phosphorylation by 2.5 to 3-fold. Okadaic acid has no effect on the intracellular ATP levels and no toxic effects in short term incubation. Proteins whose

phosphorylation was increased in hepatocytes and adipocytes included the rate limiting enzymes of glycogen and lipid metabolism. Okadaic acid increased glucose output and lipolysis and inhibited the conversion of acetate to long chain fatty acids. It has been shown that PP1 and PP2A are the major protein phosphatases acting on many phosphoproteins in vivo.

Many studies using okadaic acid have indicated that there are novel processes controlled by phosphorylation/dephosphorylation. For example, the stimulation of glucose transport into adipocytes by insulin, the production of prostaglandin E2 in rat peritoneal macrophages, the 5HT-induced suppression of a K⁺ current in the snail *Helix aspersa* and the increase in L-type inward Ca²⁺ current in isolated cardiac myocytes just to name a few.

Okadaic acid tumour promotion and suppression

Okadaic acid is a powerful tumour promoter, and in two-stage carcinogenesis experiments on mouse skin, is of comparable potency to phorbol esters which exerts their effect by activating protein kinase C (Suganuma et al., 1988). Since PP1 and PP2A are the chief enzymes involved in reversing the action of protein kinase C, okadaic acid most likely accelerates tumour formation by increasing the phosphorylation of this enzyme. Okadaic acid is capable of reversing cell transformation by some oncogenes. NIH3T3 cells transformed by either the raf

or ret-II oncogenes partially revert to the normal phenotype after incubation for two days with 10nM okadaic acid (Sakai et al., 1989). Changes include restoration of contact inhibition of growth and anchorage dependency and recovery of fibronectin expression to the normal levels. It has also been shown that okadaic acid prevents the large T-antigen of SV40 virus from stimulating DNA replication, another example where okadaic acid suppresses the action of an oncogene.

Mechanism of phosphatase inhibition by okadaic acid

It is interesting that the sensitivity of PP1 and PP2A to okadaic acid remains so constant through eukaryotic evolution, but the mechanism of inhibition still remains unknown. PP2B, whose catalytic domain is ~ 40% identical to PP1 and PP2A, is far less sensitive to okadaic acid, while a homologous protein phosphatase encoded by the genome of bacteriophage γ is unaffected by okadaic acid (Cohen and Cohen, 1989). These observations, together with kinetic data, indicate that okadaic acid does not bind to the catalytic centre. The C-terminus of the protein is ~100 residues shorter than PP1 and PP2A, suggesting that okadaic acid may interact with the C-terminal domain of PP1 and PP2A.

THE HL-60 CELL LINE

The human acute promyelocytic leukaemic cell line, HL-60, is a model system used to study the cellular mechanisms involved in differentiation. This section looks at the characteristic properties of the HL-60 cell line comparing proliferating and differentiating cells. Mutations in HL-60 cell oncogenes, *c-myc*, *N-ras* and *p53* have shown to be associated with maintaining cells in a proliferating state. The ability of HL-60 cells to undergo differentiation in response to various inducing agents has proved valuable in understanding the mechanisms involved in differentiation. Chemotherapeutic agents such as all-trans retinoic acid (ATRA), 9-cis retinoic acid (9-RA), dimethyl sulphoxide (DMSO), phorbol-12-myristate-13-acetate (PMA), okadaic acid and genistein have both been implicated in modulating the phosphorylation process.

Leukaemic cells from patients with acute myeloid leukaemia display a seemingly unlimited proliferative capacity *in vivo*. Their life span in liquid suspension culture is usually limited to a few cell divisions. One possible explanation for such limited *in vitro* proliferation is that certain growth factors sustaining leukaemic cell division in the patient are absent from the culture environment. About 25 years ago, investigators in Dr R.C. Gallo's laboratory at the National Institutes of Health (NIH), in an attempt to identify such leukaemia-specific growth factors, cultured fresh leukaemic cells with conditioned media derived from cultures of both human embryonic cells and

mitogen-stimulated lymphocytes. The T cell mitogen interleukin 2 (TCGF) was first identified (Morgan et al., 1976) and a factor-independent immortal cell line (HL-60) with distinct myeloid characteristics was developed (Collins et al., 1977).

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, can be induced to differentiate in vitro to a number of different cell types and studies with this leukemic cell line have proved invaluable in a variety of different areas. These include: (a) providing insight into the control mechanisms of normal granulocyte/ monocyte/ macrophage differentiation; (b) suggesting new therapeutic approaches for patients with leukemia; (c) providing a ready source of cDNA for cloning of important granulocyte and monocyte enzymes and monokines; (d) providing insight into possible mechanisms of rapid regulation of gene transcription; and (e) serving as an invaluable model for studying specific cellular oncogene expression in relation to particular hematopoietic differentiative lineages.

HL-60 cell growth and proliferation

HL-60 promyelocytic leukaemic cells continuously proliferate in suspension culture with a doubling time of ~36 to 48 hours and making this an unusual characteristic among human myeloid leukemia's. Most fresh myeloid leukaemia cells when cultured in liquid suspension undergo a limited number of cell divisions prior to growth arrest and cell death, and relatively few human leukaemic cell lines display distinct myeloid or myelomonocytic characteristics.

In HL-60 cells, surface expression of transferrin and insulin receptors appears to be critical to their proliferative capacity. HL-60 cells can proliferate in serum free nutrient media (e.g. RPMI 1640) provided it is supplemented with transferrin and insulin (Breitman et al., 1980). The requirement for insulin and transferrin is absolute, since HL-60 cell proliferation immediately ceases if either of these compounds is removed from the serum free culture media. The transferrin requirements in HL-60 cells as well as in other cultured cells (Barnes and Sato, 1980) indicate that an active iron transport system is necessary for cell proliferation. The HL-60 cell expression of transferrin receptors decreases as the cells are induced to differentiate terminally (Sutherland et al., 1981). Monoclonal antibodies to the transferrin receptor have been shown to inhibit proliferation in HL-60 cells (Taetle et al., 1983). Similarly, insulin receptors are also displayed by HL-60 cells, and insulin receptor expression decreases with granulocytic differentiation. The presence of the insulin and transferrin

receptors on HL-60 cells cannot itself explain the unusual in vitro proliferative capacity of this cell line since a number of fresh leukaemic cells also display these receptors but are unable to grow continuously in culture.

The in vitro proliferation of normal myeloid progenitor cells is stimulated by the colony-stimulating factor (CSF) family of compounds. Certain CSFs also stimulate the proliferation of HL-60 cells (Ruscetti et al., 1981). HL-60 cells have been postulated to produce certain CSF-like compounds that act as autostimulators of HL-60 growth, and several compounds exhibiting such “autocrine” activity have been partially purified from HL-60 conditioned media. One of the chromosome 5 GM-CSF alleles appears to be partially deleted in HL-60 cells but there is no evidence that this gene is expressed and the effect if any, of this genomic rearrangement on the proliferation of the cells is still unknown.

Differentiation of HL-60 cells

The characteristic of HL-60 cells that has attracted the most research interest is its ability to differentiate in vitro to a variety of different cell types of the myelomonocytic lineage. Various agents induce HL-60 cells to differentiate to four general types of cells: (a) granulocytes, (b) monocytes, (c) macrophage-like cells, and (d) eosinophils.

In this study the cells were induced to differentiate along the macrophage-like pathway by using phorbol-12-myristate-13-acetate (PMA) or along the granulocytic pathway by using either, dimethyl sulfoxide (DMSO), ATRA or 9-cis RA.

Granulocytic differentiation

The HL-60 cells are predominantly promyelocytes but in most cell cultures, particularly passage 60 or less, ~5% of the cells exhibit spontaneous differentiation to morphologically mature cells including myelocytes, metamyelocytes, banded and segmented neutrophils (Collins et al., 1977). The addition of specific inducing agents such as dimethyl sulfoxide and retinoic acid markedly increases spontaneous differentiation, with most cells acquiring

morphological, functional, enzymatic and surface membrane antigen characteristics of mature granulocytes.

Some of the characteristic changes include: (a) decreased myeloperoxidase, (b) decreased insulin and transferrin receptors, (c) increases in acid phosphatase, chemotactic and complement receptors, (d) no nonspecific esterase and plastic adherence activity, (e) presence of chemotactic and phagocytic activity. Granulocytic inducing agents induce terminal differentiation of HL-60 cells, which enables them to engage in the killing of microorganisms and suggests that this function may be of therapeutic benefit in patients with myelogenous leukaemia.

RETINOIC ACID INDUCED GRANULOCYTIC DIFFERENTIATION

Two classes of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid-X receptors (RXRs), mediate the physiologic activity of retinoids. The RXRs can form biologically active heterodimers with the RARs and with other nuclear receptors, including the vitamin-D, thyroid hormone, and peroxisome proliferators-activated receptors. Thus, the RXRs may play a pivotal role in modulating the action of other hormones or ligands. The RXRs were originally classified as orphan receptors since a definitive ligand was unknown until recently, when 9-cis retinoic acid (9-cis RA) was discovered to bind directly and activate this family of receptors. Since 9-cis RA also binds and activates the RARs, it is interesting to speculate that this natural ligand may regulate a broad range of physiologic processes by mediating transcriptional activity through both RAR- and RXR-linked pathways.

Retinoic acid receptors and retinoid-X receptors

Retinoic acids (RA) are natural derivatives of vitamin A (retinol), which play a prominent role in many physiological processes, including embryonic development, epidermal differentiation, and the regulation and differentiation of various cell types (Sporn et al., 1994). Research on the mechanism of action of RAs was stimulated with the discovery of two families of nuclear RA receptors,

the retinoic acid receptors [RARs (Petkovich et al., 1987)] and the retinoid-X receptors [RXRs (Hamada et al., 1989)].

The RARs and RXRs are members of a superfamily of ligand-inducible transcription factors, which also include the steroid hormone, thyroid hormone and vitamin-D receptors (Evans, 1988; Green and Chambon, 1988) as well as a number of orphan receptors whose ligands are not yet known. Both the RARs and RXRs control cellular function by regulating gene expression, as is typical of this family of transcription factors. Upon binding of ligand, the RARs and RXRs modulate transcription through DNA response elements that are located in the regulatory region of retinoic acid-responsive genes (Leid et al., 1992).

Based on amino acid and functional similarities, six regions have been identified that characterize members of this superfamily (Figure 17; Green and Chambon, 1988). Regions A and B contain a ligand-independent transactivation function, while region C comprises a DNA-binding domain. This latter domain contains two highly conserved zinc fingers that are responsible for the recognition of specific response elements within the regulatory regions of target genes. The functions of regions D and F are poorly known; however, both regions appear to be highly conserved for a given subtype across species. Region E is particularly complex, as it contains the ligand-binding domain, a ligand-dependent transactivation function and a dimerization interface (Leid et al., 1992).

Three genes that comprise the RAR family, α , β and γ have been identified and at least one of these RAR subtypes is present in each tissue or cell type studied. Each of the RAR subtypes can also be further divided into different isoforms that arise from alternative splicing or different promoter usage. Among the RAR subtypes there is high homology in the C (>94%) and E (>84%) domains; however, the remainder of the regions within each protein are less conserved. Each subtype exhibits far more amino acid identity across species than is seen within the three RAR subtypes of a given species.

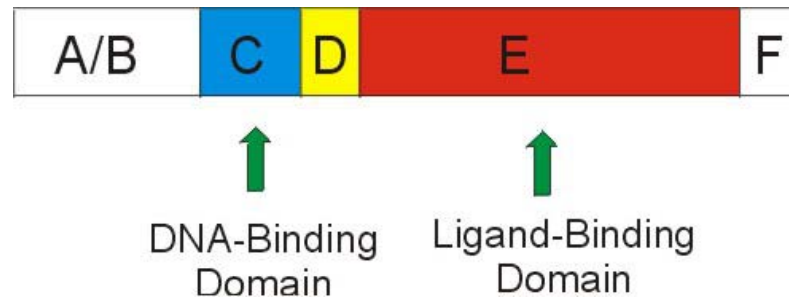


Figure 17: A model of the general structure of nuclear retinoid receptors

The specific domains of the retinoid receptors (A-F) are shown above and their functions are discussed in the text (adapted from Green and Chambon, 1988).

The RXR family also consists of three subtypes, α , β and γ . However, these receptors are substantially different in primary structure and ligand specificity from the RARs. Between the RARs and the RXRs, the most conserved region lies in the DNA-binding domain, which shows 63% homology between the two receptors. The remainder of the protein demonstrates no more than 30% homology (Leid et al., 1992).

Originally, the physiologic ligands for these receptors was thought to be all-trans-retinoic acid (ATRA; Figure 17), which binds RAR α , β and γ with very high affinity, exhibiting K_d s of 0.2, 0.4 and 0.2 nM respectively (Allenby et al., 1993). Curiously, both the RARs and the RXRs mediate transcriptional responses in the presence of ATRA; however, unlike the RARs, the RXRs exhibit little or no affinity for this isomer (Mangelsdorf et al., 1990). IC_{50} s for the competition of tritiated ATRA binding to the RARs are ~5 nM and >50,000 nM for the RXR (Allenby et al., 1993). The high divergence in the amino acid sequence between the RAR and RXR ligand binding domains (region E) suggests a potential difference in ligand specificity. The RXRs, therefore, were classified as retinoid orphan receptors whose activating ligand, X, was not known. The ability of RXRs to mediate a transcriptional response in the presence of ATRA, however, led investigators to speculate that the proximate ligand or hormone, X, for the RXRs would be a metabolite of ATRA (Mangelsdorf et al., 1990).

The discovery of 9-cis retinoic acid

Two independent methods were used to discover 9-cis retinoic acid, a stereoisomer of all-trans retinoic acid (ATRA), a proximate ligand for the RXRs (Levin et al., 1992; Heyman et al., 1992). Levin and co-workers (1992) used a technique termed nuclear receptor dependent ligand trapping in order to determine the identity of ligands bound to RXRs. This technique used COS-1 cells which were transiently transfected with an expression vector containing the cDNA for RXR α or RAR α was subsequently treated with [^{3} H] ATRA. In the experiments by Heyman and co-workers (1992) they used *Drosophila* S2 cells incubated with [^{3} H] ATRA, and then the resulting metabolites were fractionated by HPLC and identified by mass spectral analysis. Both experiments confirmed that 9-cis retinoic acid is a ligand for RXRs.

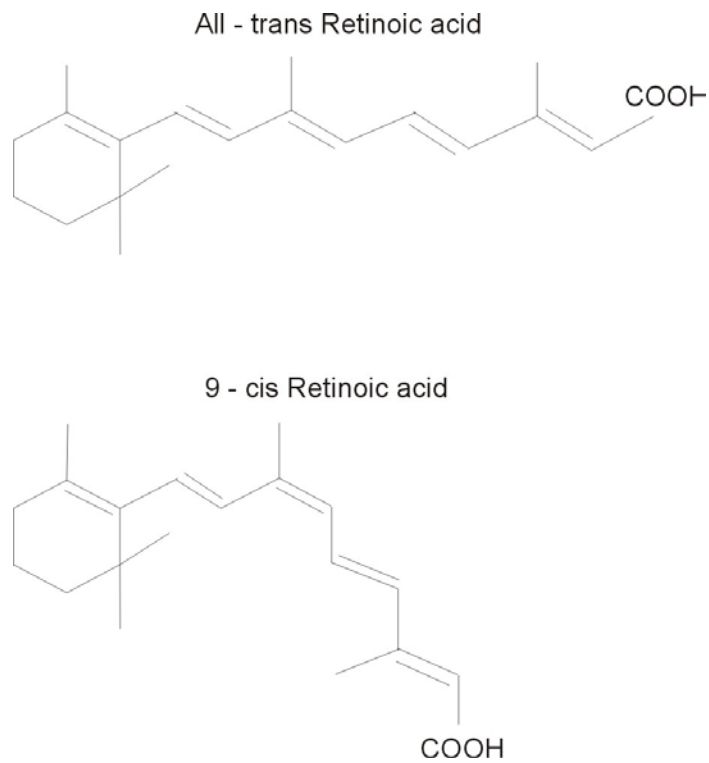


Figure 18: Graphical representation of all-trans- and 9-cis retinoic acid structures

All-trans and 9-cis retinoic acids structures (from Merck manual)

Interaction of 9-cis retinoic acid with retinoid receptors

9-cis Retinoic acid binds RXRs with relatively high affinity, exhibiting K_d s in the range of 10-20 nM for these receptors (Heyman et al., 1992; Levin et al., 1992). These results suggest that ATRA could activate RXR-mediated gene transcription after conversion to the proximate ligand, 9-cis RA. In transactivation assays, using a specific response element contained in the rCRBP II gene it was shown that activation of the gene is via the RXR α and not through RAR α (Mangelsdorf et al., 1990). Early efforts have indicated that, like ATRA, 9-cis RA can also activate transcription through the RARs. It was also shown that RARs bound both ATRA and 9-cis RA with approximately the same affinities.

RAR α , β and γ exhibited K_d s of 0.2, 0.4 and 0.2 nM for ATRA binding and 0.2, 0.4 and 0.7 nM for 9-cis RA binding. The ligand 9-cis RA bound to the RARs with higher affinity than to the RXRs. It was also shown that the affinity for 9-cis RA binding to the RAR γ receptor is lower than the affinity to both the RAR β and the RAR α receptors. This decrease in affinity appears to be due to an increase in the off-rate for 9-cis RA from the RAR γ receptor (Allenby et al., 1994).

The cellular retinoic acid binding proteins, CRABPI and CRABPII, have reduced binding capacity for 9-cis RA when compared with ATRA. Both 9-cis RA and ATRA compete with each other for binding to the RARs, indicating that they share a common binding site (Allenby et al., 1994). Recently, a secreted epididymal retinoic acid binding protein was found to be capable of binding both ATRA and 9-cis RA. Through crystallographic analysis (Newcomer et al., 1993) it was shown that ATRA could form a pseudo-cis conformation by rotation at the C8-C9 single bonds and, therefore, could fit into the same binding pocket as 9-cis RA. Such a configuration could explain how both 9-cis RA and ATRA bind with equal affinity and compete with each other for a binding pocket on the RARs. Alternatively, 9-cis RA and ATRA could interact with the RARs at distinct, but overlapping, binding sites. Truncation of a carboxy-terminal domain of RAR α yields a receptor that binds ATRA with high affinity but that exhibits no detectable binding for 9-cis RA (Tate et al., 1994), indicating that RAR α contains distinctly different high-affinity-binding determinants for these two stereoisomers.

9-cis Retinoic acid an endogenous ligand

9-cis RA has been found in several tissues, including mouse liver and kidney (Heyman et al., 1992), rat epididymal tissue (Pappas et al., 1993), neonatal rat eye and embryonic brain (CreechKraft and Juchau, 1993) and thus appears to be an endogenous derivative of vitamin A. In these tissues, 9-cis RA levels are two to six times lower than those of ATRA. While the biosynthesis of ATRA in cells occurs in vivo through oxidative reactions in which vitamin A is converted to retinaldehyde and then to retinoic acid, at present little is known about 9-cis RA production. This isomer could be formed in vivo from a 9-cis retinol precursor. Alternatively, 9-cis RA could be formed by isomerization from ATRA.

Retinoid-X receptors and 9-cis retinoic acid are key regulators

It has been shown that the RXRs form active heterodimers with other transcription factors, including the receptors for ATRA, thyroid hormone, vitamin D (Yu et al., 1991; Kliewer et al., 1992; Leid et al., 1992; Marks et al., 1992, Zhang et al., 1992) and peroxisome proliferators (Kliewer et al., 1992) (Figure 17). Data suggests that heterodimerization of these receptors with RXRs is required for effective DNA binding and function (Zhang and Pfahl, 1993). The RXRs themselves were found to bind and activate gene transcription as

homodimers on RXR response elements in the presence of 9-cis RA (Zhang et al., 1992).

Precisely how binding of 9-cis RA to the RXRs in heterodimeric complexes modulates the activity of its heterodimeric partners is currently being investigated at many research centres. In a heterodimeric complex containing two ligand-inducible transcription factors, such as RXR and the vitamin-D receptor (VDR), there are four possible states of ligand occupancy. The RXR receptor, alone can be occupied by 9-cis RA within the complex or its heterodimeric partner, alone, can be occupied by its cognate ligand. Both members of the complex can also be simultaneously liganded or unliganded (Figure 17). The role of 9-cis RA and RXR in modulating the effect of the liganded heterodimeric partner is controversial, but it is clear from the currently available data that the effect of 9-cis RA depends both on the identity of this partner and on the nature of the response element. This interaction of the RXRs and 9-cis RA with other receptors and their ligands is an example of cross talk between hormonal pathways and thus may be an important site for regulatory control.

The RXRs have been shown to form homodimers in the presence of 9-cis RA that have response elements specificities distinct from RAR-RXR heterodimers (Zhang et al., 1992). Thus, 9-cis RA induced homodimer formation may be a

mechanism by which RXR receptors are shunted away from forming active heterodimers with other nuclear receptors (Lehmann et al., 1993) and represents another means by which this ligand can control retinoid signaling. A series of retinoids have been synthesized that are selective for this RXR homodimer response pathway and conformational analysis indicates that these retinoids are spatially similar to 9-cis RA (Lehmann et al., 1992).

The discovery of a new hormone, 9-cis RA, that can bind and activate both RARs and RXRs and the ability of the RXRs to modulate the action of other ligand-inducible receptors suggest a highly sophisticated gene regulation system. A number of results have recently been described where 9-cis RA has been shown to have unique properties when compared with other retinoids. For example, 9-cis RA is more potent than ATRA as a negative regulator of activation induced T-cell apoptosis (Yang et al., 1993) and appears to be more potent in inhibiting the clonal growth of certain acute myelogenous leukemia cell lines (Sakashita et al., 1993).

DIMETHYL SULFOXIDE INDUCED GRANULOCYTIC DIFFERENTIATION

Dimethyl sulfoxide (DMSO [$(\text{CH}_3)_2\text{SO}$]) is an amphipathic molecule with a highly polar domain and two apolar groups, making it soluble in both aqueous and organic media. Despite being known since the nineteenth century, its biological properties were only discovered in the 1960s. Since then it has been used for diverse laboratory and clinical purposes. DMSO is frequently used as a solvent in biological studies and as a vehicle for drug therapy. Since its approval in 1978 by the FDA it has been used successfully in the treatment of dermatological (Burgess et al., 1998), urinary (McCammon et al., 1998), pulmonary (Iwasaki et al., 1994), rheumatic and renal manifestations of amyloidosis (Morassi et al., 1989; table 3). DMSO crosses the blood-brain barrier and has been effective in the treatment of traumatic brain oedema through its anti-inflammatory and reactive oxygen species scavenger action (Ikeda and Long, 1990; table 4).

DMSO is a hydrogen-bound disrupter, cell-differentiating agent, hydroxyl radical scavenger, intercellular electrical uncoupler, intracellular low-density lipoprotein-derived cholesterol mobilizing agent, cryoprotectant, solubilizing agent used in sample preparation for electron microscopy, antidote to the extravasation of vesicant anticancer agents and topical analgesic (Santos et al., 2003).

Table 3: Brief summary of the reported effects of DMSO in the treatment of different human and experimental animal model diseases (adapted from Santos et al., 2003)

Disease	Actions/effects
Interstitial cystitis	Improves treatment, but the effect is not related with mast cell release of histamine
Amyloidosis	Helps to control pruritus in macular and lichen amyloidosis, avoiding the recurrent deposition of amyloid in areas of trauma of the epidermis Causes regression of hyaline deposits in lipoid proteinosis Improves primary amyloidosis of the bladder (instillation) Reduces pulmonary infiltration and improves arterial blood gas levels in pulmonary amyloidosis associated with multiple myeloma Is effective in the treatment of some cases of primary amyloidosis when combined with cytoreductive chemotherapy Induces a decrease of the inflammatory activity of rheumatoid arthritis and an improvement of renal function, following 3–6 months of therapy, in patients with secondary amyloidosis Induces growth and blackish turn of scalp hair and beard
Gastrointestinal disorders	Has an anti-inflammatory effect in ulcerative colitis Associated with H2 receptor blockers, is more effective for healing acute duodenal ulceration and prevention of its recurrence than H2 receptor blockers alone Improves survival in patients bearing colon carcinoma Has a beneficial therapeutic effect in the mechanism of abdominal pain caused by alcohol-induced chronic pancreatitis Gives protection in stress-induced acute gastric mucosal injury
Brain edema	Has a beneficial effect in the treatment of traumatic brain edema
Rheumatologic disorders	Has a beneficial effect in the treatment of rheumatoid arthritis, reducing the associated pain Enhances the effect of glucocorticoids in the treatment of rheumatoid synovitis
Chronic prostatitis	Induces a positive result in the treatment of human chronic prostatitis
Schizophrenia	Has an antipsychotic action
Pulmonary adenocarcinoma	Improves the antiproliferative effect of interferon- α on human lung adenocarcinoma cells through an unknown mechanism
Dermatologic disorders	Has a beneficial effect in the topical treatment of herpes zoster Enhances the efficacy of fungicides in the treatment of dermatologic mycosis Is an effective and safe antidote that may be used with local cooling after the extravasation of certain drugs used in chemotherapy given intravenously, preventing the necrosis of tissues and ulceration
Experimental animal model	Inhibits collagen a1 gene expression, increase in tissue collagen content, hepatic stellate cell activation, synthesis of tissue inhibitors of metalloproteinase-2 and, consequently, the development of liver cirrhosis in mice Prevents the development of liver cirrhosis induced by thioacetamide

Table 4: Brief summary of the reported effects of DMSO on cell cycle, differentiation, and apoptosis studies, observed in experimental models
(adapted from Santo et al., 2003)

Type of study	Actions/effects Reference(s)
Cell cycle	Inhibits c-myc expression, in different cell types Inhibits c-myc expression during the G1 to S transition in adherent fibroblast cells arrested at G0/1 by serum starvation Arrests the cell cycle of several human and mouse lymphoid cell lines at the G1 phase Arrests the cell cycle of a transformed human B-cell line at G1 phase and suppresses interleukin-6-induced differentiation into IgM-producing cells at a concentration lower than that affecting cell proliferation, suggesting that cell proliferation and differentiation are independent of each other
Differentiation	Down-regulates c-myc protein in the G1/0 specific phase prior to the appearance of differentiation-associated markers May induce differentiation of malignant cells present in the marrow or alternatively in the body when it is infused back with the transplanted marrow Enhances the differentiation of leukemic cells to mature myelocytes Prevent dedifferentiation of normal cells. Cultured adult rat hepatocytes can be maintained and made to secrete albumin for more than 40 days Leads to the cessation of the proliferation of murine erythroleukemia cells and the production of a number of erythrocyte markers, such as hemoglobin
Apoptosis	Leads to the collapse of mitochondrial membrane potential, release of cytochrome c from the mitochondria, and activation of caspase-9 and -3, but not of caspase-8 (caspase cascade of mitochondrial apoptotic pathway is indispensable for DMSO-induced apoptosis). Induces apoptotic changes in a murine lymphoma cell line (concentration- and time-dependent effect) and down-regulation of Bcl-2 Prevents apoptosis in lymphoma cells

MONOCYTIC DIFFERENTIATION OF HL-60 CELLS

Many compounds such as vitamin D₃, tumour necrosis factor- α , interferon- γ , sodium butyrate and lymphocyte-conditioned media have all been shown to induce monocytic differentiation in HL-60 cells (Collins, 1987). These cells can morphologically be confused with immature HL-60 granulocytes since they share many important functional characteristics. They can, however, be distinguished from granulocytes using positive staining such as α -naphthyl acetate esterase (Yam et al., 1971), by the presence of monocyte specific cell membrane antigens (Dayton et al., 1983) and by their capacity for mediating antibody-dependent cytotoxicity (Miyaura et al., 1981).

Therapeutically, monocytic differentiation of HL-60, unlike granulocytic differentiation, is not necessarily accompanied by the loss of proliferative capacity of the induced cells (i.e, monocytic HL-60 differentiation is not necessarily terminal differentiation). For example, recombinant interferon- γ (INF- γ) alone as well as INF- γ plus retinoic acid induces monocytic characteristic of HL-60 cells, but they have little effect on inhibiting HL-60 cell proliferation (Hemmi and Breitman, 1987). Elias and co-workers (1985) have identified a factor in mitogen-stimulated lymphocyte conditioned media that markedly enhances thymidine incorporation of HL-60 cell. An early stimulatory effect of tumor necrosis factor on HL-60 thymidine incorporation has been observed (Trinchieri et al., 1986). Thus, monocytic differentiation of HL-60

cells may in certain cases be preceded by enhanced cell proliferation, and cells induced to acquire particular monocytic characteristics have not necessarily lost the capacity to proliferate.

MACROPHAGELIKE DIFFERENTIATION OF HL-60 CELLS

The primary inducer of macrophage-like HL-60 cells is the phorbol ester tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). This TPA-induced macrophagelike phenotype is frequently combined with the “monocytic” phenotype in discussions of HL-60 differentiation since these induced cell types share many of the same enzymatic, histochemical and functional characteristics (Collin, 1987). Several important phenotypic characteristics clearly distinguish the HL-60 macrophage-like differentiation from HL-60 monocytic differentiation. Firstly, TPA-treated HL-60 cells are markedly more adherent than monocytes since they have prominent pseudopodia and adhere to each other (Rovera et al., 1979). Secondly, several specific surface antigens present on normal and HL-60 monocytes are not induced in TPA-treated HL-60 cells (Tanaka et al., 1983). Thirdly, no induction of chemotactic peptide receptors or of NBT reduction activity occurs in TPA-treated HL-60 cells, whereas these characteristics are readily demonstrated in HL-60 monocytes (Olsson et al., 1981). Fourthly, TPA induction of HL-60 cells is associated with a rapid and profound loss in proliferative capacity and few cells are able to enter the S-phase (Rovera et al., 1979). Fifthly, in contrast to the HL-60 granulocyte and monocyte inducers, prolonged exposure to TPA is not necessary to induce macrophage-like differentiation since even after 20 minutes of exposure differentiation has been demonstrated (Rovera et al., 1979).

EOSINOPHILIC DIFFERENTIATION OF HL-60 CELLS

Several researches have noted a small percentage of HL-60 cells (4% to 7%) to stain positive for eosinophils using Luxol-fast-blue (Lu et al., 1981). The proportion of colonies containing such cells increases with the addition of GM-CSF or growing cells in slightly alkaline media (pH 7.6 to 7.8) (Fischkoff et al., 1986). These cells have been extensively characterized and demonstrate multiple eosinophilic granule proteins, including the eosinophil major basic protein (MBP), the Charcot-Leyden Crystal (CLC) protein, eosinophil peroxidase, acid phosphatase and arylsulfatase (Fischkoff et al., 1986). The amount of eosinophil major basic protein markedly diminishes when cultures are treated with granulocytic inducers such as dimethyl sulfoxide (Fischkoff et al., 1984).

HL-60 ONCOGENE STRUCTURE AND EXPRESSION

HL-60 cells provide an invaluable model system for studying the relationship of oncogene structure and expression to normal and leukaemic cell proliferation and differentiation. Several mutations involving certain cellular oncogenes have been noted in HL-60 cells, reinforcing the concept that the development of malignancy is a multistage phenomenon and cancer results not from a single mutation but from a series of mutations. HL-60 cells exhibit specific genomic mutations involving the cellular oncogenes *c-myc* and *N-ras* and tumour suppressor protein, p53.

c-myc

The human *c-myc* gene is defined by virtue of its homology to *v-myc*, the transforming gene of the avian myelocytoma virus, which induces myeloid tumours in chicks. The protein product of this cellular oncogene is localized in the nucleus (Hann et al., 1983), and enhanced expression of the *c-myc* gene is rapidly induced following mitogenic stimulation of resting lymphocytes or fibroblasts. This oncogene also acts together with other oncogenes, particularly those in the *ras* gene family. HL-60 cells exhibit a 15- to 30-fold genomic amplification of *c-myc* as compared with normal cells (Collins and Groudine, 1982).

HL-60 cells exhibit high levels of steady state c-myc RNA. These levels decrease significantly as HL-60 cells are induced to differentiate terminally with granulocytic inducers. In addition, monocytic and macrophagelike differentiation of HL-60 cells is also associated with a marked decrease in c-myc RNA levels. The decrease in c-myc expression mimics that which occurs during normal hematopoiesis. Decreased c-myc transcription, rather than enhanced c-myc mRNA degradation, appears to account for the diminished steady-state c-myc RNA in differentiated HL-60 cells. In retinoic acid-treated HL-60 cells this transcriptional down regulation occurs as a block in elongation of the c-myc RNA transcription between axons 1 and 2 rather than as decreased transcript initiation.

N-ras

HL-60 genomic DNA is capable of malignantly transforming NIH3T3 cells in transfection assays, and the HL-60 gene that mediates this transformation has been identified as the N-ras gene (Murray et al., 1983), a member of the ras family of oncogenes which also includes Kirsten and Harvey ras. A point mutation involving the second nucleotide of N-ras codon 61 has been shown in HL-60 cells (Bos et al., 1984). Other acute myeloid leukaemia cells also exhibit NIH3T3 transforming activity and N-ras point mutations (Needleman et al., 1986). In contrast to c-myc gene expression, expression of the N-ras gene does not appear to change significantly as HL-60 cells are induced to differentiate.

An approximately two fold decrease in N-ras RNA was noted in dimethyl sulfoxide differentiated HL-60 cells (Filmus and Buick, 1985), whereas no change in N-ras expression was noted in TPA-treated cells (Sariban et al., 1985).

c-fos

The *c-fos* gene is the homolog of *v-fos*, the transforming gene of the FBJ-murine sarcoma virus, which induces osteosarcomas in mice. The *c-fos* gene product is localized in the nucleus (Curran et al., 1984). Mitogen stimulation of resting fibroblasts induces the rapid onset of *c-fos* mRNA and protein product, suggesting that *c-fos* may play a role in rendering resting cells competent to divide (Greenberg and Ziff, 1984). Enhanced *c-fos* expression has also been noted during the monocytic differentiation of the mouse WEHI-3B cell line (Gonda and Metcalf, 1984), as well as in normal monocytes and macrophages (Muller et al., 1984). HL-60 cells induced to monocytes with vitamin D₃ also exhibit enhanced *c-fos* expression, although such *c-fos* expression occurs more gradually without the rapid initial *c-fos* expression noted in TPA-treated HL-60 cells. *C-fos* is an immediate-early gene and is a member of the bZip transcription factor family. Transcriptional activity is initiated by *c-fos* phosphorylation in response to activation of the ras signaling pathway by growth factor stimulation (Buckle et al., 1996).

c-fms

The *c-fms* gene is the cellular homolog of the transforming gene of the McDonough strain of the feline sarcoma virus. The *c-fms* gene product appears to be identical to the cell membrane receptor for the monocyte/ macrophage colony-stimulating factor (CSF-1) (Sherr et al., 1985). HL-60 cells induced to differentiate to monocytes with vitamin D₃ or to macrophagelike cells with TPA express enhanced *c-fms* RNA levels as compared with uninduced HL-60 cells. The kinetics of this enhanced expression is considerably slower than that induced by *c-fos*, with *c-fms* transcripts initially noted ~6 hours after inducer treatment and peaking at 24 to 48 hours following induction. By means of immunologic reagents CSF-1 receptors have been detected on monocyte induced HL-60 cells, but their numbers appear relatively small when compared with normal monocytes /macrophages. No enhanced *fms* expression is noted in HL-60 cells treated with granulocytic inducers (Sariban et al., 1985).

c-src

The *c-src* gene is the cellular homolog of the transforming gene of the Rous sarcoma virus and codes for a cell membrane-associated protein (pp60^{*c-src*}) with tyrosine kinase activity. The level of pp60^{*c-src*} kinase activity is increased in HL-60 cells induced to macrophages, monocytes or granulocytes with TPA, vitamin D₃ and DMSO respectively (Gee et al., 1986). Terminally differentiated

rat CNS neurons similarly exhibit relatively high levels of c-src (Brugge et al., 1985). There is evidence that c-src family members also interact with the B-cell receptor (BCR), the T-cell receptor (TCR) and receptors for the Fc portions of IgG and IgE by a common mechanism (Brickell, 1996).

c-fgr

C-fgr is the cellular homolog of the transforming gene of the Gardner-Rasheed feline sarcoma virus. This gene is a member of the src family of oncogenes and codes for a cell membrane-associated protein with tyrosine kinase activity (Naharro et al., 1984). This gene is expressed in peripheral blood leukocytes with preferential expression in mature monocytes and granulocytes and little expression in B and T lymphocytes. Induction of HL-60 granulocytic differentiation is associated with a marked increase in the level of expression of c-fgr (Collins, 1987).

Tumour suppressor gene p53

The p53 protein is frequently found at elevated levels in various types of tumour cells. This protein shares some characteristics with the c-myc product. Both are localized to the nucleus, and p53 can substitute for myc in the myc-ras cooperative transformation of primary rodent fibroblast cultures (Eliyaku et al.,

1984). The p53 locus is on the short arm of chromosome 17 (Miller et al., 1986), and this gene is extensively deleted in HL-60 cells associated with absent p53 expression (Wolf and Rotter, 1985). Since c-myc and p53 share similar physiological function it has been suggested that the loss of p53 in progenitors of HL-60 may be responsible for the subsequent selection of cells harbouring amplification of c-myc.

OBJECTIVE OF THIS STUDY

The objective of this study was to gain further understanding of the differentiation process and the effects of malignant transformation at the molecular level by investigating temporal variations in the expression of PP1, PP2A and PTP-1B enzyme proteins and PTP-1B mRNA during proliferation and differentiation of HL-60 cells.

HL-60 cells were induced to differentiate along the macrophage pathway using PMA and along the granulocytic pathway using either DMSO, ATRA or 9-cis RA. This project aimed to gain insight into the mechanisms of action of these inducers of differentiation by analyzing their effect on the amplitude, frequency or phasing of the phosphatase rhythms. This may have important implications with respect to design of drugs and treatment of disease. A further aspect of this study was to clone the PTP-1B mRNA to produce recombinant protein.