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Beautiful is what you see
More beautiful is what you know
Most beautiful is what you don't comprehend

NIELS STEENSEN
(Nicholas Steno, 1638-1685)

THE ROSA LOWIE AWARD

Corinna Bialojan and Akira Takai have been awarded the Rosa Lowie Award for their contribution to volume 4 of Advances in Protein Phosphatases entiteld: "Inhibition of protein phosphatase activity and actin-myosin interaction by black sponge toxin".

A.Takai was born on September 26, 1954, in Gifu, Japan, and he was previously working on problems of EC-coupling and metabolic regulation in smooth muscle with Prof. Tomita in Nagoya. In 1986, he was granted a Humboldt-Fellowship and joined the Department of Physiology II of the University of Heidelberg where he started investigating the effect of the Japanese black sponge poison okadaic acid on skinned fibres of smooth muscle.

The skinned fibre studies revealed that the activation of this simplified contractile system by okadaic acid could not be ascribed to an effect on myosin light chain kinase or calmodulin and yet it was associated with an increased extent of myosin phosphorylation. This increased phosphorylation was puzzling. In search for an answer, Dr. Takai was fortunate to collaborate with Corinna Bialojan who had purified the myosin phosphatase and developed a sensitive system for assaying its activity. Jointly, they found that indeed okadaic acid inhibited the phosphatase activity in a purified enzyme sytem as well as within skinned fibres.

Corinna Bialojan was born on January 29, 1957, in Hannover, Germany, and had already been awarded the distinguished scholarship of the "Studienstiftung des Deutschen Volkes". Since 1982 she has been working in the Department of Physiology II where she received her Ph.D.-degree for her work on myosin phosphatase and its effect on contractility of smooth muscle, and she continued these studies after obtaining her degree in 1984.

In the hands of Drs. Bialojan and Takai, okadaic acid proved to be an invaluable tool to manipulate the state of myosin phosphorylation in contractile systems with the aim of understanding the role of phosphorylation in smooth muscle contraction. Conceivably, this first potent and specific poison may prove to be most important for the elucidation of cell motility and ion channels and many other regulatory processes of the cell.

Professor Dr J.C. Rüegg
Chairman of Physiology

PRAEMIVM ROSA LOWIE

COLLEGIVM PRAEMIO **ROSA LOWIE** DECERNENDO A FACVLTATE STRICTA MEDICINAE STVDIORVM VNIVERSITATIS CATHOLICAE LOVANIENSIS IN VRBE LOVANIENSI RITE CONSTITVTVM OMNIBVS PRAESENTES LITTERAS INSPECTVRIS SALVTEM.

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COMMENTATIONEM A

Corinna BIALOJAN et Akira TAKAI

CONSCRIPTAM, CVI TITVLVS EST:

INHIBITION OF PROTEIN PHOSPHATASE ACTIVITY AND ACTIN-MYOSIN INTERACTION BY BLACK SPONGE TOXIN

CONSPICVIS DOCTRINAE VIRTVTIBVS CLARERE CETERISQVE MANIFESTO PRAESTARE CONFIRMAVIT.

QVAE CVM ITA ESSENT, AVCTOREM PRAEMIO A NOMINE **ROSA LOWIE** VOCATO IVRE MERITOQVE HONORANDVM ESSE DECREVIT ET, RELATIONE AD PRELVM MISSA, HONORATVM RENVNTIAVIT.

CVIVS REI VT FIRMA SIT ATQVE TESTATA FIDES, DIPLOMA HOC PVBLICVM A SINGVLIS COLLEGII IVDICANTIS SODALIBVS SVBSCRIPTVM AVCTORI DATVM EST HONORATO.

LOVANII, KALENDIS IVLIIS ANNI *1988*

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THE CONTROL OF PROTEIN PHOSPHATASE 1 BY TARGETTING SUBUNITS

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Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Tayside, Scotland, United Kingdom

INTRODUCTION

Protein phosphatase 1 (PP1) is one of the four principal serine/threonine-specific protein phosphatase catalytic subunits of eukaryotic cells. It is distinguished from type 2 protein phosphatases in several ways, but most conveniently by its specificity for the ß-subunit of phosphorylase kinase and sensitivity to the thermostable proteins inhibitor 1 and inhibitor 2 (reviewed in [1]). Type 1 protein phosphatases with these properties have been identified in mammals, insects [2,3], echinoderms [4], protozoa [5] and yeast (P.Cohen, unpublished work), while recent cDNA cloning studies have revealed that the structure of the type 1 catalytic subunit is remarkably conserved during evolution, the Drosophila head enzyme showing 92% identity at the amino acid level with PP1 from rabbit skeletal muscle [6].

Protein phosphatase 1, like protein phosphatases 2A (PP2A) and 2C (PP2C), is capable of dephosphorylating many proteins *in vitro* [1]. A critical problem in protein phosphatase research is therefore to try and discover which proteins are dephosphorylated by which enzyme *in vivo*. At physiological concentrations of Mg^{2+} (≈1mM), PP2C accounts for a relatively small proportion of the phosphatase activity in mammalian tissue extracts towards many phosphoproteins [7], and recent studies on intact cells with a protein phosphatase inhibitor, okadaic acid, support the view that PP1 and/or PP2A are the dominant catalytic subunits acting on many phosphoproteins *in vivo* [8].

Okadaic acid is a polyether derivative of a 38 carbon fatty acid synthesized by marine dinoflagellates, which accumulates in sponges and in the digestive glands of molluscs [9]. It is implicated as the causative agent of diarrhetic shellfish poisoning [10] and is a potent tumour promoter in two stage mouse skin carcinogenesis experiments [11]. Okadaic acid is a potent inhibitor of PP1 (I_{50}=20nM) and PP2A (I_{50}≈0.2nM) *in vitro* [8,12]. Protein phosphatase 2B

(PP2B) is inhibited at much higher concentrations [12], while PP2C [12], pyruvate dehydrogenase phosphatase [8], acid and alkaline phosphatases [12], protein tyrosine phosphatases [12] and eight different protein kinases tested [8] (including protein kinase C [11]) are unaffected. When added to intact adipocytes and hepatocytes, okadaic acid (0.1-1μM) rapidly produces marked increases in the phosphorylation states of many proteins, as judged by increased ^{32}P-labelling , without changing the specific radioactivity of intracellular ATP or the ATP/ADP ratio [8]. A number of these proteins are enzymes involved in the regulation of carbohydrate and lipid metabolism, including acetyl-CoA carboxylase and ATP-citrate lyase (adipocytes and hepatocytes), glycogen phosphorylase, glycogen synthase, 6-phosphofructo 2-kinase/fructose 2,6 bisphosphatase and pyruvate kinase (hepatocytes). Consistent with these findings, okadaic acid suppresses the incorporation of acetate into lipid in adipocytes and mimics the effect of glucagon in increasing the rate of glucose output and the conversion of lactate to glucose in hepatocytes. It increases basal lipolysis in adipocytes (consistent with increased phosphorylation of hormone-sensitive lipase) and prevents insulin from antagonizing the ß-adrenergic activation of lipolysis [8]. An unexpected finding is that okadaic acid mimics the effect of insulin in dramatically stimulating the uptake of glucose into adipocytes, providing evidence that this process is stimulated by a protein phosphorylation event [8].

Since PP1 and PP2A are likely to be the major enzymes that reverse the actions of protein kinase C, it is not surprising that a permeant phosphatase inhibitor should be a tumour promoter, akin to activators of protein kinase C. Tumourigenesis is presumably favoured by nett phosphorylation of one or more proteins that are phosphorylated by protein kinase C and dephosphorylated by PP1/PP2A. The effects of okadaic acid on protein phosphorylation in isolated cells emphasize what a powerful tumour-suppressing effect PP1 and/or PP2A must have on normal cells.

Although the effects of okadaic acid implicate PP1 and/or PP2A as the major phosphatases acting on many phosphoproteins *in vivo* , they do not identify which enzyme dephosphorylates which substrate. A further clue to the *in vivo* specificity of PP1 has stemmed from the realisation that the active forms of this enzyme are largely particulate (reviewed in [1]). For example, in mammalian skeletal muscle nearly all the active PP1 is associated with glycogen particles [13], the sarcoplasmic reticulum (SR) [14] and myofibrils [15], while in the liver PP1 is greatly enriched in glycogen-particles [16], microsomes [16] and nuclei [17]. PP1 is the major phosphatase associated with ribosomes in

reticulocytes [18], and with postsynaptic densities in the brain [19]. Even in invertebrate tissues, such as starfish oocytes, most of the PP1 activity can be sedimented at 100,000xg, whereas PP2A and PP2C are mainly in the 100,000xg supernatant [4].

The remainder of this article will briefly review our current knowledge of the active forms of PP1 that are associated with subcellular fractions and organelles. The enzyme associated with the glycogen-particles of rabbit skeletal muscle will be reviewed in detail since it is the analysis of this enzyme which has introduced the concept of targetting subunits as a major mechanism for determining the *in vivo* specificity and regulation of PP1.

THE GLYCOGEN-BOUND FORM OF PROTEIN PHOSPHATASE 1 IN SKELETAL MUSCLE.

When skeletal muscle homogenates are centrifuged at low speed to remove the myofibrils, most of the PP1 activity remaining in the supernatant can be sedimented at 100,000xg in the fraction containing glycogen-protein particles and elements of the SR [e.g.13,16]. About 75% of this activity is solubilised by digestion with α-amylase, indicating that it is bound to glycogen. The glycogen-associated form of PP1 (termed $PP1_G$) was purified to homogeneity by Strålfors *et al.* [20] who showed it to be a heterodimer composed of the 37kDa PP1 catalytic (C) subunit and a 103kDa protein. Several lines of evidence demonstrated that the 103kDa protein specifically associates with the C subunit. Firstly, both components comigrated during glycerol density gradient centrifugation and gel-filtration, even though no fractionation based on size had been used during the purification [20]. Secondly, the 37kDa and 103kDa components could be dissociated by gel-filtration in the presence of 1M NaCl and recombined at low ionic strength [20]. Thirdly, antibodies prepared against a synthetic peptide corresponding to a sequence present in the 103kDa protein precipitated PP1 activity quantitatively [21].

The free C subunit of PP1 does not bind to glycogen [20,22], indicating that the 103kDa subunit is the component responsible for interaction with glycogen. It is therefore termed the G subunit. The $PP1_G$ holoenzyme is released from purified glycogen-protein particles by dilution, with a dissociation half point corresponding to about 10nM $PP1_G$ (Fig.1). Dissociation of PP1 from glycogen upon dilution is at the level of the G subunit-glycogen interaction and not between the G and C subunits, because even at the highest dilutions examined (0.4nM PP1) the released activity is immunoprecipitated quantitatively by antibodies to the G subunit [22].

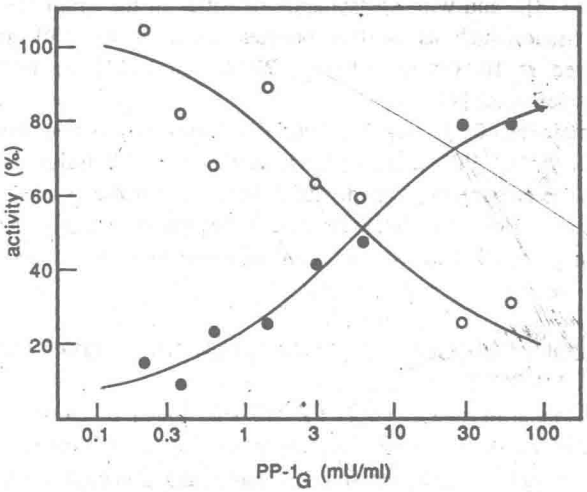

Figure 1. Dissociation of the $PP1_G$ holoenzyme from glycogen-protein particles upon dilution [22]. The percentage of PP1 activity associated with sedimented glycogen (closed circles) and the supernatant (open circles) is shown as a function of total PP1 activity. The dissociation half point, 5mU/ml, corresponds to about 10nM $PP1_G$ [22].

Purified $PP1_G$ binds to deproteinized glycogen with high affinity (Fig.2). The data are consistent with a bimolecular process, with K_{app} values of about 0.1mg/ml glycogen and 4nM $PP1_G$ [22]. A glycogen concentration of 0.1mg/ml corresponds to 8nM if the molecular mass of rabbit skeletal muscle glycogen is taken as $1.2x10^4$ kDa [23]. At saturating concentrations of glycogen (5mg/ml) and $PP1_G$ (40nM), virtually all the activity is sedimented with glycogen (Fig.2). The apparent affinity of $PP1_G$ for glycogen is about 10-fold higher than that reported for glycogen phosphorylase [24].

Binding to glycogen is essentially unaffected by the presence of 0.5M NaCl or variation in pH from 6 to 8 [22], indicating that it is not likely to be much affected by the acidification that accompanies sustained muscle contraction. Since the inferred concentration of $PP1_G$ in muscle cytosol is about 0.2μM [20] and the glycogen concentration in exhaustively exercised rat skeletal muscle is still about 4 mg/ml [25], $PP1_G$ should be almost entirely bound to glycogen *in vivo* , even after exercise (except during adrenergic stimulation - see below).

Immunoblotting studies using either the antipeptide antibodies referred to above [21], or antibodies prepared against the 103kDa subunit [26], have

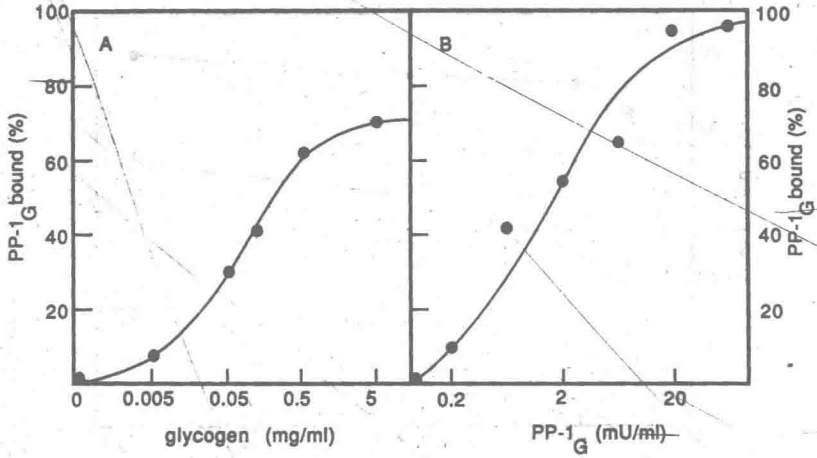

Figure 2. Binding of purified $PP1_G$ to glycogen [22]. A. Dependence of $PP1_G$ (5mU/ml) binding on glycogen concentration. B. Effect of $PP1_G$ concentration on binding to a saturating concentration (5mg/ml) of glycogen. The data indicate a high affinity bimolecular interaction between $PP1_G$ and glycogen.

revealed that the native form of the G component present in muscle extracts or glycogen-protein particles has an apparent molecular mass of 161kDa [22,26]. Conversion to the 103kDa component occurs as a result of cleavage by an endogenous proteinase, largely during chromatography on DEAE-Sepharose [22]. However, a rapid purification procedure has been developed in which the final product includes small amounts of the intact 161kDa subunit [22]. The 161kDa protein is now termed the G subunit and the "nicked" 103kDa species the G' subunit. The G' subunit is frequently degraded to even smaller fragments of 40-80kDa during purification [21]. These fragments retain the high affinity for both the C subunit and for glycogen [21,22], as well as the two sites phosphorylated by A-kinase (see below). Fragmentation of the G subunit and dissociation of $PP1_G$ from glycogen particles upon dilution explain the wide variation in gel-filtration behaviour of rabbit skeletal muscle PP1 reported over many years and the failure to demonstrate this component until recently (discussed in [21,27]).

The G subunit is phosphorylated by cyclic AMP-dependent protein kinase (A-kinase) at a comparable rate to other physiological substrates, such as glycogen synthase [20]. Initial protein sequencing studies revealed a single phosphoserine [28], termed Site 1, but subsequent work has shown that a second serine (Site 2) is also phosphorylated [21,22]. Site 2 was missed in the

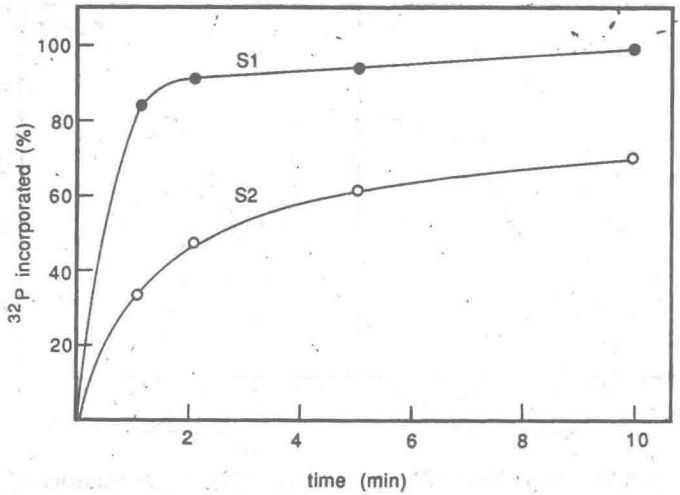

Figure 3. Phosphorylation of $PP1_G$ at Site 1 and Site 2. $PP1_G$ was incubated with the catalytic subunit of A-kinase and [γ-^{32}P]ATP for the indicated times and incorporation of ^{32}P into Site 1 (S1) and Site 2 (S2) was quantitated after separation of the tryptic peptides by reverse phase HPLC. [22]. Incorporation into Site 1 approaches 1mol/mol at 10min.

initial studies for several reasons. Its phosphorylation requires high concentrations of A-kinase in $PP1_G$ preparations containing extensively degraded G' subunit. Site 2 undergoes autodephosphorylation by PP1, whereas Site 1 does not [22]. The tryptic peptide containing Site 2 is insoluble in 5% trichloroacetic acid [29], very hydrophobic and easily lost on surfaces [22]. Using $PP1_G$ containing relatively undegraded G subunit and a modified purification protocol giving a high yield of the Site 2 tryptic peptide, this second serine is phosphorylated at a similar rate to Site 1 and to near stoichiometric levels (Fig 3).

Primary structural analysis has revealed that Site 2 is located only 19 residues C-terminal to Site 1 [29]. Both phosphorylation sites have the structure Arg-Arg-X-Ser(P)-Y, frequently found at the phosphorylation sites of physiological substrates for A-kinase [1]. If the phosphorylation reaction is stopped by addition of EDTA, Site 2 is autodephosphorylated by PP1 with a half time of 30 minutes under the conditions used (Fig 4). This rate is very slow considering that 10μM phosphorylase would be dephosphorylated with a $t_{0.5}$ of <1s by this concentration of $PP1_G$. Autodephosphorylation of Site 1 is

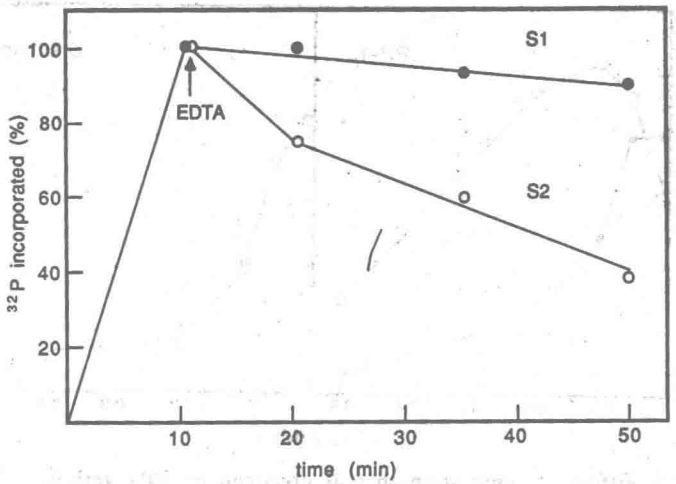

Figure 4. Autodephosphorylation of Site 1 and Site 2 [22]. $PP1_G$ was phosphorylated as described for Fig.3, then the kinase reaction stopped by adding EDTA. Loss of ^{32}P-label from Site 1 (S1) and Site 2 (S2) during subsequent incubation at 30°C was determined as described for Fig.3 [22].

negligible. These observations suggest that Site 1 and Site 2 may be dephosphorylated by other protein phosphatases *in vivo*.

$PP1_G$ is inhibited by inhibitor 1 and inhibitor 2, but unlike the free C subunit (which is inhibited almost instantaneously by these proteins), inactivation is time dependent ($t_{0.5}$ = 5 min in the standard assay) [20]. Initial studies indicated that phosphorylation of the G subunit increased the rate of inactivation by inhibitor 1 [20] and decreased the proportion of PP1 that sedimented with glycogen [30]. These observations are now explained by the finding that phosphorylation of the G subunit by A-kinase causes its dissociation from the C subunit *in vitro*. This has been demonstrated by gel-filtration experiments and by the failure of PP1 activity (but not the G subunit) to bind to glycogen after phosphorylation [29].

Phosphorylation of $PP1_G$ does not alter its ability to dephosphorylate glycogen phosphorylase in the standard assay, which is carried out in 50mM Tris-HCl pH 7.0 in the absence of glycogen. However, in the presence of physiological concentrations of KCl, phosphorylase phosphatase activity is strongly (70-80%) suppressed in the absence of glycogen, but not in its presence (Fig 5). This is a consequence of $PP1_G$ binding to glycogen since the activity of the free C subunit is inhibited in a similar manner by KCl in the presence or