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The use of erythrocytic and animal models in the study of protein phosphorylation

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Abstract

Phosphorylation processes are common post-transductional mechanisms, by which it is possible to modulate a number of metabolic pathways. Proteins are highly sensitive to phosphorylation, which governs many protein–protein interactions. The enzymatic activity of some protein tyrosine-kinases is under tyrosine-phosphorylation control, as well as several transmembrane anion-fluxes and cation exchanges. In addition, phosphorylation reactions are involved in intra and extra-cellular 'cross-talk' processes. Early studies adopted laboratory animals to study these little known phosphorylation processes. The main difficulty encountered with these animal techniques was obtaining sufficient kinase or phosphatase activity suitable for studying the enzymatic process. Large amounts of biological material from organs, such as the liver and spleen were necessary to conduct such work with protein kinases. Subsequent studies revealed the ubiquity and complexity of phosphorylation processes and techniques evolved from early rat studies to the adaptation of more rewarding in vitro models. These involved human erythrocytes, which are a convenient source both for the enzymes, we investigated and for their substrates. This preliminary work facilitated the development of more advanced phosphorylative models that are based on cell lines.

Keywords: Phosphorylation; Human erythrocytes; Rat liver mitochondria; Nephrolithiasis

1. Introduction

The discovery of the possible regulatory function of protein phosphorylation was in the late 1930s when Carl and Gerty Cory identified two forms of glycogen phosphorylase (a and b). Through the 1970s and early 1980s, the general significance of protein phosphorylation came to be appreciated, thanks to the study on the inhibitory effect of phosphorylation on the mitochondrial pyruvate dehydrogenase complex (Linn et al., 1969). Subsequent studies revealed a considerable number of protein kinase and phosphatase enzymes, whose activity can be resolved into two separate biochemical pathways: the first, involving the phosphorylation of serine/threonine (Ser/Thr)-residues and the second of the tyrosine (Tyr)-residues of proteins (Cohen, 2002). Post-translational modification by phosphorylation is a ubiquitous regulatory mechanism triggered in response to extracellular signals and provides a mechanism to modulate many diverse processes, such as various metabolic pathways, kinase cascade activation, membrane transport, gene transcription and motor mechanisms.

2. Initial animal studies

Our initial studies were directed at searching for possible substrates of casein kinases. In the 1970s, one of the best sources of both casein kinases and their relative substrates was rat liver cells, which were inexpensive, easy to use and readily available (Pinna et al., 1969, 1971, 1976; Clari et al., 1971, 1975, 1976; Moret et al., 1975). However, for the most part, in purification studies involving rat liver, much material was lost during the numerous processing steps (Fig. 1). This necessitated the use of large numbers of animals for kinase studies.

Liver mitochondria from 20 rats homogenate were subjected to three different purification steps including chromatography with DEAE-cellulose at step 1, P-cellulose at step 2 and Sephadex G-100 at step 3. Kinase activity recovered at the end of the purification process represents only about the 2.5–5% of the total activity in the crude homogenate.

For this work, up to 23 rat livers were isolated and both cytosol and mitochondria underwent purification processes to

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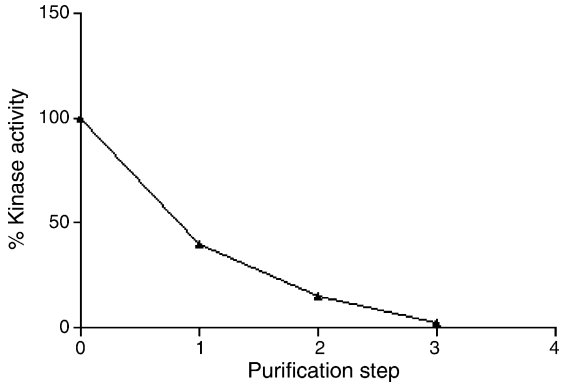

Fig. 1. Kinase activity obtained after three purification steps.

analyze casein kinase, phosphatase and tyrosine-protein kinase activities. Unfortunately, every purification step caused a partial loss of enzyme activity, probably because by isolating the enzyme from its normal enveloping cellular components, an alteration was induced in the tertiary and quaternary structure of the enzyme, thus causing loss of function. Besides, the presence of a concentration of high protease activity together with the long timeframes of the purification steps tended to exacerbate the situation.

3. In vitro erythrocytic studies

Eventually, subsequent work revealed the suitability of erythrocytes for phosphorylation studies (Clari et al., 1978). These cells represent one of the simplest cellular approaches for a scientific investigation, since they are commonly available and their manipulation does not require all the techniques needed for the separation of sub-cellular organelles. Utilizing erythrocytes we have been investigating the complex system of the phosphorylation of membrane proteins and their influence on protein–protein regulation (Clari et al., 1978, 1981, 1991, 1992; Clari and Ferrari, 1983; Bordin et al., 1992, 2002; Brunati et al., 1996, 2000). Thanks to the simple cellular organization of erythrocytes, it was possible, within a shorter time period compared with animal studies, to evolve several ideas and hypotheses in these cellular models (Brunati et al., 2000; Bordin et al., 2002), that could be continually updated and adapted.

Protein kinases and protein phosphatases were identified as a part of a complex cellular mechanism responsible for maintaining constant cellular levels of both Ser/Thr- and Tyr-phosphorylation. Any alteration in these sensitive equilibria may have serious toxicological consequences. There are many cases of anomalous erythrocytic phenotypes in nature; these are possessed of morphological (Olivieri et al., 1997) as well as metabolic defects (Baggio et al., 1984a) and studies with these cells improved our hypothetical models and facilitated understanding of the relevance of phosphorylative processes in the maintenance of normal erythrocyte function. Through the use of erythrocytes, it has been possible to ascribe two distinct different physiological roles to Ser/Thr and Tyr-phosphorylation in cell homeostasis. In addition, it was also found that Band-3 protein, a constituent of erythrocyte membranes, plays a key role in cell survival and function.

Following a screening and evaluation process of human erythocytic conditions, we identified a pathological increase of band-3 Ser/Thr-phosphorylation, which was a marker of idiopathic calcium oxalate nephrolithiasis. Oxalate is a major component of two thirds of all kidney stones and small changes in its urinary concentration critically influence calcium oxalate stone formation (Robertson and Nording, 1969). Nephrolithiasis is certainly a multifactorial disease and oxalate is not the only factor. However, the observation of abnormally high urinary excretion and intestinal absorption of oxalate in idiopathic calcium oxalate nephrolithiasis (Marangella et al., 1982; Baggio et al., 1983) seems to suggest a pivotal role for this anion in stone formation. The high frequency of a positive family history in patients with idiopathic calcium oxalate urolithiasis (McGeown, 1960; Ljunghall, 1979) strengthened the consideration that this disease may be a metabolic disorder characterized by a defect in cellular oxalate transport. It had been previously noted that transmembrane oxalate flux in erythrocytes from patients with idiopathic calcium oxalate renal stone formation is faster than in subject without stones (Baggio et al., 1984b). Band-3 is one of the major transmembrane proteins known to display multiple functions, including the ability to transport anions across the membrane. Indeed, the cytoplasmic domain of band-3, containing all the protein phosphorylatable sites, is the critical area of the protein as far as anionic transport through cellular membrane is concerned. Essentially, this domain acts as a gate that Ser/Thr-phosphorylation level can open or shut in accordance with cell physiological state (Baggio et al., 1993a,b, 1994, 1999). What is noteworthy, is that under simple in vitro conditions, we can mimic in normal erythrocytes the same up-regulation obtained from nephrolythiasic patients, confirming this important activity of Ser/Thr-phosphorylation level of band-3 (Baggio et al., 1993a). The rapid responsiveness of this simple cell model together with the univocal identification of the enzymes responsible of this phosphorylative level prompted us to quickly address the balance of their relative activities by biological effectors. It was found that that arachidonic acid, which is ubiquitous in the diet of developed countries, was linked with an increased incidence in lithogenesis in these countries (Baggio et al., 1996, 1999; Gambaro et al., 2000).

There are a number of homeostatic mechanisms linked to Tyr-phosphorylation, which involve band-3 protein. In normal erythrocytes, no Tyr-phosphorylation activity is usually present under resting conditions. Oxidative or hyperosmotic stresses, as well as impairment of Tyr-phosphorylation balance, such as selective inhibition of protein tyrosine-phosphatase activity, can increase band-3 Tyr-phosphorylation level triggering a two step catalysis. At first, $p72^{Syk}$, a Tyr-kinase belonging to the Syk family, catalyzes the so-called primary phosphorylation of tyrosines 8 and 21 and generates P-Tyr-residues recognized by the SH2 domain of a second Tyr-kinase, 'Lyn' which belongs to the Src family. Once Lyn is recruited, the secondary phosphorylation of residues 359 and 904 is catalyzed. P-Tyr-359 represents the docking site for the protein tyrosine-phosphatase SHP-2-SH2 domain, which once bound to band-3, dephosphorylates