

PROTEIN PHOSPHORYLATION AND CELLULAR REGULATION, II

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by

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In the talk he just gave, Ed Krebs retraced for you the early history of glycogen phosphorylase and its regulation by reversible phosphorylation. In retrospect, we were extraordinarily lucky in more ways than one to have selected this particular enzyme to work on. First, it is extremely abundant in skeletal muscle so that material was never limiting. In fact, when 20 years later, we undertook its total amino acid sequence with Ko Titani and Ken Walsh (1), we used no less than 10 grams of the crystalline enzyme to complete the analysis. Second, the phosphorylation reaction was unambiguous, converting a totally inactive enzyme into a fully active species. Third, the phosphorylation ran to completion, introducing 1 mole of phosphate per mole of enzyme subunit. Fourth, only one site became phosphorylated; and fifth, this site occurred within a loose N-terminal arm of the molecule that could be easily cleaved by limited proteolysis, leaving behind the bulk of the enzyme intact. This made it easy to separate the phosphopeptide and determine its structure (2), though it took a long time since in the mid fifties sequences had to be carried out by paper chromatography. We could show that a single seryl residue had been phosphorylated (Fig. 1). Incidentally, it took another five years to extend this sequence by 9 residues (3). The longer structure showed the presence of several positive charges, mainly upstream, and forming those recognition motifs whose significance became apparent only later (4,5).

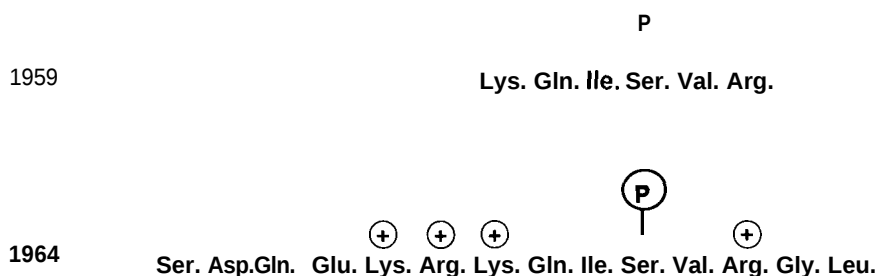


Fig. 1: Sequence of the site phosphorylated during the phosphorylase b to a reaction.

The phosphorylation reaction was so straightforward and simple that there was no doubt in our minds that it would represent the prototype for such kinds of interconversions. As it turned out, it was really the exception. Six years went by before Joe Larner identified the next enzyme to be regulated by phosphorylation-dephosphorylation, namely, glycogen synthase (6). He found that this enzyme was inactivated rather than activated by phosphorylation (7), which made sense: closing off the backward reaction when the forward one is turned on guarantees that there would be no recycling of the system that would inevitably result in a useless expenditure of energy. Joe Larner rapidly found, however, that far more than one phosphate was introduced during the interconversion (8); about six per mole of synthase. At that time, the idea that a single phosphorylation event was all that was needed to alter the state of activity of an enzyme was so ingrained that I remember that we thought: "How can this be possible? It would imply that glycogen synthase is made up of 6 identical subunits, each of approximately 15,000 MW." And Joe Larner himself thought along these lines (8,9). Of course, we know today from his work as well as that of Phil Cohen (10), Peter Roach (11) and others, that the enzyme is phosphorylated on no less than seven sites by seven different protein kinases, all totally unknown at that time (Fig. 2). Furthermore, some of these phosphorylation events follow a most complicated program of successive reactions that have to proceed in a strictly prescribed order. The enzyme is inhibited by phosphorylation by glycogen synthase kinase 3 (or GSK-3) but not until a first phosphate is introduced by casein kinase 2 (CK-2). This then allows phosphorylation of the next residue, then the next and the next after that until all sites are finally occupied (12). Imagine the incredible difficulties we would have encountered had we decided to study this enzyme rather than phosphorylase.

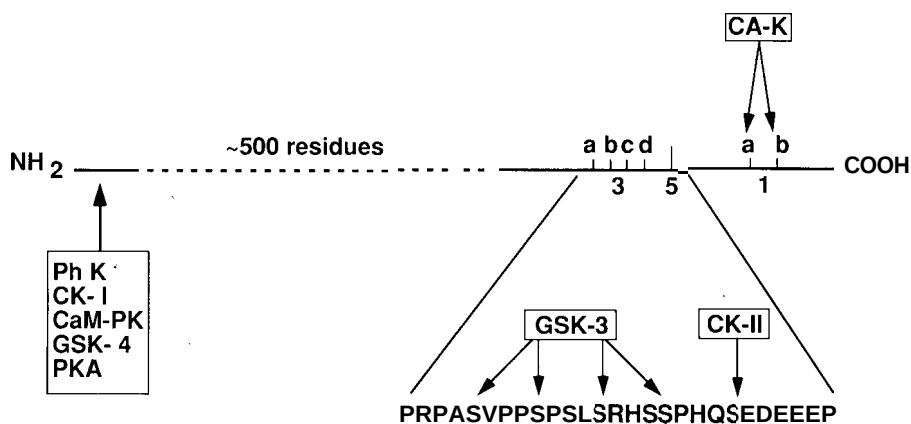
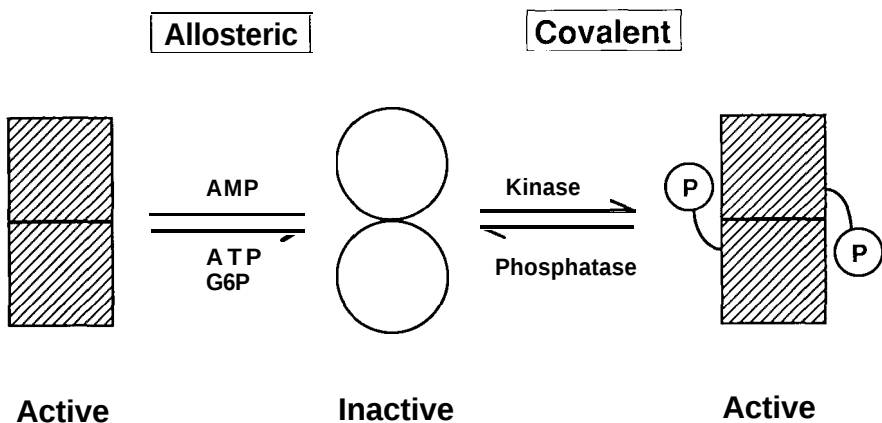


Fig. 2: Major phosphorylation sites in glycogen synthase. PhK, phosphorylase kinase; CK casein kinase; CaM-PK, multifunctional calcium/calmodulin-dependent kinase; GSK, glycogen synthase kinase; PKA or CA-K, CAMP-dependent protein kinase (from P. Roach).

We did not know at that time whether the phosphorylation reaction was a unique occurrence, a rare event restricted to the control of these two enzymes or perhaps to carbohydrate metabolism. It was well known, for example, that during glycogenolysis, inorganic phosphate was picked up from the medium and used for the production of many sugar phosphate intermediates. Could nitrogen metabolism be regulated by another type of covalent modification, for instance, amidation / deamidation, or lipid metabolism by acetylation / deacetylation? Or had we discovered a more general type of reaction that would apply widely to many different systems. Once again, as luck would have it, reversible protein phosphorylation turned out to be one of the most widespread mechanisms by which cellular processes can be regulated (13,14).

Allosteric and Covalent Regulation

Here, a question should be raised. Ed Krebs already told you that phosphorylase could also be activated by AMP. Why are two mechanisms required to control the activity of an enzyme when, in both cases, they lead to an active conformation (Fig. 3)? According to the allosteric model proposed by Jacques Monod in the early 1960s, the enzyme responds to effectors that are generated during the normal maintenance of the cell and reflect its overall internal condition: whether it is proliferating or quiescent, actively metabolizing or not, its energy balance, i.e., its ratio of AMP to ATP, etc. According to the rule that enzymes are subjected to end-product or feedback inhibition, phosphorylase would be expected to be inhibited by G6P that accumulates during its reaction, and by ATP, the ultimate end product of carbohydrate metabolism. By the same token, it would be activated by AMP as indeed it is. But then, many enzymes of carbohydrate metabolism would be similarly affected by the same effectors; in proceeding down the metabolic path, all these “doors” would become open at the same time. By contrast, the kinase reaction is highly specific: it allows the activation of phosphorylase only without affecting the activity of any other enzyme.



Rg. 3: Allosteric and covalent regulation of phosphorylase

Furthermore, and this is perhaps one of the major lessons we have learned over the last 30 years, covalent regulation responds mainly to extracellular signals (Fig. 4). These external signals come in the form of hormones, or neurotransmitters, growth factors and other stimuli such as drugs, light, odorants, and perhaps touch in plants. Each will act on its own membrane receptor and either cause the release of second messengers (cA, cG, Ca²⁺, DAG, IP₃, etc.) in reactions regulated by G-proteins, or induce the intrinsic tyrosine kinase activity of the receptors themselves. These second messengers or internal signals will act on kinases or phosphatases, then on target enzymes to finally elicit a physiological response. One finds here all the elements of a cascade system such as those described by Ed Krebs earlier: enzymes acting on enzymes, resulting in the enormous amplification of an external signal. In addition, it allows the coordinate regulation of different physiological events through the pleiotropic action of some of the enzymes involved.

The Protein Kinases

Today, one knows several hundred protein kinases that can be classified according to their mode of regulation or substrate specificity (15) (Table I). Among the large family of Ser/Thr kinases, some are dependent on the second messengers mentioned above, others on specific components of the system that they are called upon to regulate. Such is the case of the heme-regulated kinase that blocks the initiation of globin synthesis when heme or iron comes to be missing, or the double-stranded RNA-dependent kinase that is induced by interferon in cells under viral attack. There are the so-called “independent kinases” such as the casein kinases for which no precise mode of control has been elucidated as yet.

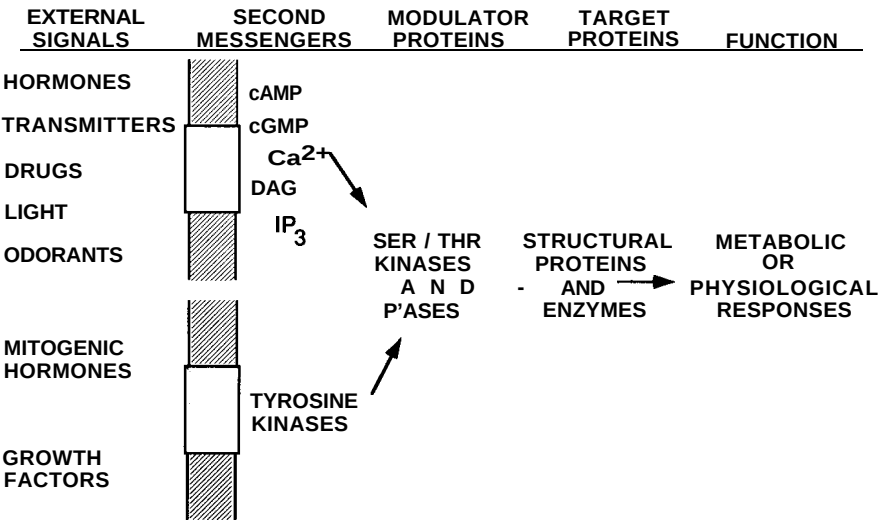


Fig. 4: Control of cellular processes by protein phosphorylation. Cascade systems triggered by extracellular stimuli.

Next come the mixed function kinases that can phosphorylate their substrates on both tyrosine or serine/threonine, or that can be regulated by tyrosine and serine/threonine phosphorylation. This is the case for the enzymes that Ed Krebs has just described (16): MAP kinase, the MAP kinase kinase, perhaps Raf, or the cell cycle kinases such as p34cdc2 (17). They stand guard at crucial crossroads of signal transduction. They form the link between a signaling system that originates at the membrane level and relies on tyrosine phosphorylation, and the more widespread serine/threonine phosphorylation reactions that occur downstream. Their dual control and specificity would ensure that no accidental initiation of important cellular events occurs at inappropriate times, just as one would need two keys to get access to a safety deposit box.

Finally, there is the large class of tyrosine kinases which will be discussed later. Not listed are the less common histidine kinases such as those involved in bacterial chemotaxis (18) and the double-headed kinase/phosphatase that regulates bacterial isocitrate dehydrogenase (19).

Regulation of Protein Kinases

All these enzymes have homologous catalytic domains but vary greatly in the structure of their regulatory segments. They have consensus sequences,

I. Second Messenger-dependent Ser / Thr Kinases

A. Cyclic Nucleotides: cAMP, cGMP PKs

B. Ca^{2+} / CaM : Phos. Kinase, MLCK, CaM Kinase II

C. DAG / Ca^{2+} : PKC

II. Second Messenger-independent Ser / Thr PKs

A. Heme-, ds RNA-, (INF)- dep. eIF2 Kinases

B. CK-I, CK-II, GSK-3, S6 Kinases

III. Dual Specificity (Ser / Thr and Tyr) PKs

MAPK, MAPKK, Raf (?)

p34^{Cdc2}

IV. Protein Tyrosine Kinases (PTK's)

A. Cellular or viral (oncogenic) PTK's

B. Receptor- linked PTK's

such as the motifs that are involved in the binding of ATP, by which they can be identified by searching the database (20,21). Most are regulated by segments that block their activity, often by virtue of the fact that they contain pseudo-substrate motifs that interact with, and shield, their catalytic sites. These autoinhibitory domains can exist on separate subunits as in the CAMP-dependent protein kinase (cA PK) first characterized by Ed Krebs and Don Walsh (22), or within the same peptide chain, as in the cG PK, where the two segments have become fused in the course of evolution. Initially, for the cA PK, the reaction seemed simple enough: the enzyme exists as an inactive complex between catalytic and regulatory subunits; cAMP induces a change in conformation in the regulatory subunits resulting in the dissociation of the enzyme and the liberation of active catalytic subunits. It soon became apparent, however, that the inactive complex had a more substantial purpose, namely, to prevent the translocation of the free catalytic subunits to other compartments of the cell, particularly the nucleus (23). But we know today that the regulation of this enzyme is even more sophisticated: the regulatory subunits themselves contain structural determinants that allow them to recognize and bind with high affinity to anchoring proteins distributed at specific locations within the cell (24-27). More than two dozen of these have been identified; they are particularly abundant in brain and the thyroid. Conceivably, some of these could co-localize with particular CAMP-generating receptor. This would confer a certain degree of selectivity to the hormonal response by targeting the kinase toward a given set of substrates (27) (Fig. 5).

Enzyme translocation may also play an important role in the regulation of protein kinase C first described by Nishizuka (28, 29). Depending on which subspecies of PKC is involved, the enzyme contains up to three regulatory

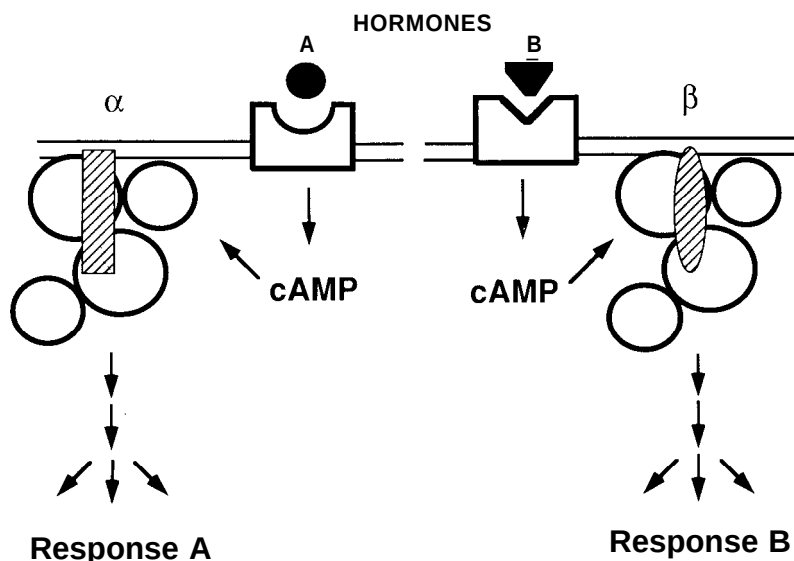


Fig. 5: Hypothetical selectivity in hormonal response by juxtaposition of CAMP-dependent protein kinase anchoring proteins with specific adenylate cyclase receptors.

domains responsible for the binding of Ca^{2+} , diacylglycerol and phospholipids. Binding of these allosteric effectors can promote the translocation of the enzyme to the plasma membrane where it could bind to specific anchoring proteins (30). Once again, this could determine which particular signal pathway would become affected.

Enzyme translocation might also be one of the functions of the cyclins, those regulatory subunits that are transiently expressed during various phases of the cell cycle. They associate strongly with the cell cycle-dependent kinases and operate particularly at the G1/S and G2/M transitions (31-33). But in *S. cerevisiae*, for instance, between 4 and 5 dozen cyclins have been identified (34). While their multiplicity would provide the cell with the redundancy it needs to protect itself from accidental failures, it would seem unlikely that their sole purpose would be to modulate the activity of the kinases. Some of these complexes must become operative at other set points along the cell cycle. More importantly, perhaps, they could be essential to target the enzymes toward those elements that become operative during the profound cytoskeletal reorganizations that accompany cell division.

Targeting of Serine/Threonine Phosphatases

Targeting subunits are particularly crucial for serine/threonine phosphatases because these enzymes are not geared to recognize specific sequences, or structural determinants within their substrates. Furthermore, unlike the kinases, they consist of just a few types of enzymes that have broad and overlapping specificities (35-37). Thus they have to depend on regulatory subunits or binding proteins to direct them toward particular compartments of the cell where they will encounter particular substrates. That is the case, for instance, of the type 1 phosphatase whose catalytic subunit can bind to a glycogen-recognizing subunit, a myosin-recognizing subunit or an inhibitory molecule called Inhibitor 2. In each of these forms, the enzyme recognizes a particular set of substrates. Formation or dissociation of these complexes is under hormonal control (38-41). This is the theme that I propose to develop in the second part of this talk devoted to the role of regulatory/localization domains in the function of tyrosine phosphatases.

Protein Tyrosine Phosphorylation

Three remarkable discoveries, 14 years ago, provided considerable excitement to the field of cellular regulation by protein phosphorylation. First, the finding by the groups of Ray Erickson (42,43) and Varmus-Bishop (44) that the product of the src gene responsible for the oncogenicity of Rous sarcoma virus was a protein kinase they designated as pp60^{src}. Second, the unexpected report by Tony Hunter and Bart Sefton that this kinase, unlike all previously known enzymes, phosphorylated its protein substrates exclusively on tyrosyl residues (45). Third, the identification of the non-transforming homolog of v-src (46-50), i.e., the cellular c-src. c-src encodes a product, (pp60^{c-src}) which differs from its oncogenic viral counterpart by

having, among other discrete mutations, a short extension at the C-terminus. This extension carries a phosphotyrosyl residue that keeps the activity of the enzyme under control. Today we know more than a dozen tyrosine kinases of cellular or viral origin and their number continues to grow (15,50).

Finally came the seminal discovery from the laboratory of Stanley Cohen (51,52) that the receptor for epidermal growth factor was itself a tyrosine kinase whose activity was induced by binding of the ligand. Since then, many families of receptors with tyrosine kinase activities have been identified (53). They all have an external, ligand-binding domain, some with cystein rich regions, a single transmembrane segment and a cytoplasmic tyrosine kinase domain (Fig. 6).

Just as mutation of the intracellular tyrosine kinases can lead to cell transformation, mutation of the growth factor receptors can lead to oncogenic products. The first to be identified was the retroviral oncogene v-erb B (54,55) generated by a truncation of the external domain of the EGF receptor, its cellular progenitor. Many others have since been cloned and characterized; they usually result from truncation of the molecule at one end or the other or both, fusion with certain viral elements and other kinds of mutations.

With accumulating evidence implicating tyrosine phosphorylation in cell proliferation and transformation, it is hardly surprising that many groups would become interested in the enzymes that would catalyze the reverse reaction, namely, the protein tyrosine phosphatases.

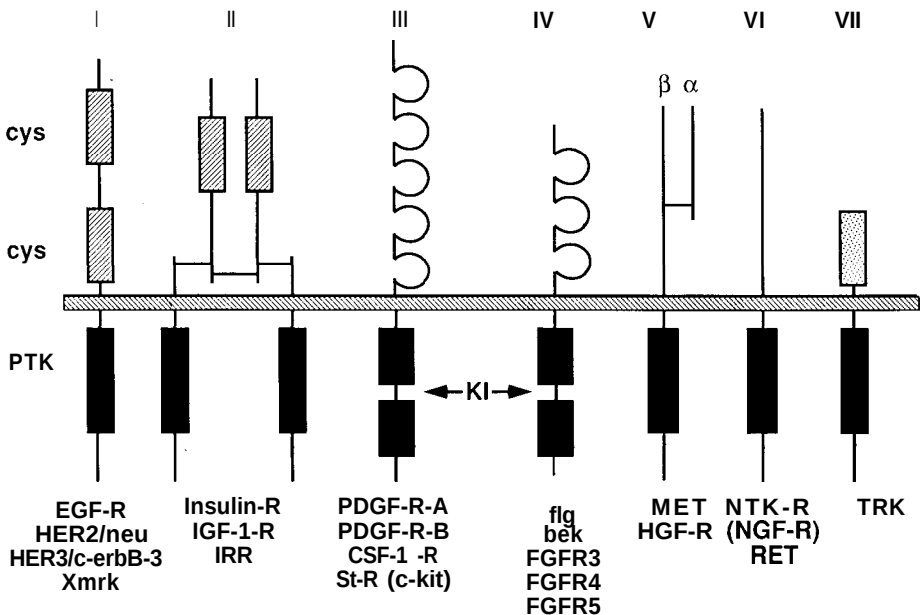


Fig. 6: Growth factor tyrosine kinase receptors.

Protein Tyrosine Phosphatases

First evidence for phosphotyrosine dephosphorylation was obtained by Graham Carpenter and Stanley Cohen (51,56) using A431 cell membranes overexpressing the EGF receptor, allowing these to undergo autophosphorylation, and then monitoring their rate of dephosphorylation. Similar observations were made by Bart Sefton and Tony Hunter using cells transformed with a temperature sensitive mutant of Rous sarcoma virus (57). Then followed a flurry of studies by a number of groups, including our own six years ago (58-60). The work was originated with Nick Tonks, a superb Post Doctoral Fellow and second generation Seattleite since he had just obtained his PhD degree with Phil Cohen at Dundee who himself had been in our laboratory some 20 odd years before. When we started, we also assumed that if transformation could be brought about by overexpression of the tyrosine kinases, or mutations that would render them constitutively active, then overexpression of the phosphatases might block or reverse these reactions. This assumption was too simplistic.

Within a couple of years, a tyrosine phosphatase was isolated in homogeneous form from human placenta (61,62). The enzyme was totally specific for phosphotyrosyl residues and extremely active. It had a specific activity about one order of magnitude higher than most viral tyrosine kinases and up to 3 orders of magnitude higher than certain receptor tyrosine kinases. This high activity suggested that it had to be tightly regulated to allow for those mitogenic signals that are necessary for normal cell development.

The Leukocyte Common Antigen, CD45: a Tyrosine Phosphatase

The surprise came when the amino acid sequence of the enzyme was determined by Ken Walsh and Harry Charbonneau because it showed no homology with any of the other protein phosphatases (63). However, a search of the data base indicated that the enzyme was structurally related to an abundant and already well-known surface antigen, the leukocyte common antigen also designated as CD45 (64) (Fig. 7). The leukocyte common antigen represents a broad family of membrane-spanning molecules found in all hematopoietic cells except mature erythrocytes (65). Their intracellular moiety is highly conserved and contains two internally homologous domains of approximately 30 kDa each. It is those two domains that are structurally related to the placenta phosphatase. CD45 has been implicated in the regulation of lymphocyte function, including cytotoxicity, proliferation and differentiation and in modulating IL2 receptor expression.

Here, I must open a parenthesis with biblical overtone. Had we obtained the sequence of the placenta phosphatase before that of CD45 was determined, nobody would have paid much attention - and we neither - because it would just have been the sequence of another enzyme. And it is those working on CD45 - Ian Trowbridge, Allen Williams, Matt Thomas, etc. who, once they got their sequence and searched it through the data base, would have made the surprising observation that their receptor was, in fact, a tyrosine phosphatase. What this means is that in this business, it really pays

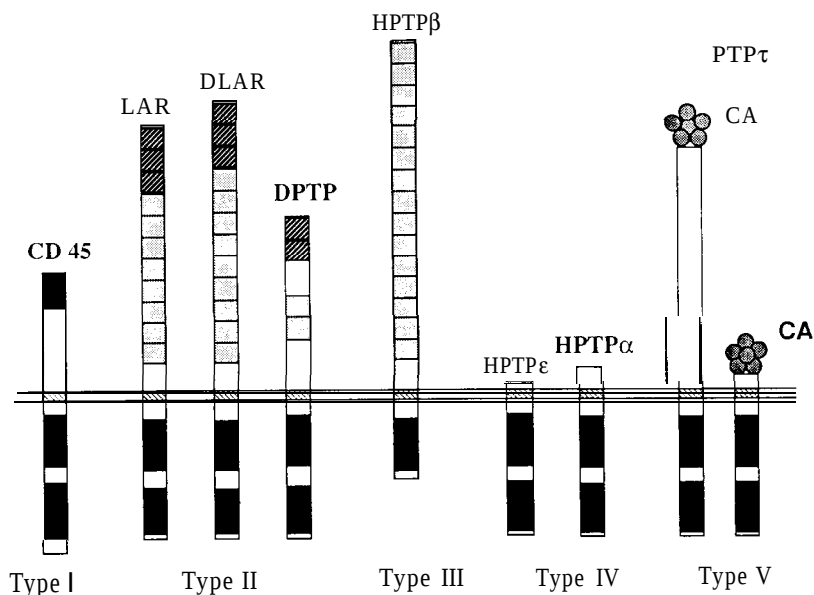


Fig. 7: Tyrosine phosphatase receptors

to be last, precisely as recorded in the Gospel which states that “The last shall be first and the first last”.

Since then, a great variety of receptor forms have been identified (58-60). All but one display the same double catalytic domains in their cytoplasmic portion but considerable diversity in their external segments (Fig. 7). Some have structural characteristics of cell adhesion molecules such as the leukocyte common antigen-related LARs first cloned by Saito and Schlossman. They are related to the N-CAMs (Neural Cell Adhesion Molecules) or fasciclin II suggesting that they might be involved in homophilic cell-cell interactions and perhaps modulate morphogenesis and tissue development. Others contain fibronectin type III repeats and might be involved in cell-cell or cell-matrix signaling. Some have very short external domains. Perhaps the most intriguing receptors are the ones recently cloned independently by Schlessinger (66) and Saito (67). At the end of an external segment, one finds a large globular domain almost identical to carbonic anhydrase except that it contains only one of the 3 histidines involved in the binding of Zn^{2+} . Except for CD45, no ligand has been found for any of these structures.

The Intracellular Tyrosine Phosphatases in cell cycle progression and transformation

Likewise, the low molecular weight, intracellular tyrosine phosphatases display a great diversity of structures, either preceding or following a highly conserved catalytic core. These are undoubtedly involved in the regulation

and localization of the enzymes.(Fig. 8). Some PTPs have segments homologous to cytoskeletal proteins such as band 4.1, ezrin and talin; others contain two SH2 (src-homology 2) domains which might allow them to interact with phosphotyrosyl residues at sites of autophosphorylation of growth factor receptors. Tyrosine phosphatases are also found as the gene products (YOPs) of virulence plasmids from bacteria of the genus *Yersinia* (such as *Y. pestis* responsible for the bubonic plague).

I would like to discuss now the role that these regulatory domains might play in enzyme localization and function, focussing on the human T-cell enzyme cloned by Debbie Cool (68) (Fig. 9). The regulatory domain is entirely hydrophilic until one reaches the last 19 residues that are very hydrophobic, reminiscent of a transmembrane domain. There is also a short stretch of 5 basic residues that could serve as a nuclear localization signal (67).

The 11 kDa segment of the enzyme was mutated, as well as other segments of putative physiological importance. As a first step, a premature stop codon was introduced after the catalytic domain to delete the entire C-terminal tail. When this is done, the truncated enzyme becomes soluble whereas the wild-type protein is particulate (69, 70), localizing with the endoplasmic reticulum (ER). Expression of the C-terminal tail as a fusion protein with β -galactosidase shows that the 'soluble β -galactosidase now becomes attached to the ER. Finally, if one deletes just the C-terminal hydrophobic stretch, the enzyme localizes in the nucleus (J. Lorenzen, unpublished data).

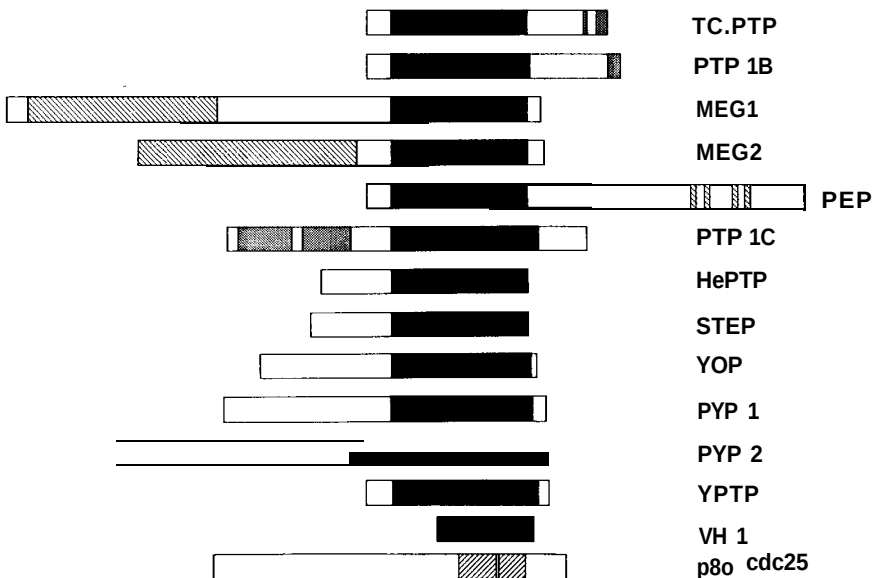


Fig. 8: Intracellular tyrosine phosphatases , aligned on the basis of their conserved, catalytic domains.

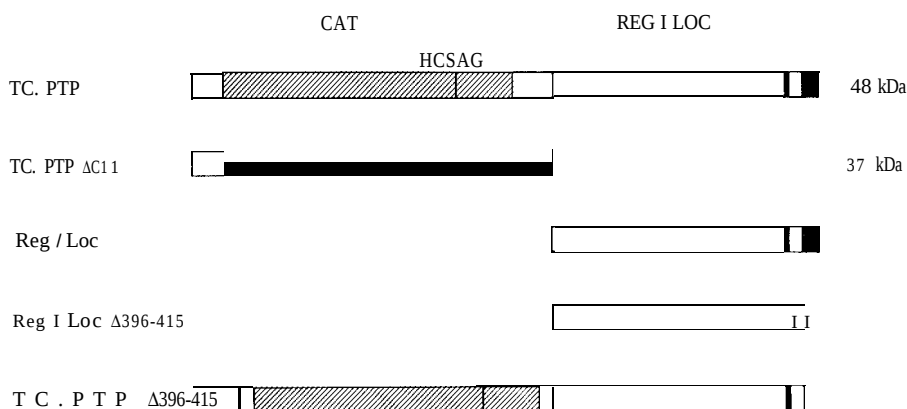


Fig. 9: Schematic representation of the human T-cell tyrosine phosphatase (TC-20) and some of its mutated forms. Illustrated are the full-length, wild-type 48 kDa enzyme, the 37 kDa truncated form containing only the catalytic domain; the 11 kDa regulatory/localization domain; and two mutant forms in which the hydrophobic IO-residue segment at the C-terminus (dark band) has been deleted.

Because of lack of time, I will only discuss the differences one observes in cell cycle progression and transformation when one expresses the wild-type enzyme vs. its truncated form obtained by introducing a premature stop codon after the catalytic domain. In BHK cells in which the wild-type enzyme is overexpressed, there is no obvious change in cell morphology. By contrast, 60-70% of the cells expressing the truncated form become multinucleated (71) due to a failure in cytokinesis.

Multinucleation is not unusual; it can occur by cell fusion, with certain drugs or with antibodies against myosin ATPase since cytokinesis is an actomyosin-dependent process (72,73). But in all these instances, when nuclear division goes on, it goes on synchronously. What is unusual in these BHK cells is that nuclear division is more often than not asynchronous (Fig. 10): that is, one nucleus will divide while the other will not. Therefore, one will see cells with nuclei at all phases of cell cycle. At this time, we don't know which inter nuclei signals have been disrupted.

Differences in cell behavior brought about by expression of the wild-type vs. truncated T-cell enzyme can also be seen in cell transformation, using the same highly tumorigenic BHK cell line. Transformation is the change a cell undergoes when it becomes malignant and no longer abides by the constraints under which a normal cell must operate. A normal cell does not grow on soft agar as it needs a solid support to which it can adhere, whereas a transformed cell will. As expected, the transformed BHK cell line grows readily on soft agar but it grows just as well, if not better, when transfected with the wild-type enzyme. By contrast, overexpression of the truncated form almost abolishes growth under these conditions, as if transformation had been suppressed (D. Cool, unpublished data).

A similar enhancement in tumorigenicity by overexpression of the full-length phosphatase is observed when these cells are injected into athymic

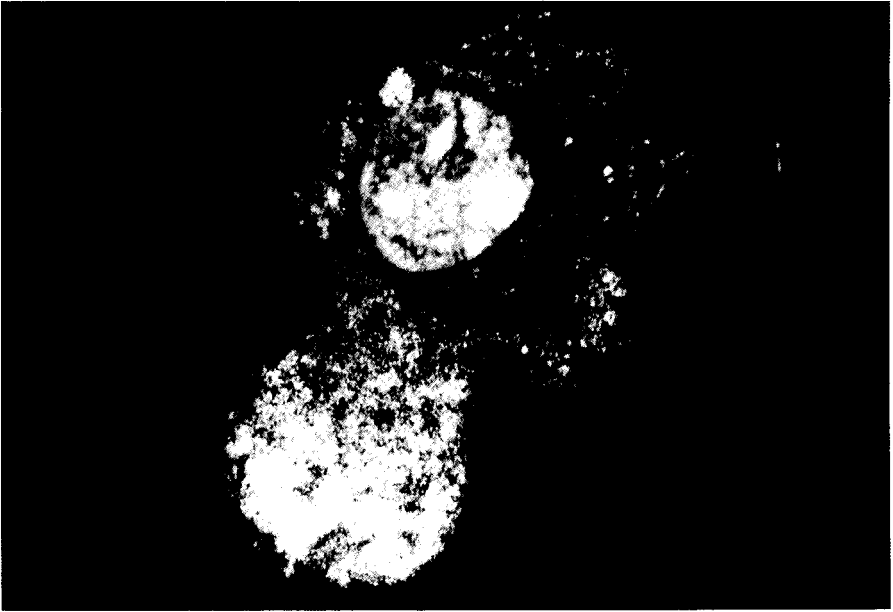


Fig. 10: BHK cells overexpressing the truncated 37 kDa form of the T-cell tyrosine phosphatase showing asynchronous nuclear division. The cell has rounded up around the lower nucleus ready to undergo mitosis. The nucleus on top is in an interphase configuration (71).

nude mice. The tumors produced are highly vascularized, as compared to tumors formed from control BHK cells. On the other hand, tumor formation is greatly reduced if not suppressed with BHK cells containing the truncated form. In several animals, no tumor was detected.

Since the nature of the transforming agent in these BHK cells was not defined, these studies were repeated in embryonic Rat-2 cells transformed with the well-characterized viral oncogene *v-fms* (74). *v-fms* was first isolated from a feline sarcoma virus and belongs to the platelet-derived growth factor (PDGF) family of tyrosine kinase receptors (75). Its non-transforming progenitor, the cellular protooncogene *c-fms*, encodes the receptor for CSF-1, the macrophage colony-stimulating factor. Binding of CSF-1 triggers signaling events that lead to the transcription of CSF-1 genes necessary for mononuclear phagocyte growth, differentiation and survival (76).

Control Rat-2 cells display typical, non-transformed, cobblestone morphology – as opposed to the spindle-shape *v-fms* transformed cells (74). Cells overexpressing the full-length enzyme have the same stringy transformed appearance, whereas clones containing the truncated form have the Rat-2, non-transformed cobblestone phenotype (Fig. 11). The transformed cells will grow on soft agar, the others will not. To achieve a high level of expression of the tyrosine phosphatase, the enzyme was packaged in retroviruses with which the cells were infected. None of the cells containing the truncated enzyme grew very well on soft agar. That these cells had reverted to a non-transformed state was further demonstrated by the appearance of

their cytoskeleton (Fig. 12). Transformed cells exhibit a disruption of actin microfilaments and a loss of focal adhesions; i.e., the structures through which normal cells adhere to their substratum. The actin fibers are restored in cells expressing the truncated enzyme and many more focal adhesions can be seen (D. Cool, unpublished results).

Finally, the same differences were observed in the ability of these two types of cells to form tumors when injected into nude mice. All cells containing the *v-fms* oncogenes produced large tumors, as those co-expressing the wild-type phosphatase. As observed in BHK cells, tumor formation was abolished in most cells transfected with the truncated enzyme.

Concluding Remarks

The above data, plus others I did not have the time to develop, indicate that phosphatases cannot be viewed simply as providing an "off" switch in an "on/off" kinase/phosphatase system. In certain cases and depending on the form of the enzyme involved, tyrosine phosphatases can clearly act synergistically with the kinases to bring about a particular physiological

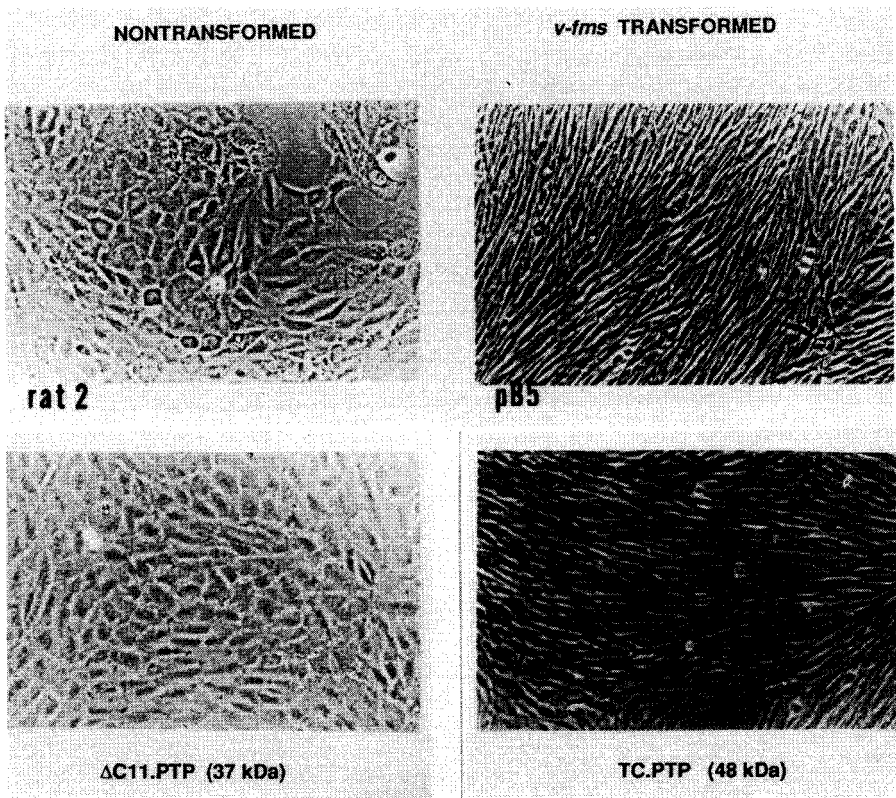


Fig. 11: Morphology of non-transformed Rat-2 cells (upper left) or cells transformed with *v-fms* (upper right). The *v-fms*-transformed cell lines were co-transfected with full-length T-cell tyrosine phosphatase (lower right) or its 37 kDa truncated form (lower left). The data indicate that while the full-length enzyme has not altered the transformed (spindle-shape) morphology, cells containing the truncated enzymes display a non-transformed phenotype.

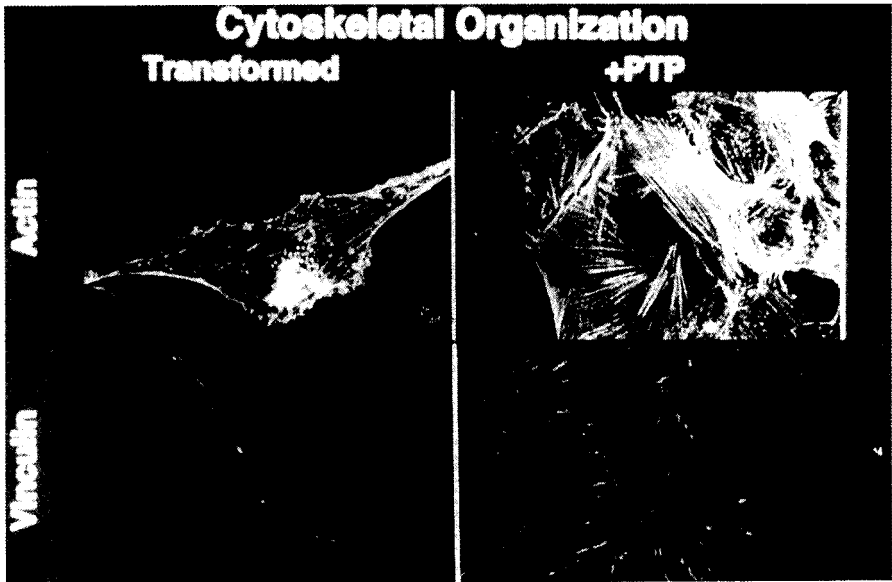


Fig. 12: Cytoskeleton morphology of v-fms transformed Rat-2 cells before (left) and after detransformation by co-expression of the truncated (37 kDa) form of the T-cell enzyme. Upper frames: actin stained with rhodamine-labeled phalloidin; lower frames: stained with anti-vinculin antibodies to mark the focal adhesions (bright spots).

response. An obvious way by which they could do that would be by activating the src family kinases which are repressed by phosphorylation at the C-terminus (Fig. 13). Furthermore, the factors that would determine whether a phosphatase would enhance or oppose a kinase reaction would seem to depend less on its state of activity than on its subcellular localization. An analogous situation is found with pp60^{v-src}. Removal of the myristoyl group required for its binding to the plasma membrane does not affect its enzymatic properties but abolishes its transforming abilities (77). Association with the membrane is, therefore, essential for its oncogenicity. The above data would indicate that if one wanted to control transformation through the phosphatases, one should try to manipulate the segments involved in their localization - or whatever anchoring proteins to which they may bind - rather than their catalytic domains. There are, of course, many questions that need to be answered. To mention a few:

- a) What controls the activity of phosphatases under normal conditions? It may be easier to achieve high level expression of these enzymes in transformed lines in which the signal pathways are turned on, rather than in normal cells; as if a balance existed between kinases and phosphatases.
- b) Do the differences between wild-type and truncated enzymes depend solely on their localization or is enzyme specificity also implicated?
- c) Would the same effects be observed with any oncogene, including those that are not tyrosine kinases (such as ras, raf, mos, etc.)?
- d) Which are the steps in the signaling pathway that are specifically affected by the enzymes? And finally,

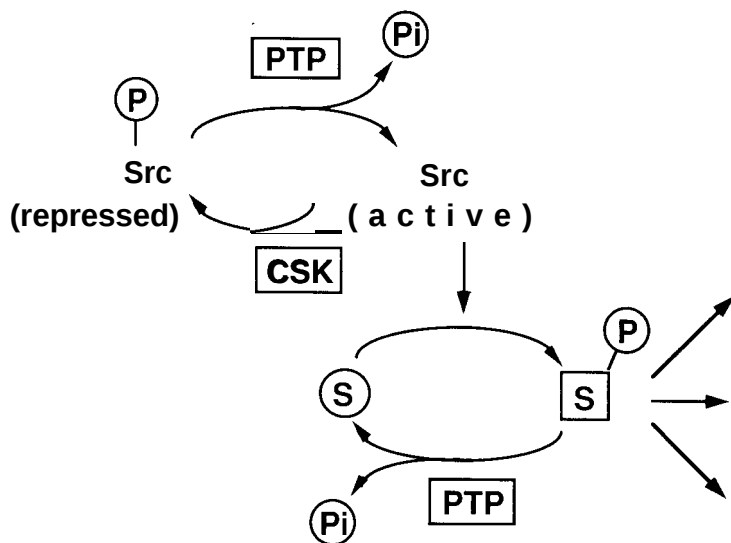


Fig. 13: Possible dual role of tyrosine phosphatases in signal transduction. Upper reaction, activation of src family tyrosine kinases by dephosphorylation of the C-terminal phosphotyrosyl residue. Lower reaction, dephosphorylation of other tyrosine phosphorylated substrate to return the system to its original state. csk is the cellular src kinase that represses enzymatic activity by tyrosine phosphorylation at the C-terminus.

e) If oncogenicity can result from an overexpression of tyrosine kinases, could oncogenicity also result from an underexpression of certain phosphatases?

All the processes Ed Krebs and I have described today have had something in common; they followed a similar pattern at the molecular level. Under the influence of an external stimulus, the state of phosphorylation of a protein or enzyme has been altered. This, in turn, has triggered a given cellular response. The finding that certain oncogenic agents may operate along the same principles may provide some clues as to the very mechanism of cell transformation. And who knows, perhaps with a little luck, a deeper understanding of these signaling pathways may suggest new avenues by which oncogenicity can be brought under control.

ACKNOWLEDGEMENTS

My scientific career has been profoundly marked by two long-standing collaborations, first with the late Eric A. Stein on α -amylases, then with Edwin G. Krebs, with whom I share this Award, on the protein phosphorylation problem. In addition to being the most valuable of colleagues, they have been two of my closest friends. I also owe a debt of gratitude to all those who joined the laboratory over the years, contributing their skills, original ideas and maximum effort before moving on to pursue their own

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