Membrane phosphorylation: a crucial role in the action of insulin, EGF, and pp60src?

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Insulin, epidermal growth factor (EGF), and the membrane protein responsible for malignant transformation by avian sarcoma virus, pp60src, have at least two things in common. The first is an involvement with cell growth and proliferation, and the second is an ability to regulate the phosphorylation of specific membrane proteins. Covalent modification by phosphorylation of soluble proteins has been demonstrated to be an important control mechanism regulating a variety of cellular processes (Rubin & Rosen, 1975; Nimmo & Cohen, 1977; Krebs & Beavo, 1979; Weller, 1979). It is presumably then through altering the activity of these target proteins that these entities express certain of their biological effects.

Insulin (Cuatrecasas, 1974; Czech, 1977) and EGF (Carpenter & Cohen, 1979) both bind to specific receptors integrated into plasma membrane, and pp60src is also found in the plasma membrane (Willingham et al., 1979). This review explores some connections between these systems and considers spatial relationships between kinases and target proteins in the membrane.

Membrane proteins are free to undergo lateral diffusion in two dimensions of the lipid bilayer unless specifically constrained. Such proteins may be divided into two categories: these are the integral proteins, which are firmly embedded in the bilayer, and the peripheral proteins, which are associated with the membrane through electrostatic interactions. All copies of a particular integral protein exhibit an absolute asymmetry which is set up during biosynthesis, and the removal of these proteins necessitates the use of detergents. Peripheral proteins, however, can be liberated by merely adding chelating agents or by increasing the pH or ionic strength (see Warren & Houslay, 1980). Recent studies have demonstrated that integral membrane proteins can be divided into a number of subclasses based upon the degree of penetration of their globular portions into the lipid bilayer. Peripheral proteins, on the other hand, can be divided into two categories: those that interact predominantly with phospholipids and those that bind specifically to integral proteins. These are shown schematically in Fig. 1. It would seem important, then, for any study of the phosphorylation of membrane proteins to assess: i) whether peripheral or integral proteins are involved, ii) to which integral proteins the peripheral proteins are attached, iii) the asymmetrical orientation of the proteins, iv) whether free lateral diffusion is necessary to propagate the response, v) if an activated membrane-bound kinase can act on both soluble and membrane-bound targets, and vi) whether soluble kinases can act on membrane-bound...
Fig. 1. Classes of integral and peripheral proteins. Schematic representations of the various relationships of integral (I-IV) and peripheral (V-VI) proteins with the lipid bilayer. I, transmembrane globular integral protein with functioning region inserted in the bilayer, e.g. erythrocyte anion transport protein (Band 3), Ca$^{2+}$ ATPase, and bacteriorhodopsin; II, transmembrane fibrous integral protein with globular mass in aqueous compartment, e.g. glycophorin and aminopeptidase; III, globular portion in aqueous phase but anchored by an integral hydrophobic pedicle that does not span the bilayer, e.g. cytochrome $b_5$ and cytochrome $b_5$ reductase; IV, globular integral protein associated with essentially one or the other half of the bilayer. A fibrous tail may span the bilayer, e.g. adenylate cyclase (see e.g. Rothman & Lenard, 1977; Kenny & Booth, 1978; Warren & Houslay, 1980; Houslay et al., 1980); V, peripheral protein associated essentially through interactions with phospholipids and therefore able to partition between membranes, e.g. cytochrome $c$ and pyruvate oxidase; VI, peripheral protein binding to specific integral transmembrane proteins and therefore displaying a single location, e.g. erythrocyte glucose-6-phosphate dehydrogenase and ankyrin (Rothman & Lenard, 1977; Kant & Steck, 1973; Bennett & Stenbuck, 1980). Fibrous tails or anchors are shown as twists of $\alpha$-helix integrated into the phospholipid bilayer. Carbohydrate attached to proteins shown as (¶).
targets. For such questions to be answered, phosphorylation studies need to be carried out both on purified membranes and in whole cells which can subsequently be fractionated. Both types of studies need careful evaluation because of the many inherent problems.

\[ \text{pp60}^{src} \]

The RNA-containing retroviruses are natural agents of oncogenesis found widely distributed in nature. Those that induce transformation of fibroblasts in culture or sarcomas in animals insert a single gene (\(src\)), produced by the action of reverse transcriptase, into the host chromosomal DNA (Bishop, 1978). In chicken fibroblasts infected with Rous sarcoma virus (RSV), the expression of \(src\) leads to the synthesis of a single product, \(pp60^{src}\), which is a phosphorylated polypeptide, \(M_r 60,000\) (Collett & Erikson, 1978). This is related to the normal cellular protein \(pp60^{sar}\) which is found only in minute amounts (Collett et al., 1976, 1979a; Oppermann et al., 1979; Bishop, 1978).

\(pp60^{src}\) is localized to the cytosol surface of the cell plasma membrane (Willingham et al., 1979) and expresses an unusual kinase activity (Collett & Erikson, 1978; Levinson et al., 1978), in that it specifically transfers phosphate to tyrosine residues on substrate proteins (Hunter & Sefton, 1980). It is presumably through this kinase activity that RSV is able to have such dramatic effects on cell function, adhesion, and shape (Pastan & Willingham, 1978). Indeed, \(pp60^{src}\) can \textit{in vitro} phosphorylate tyrosine residues on a spectrum of proteins associated with the cell cytoskeleton as well as causing its own phosphorylation (Collett et al., 1980). A soluble cyclic AMP-dependent protein kinase can also act on \(pp60^{src}\), but this phosphorylates at a distinct site supplied by a serine residue (Collett et al., 1978, 1979b).

It is clearly of some importance to identify the natural targets for phosphorylation by \(pp60^{src}\), as alterations in the target proteins can lead to morphological revertants, even though \(pp60^{src}\) remains active (Collett et al., 1976). Recently a protein (\(M_r 34,000-36,000\)) has been identified which becomes rapidly phosphorylated after exposure of fibroblasts to RSV (Radke et al., 1980; Erikson & Erikson, 1980). The functional significance of this has yet to be elucidated. However, it appears to exist in two phosphorylated populations, one of which exhibits a phosphoserine and the other a phosphotyrosine residue (Radke et al., 1980). This suggests an interplay between the cyclic AMP-dependent and -independent phosphorylation systems. Furthermore, two plasma-membrane proteins (\(M_r 55,000\) and \(57,000\)) become dephosphorylated upon expression of the transformed phenotype (Witt & Gordon, 1980), suggesting that \(pp60^{src}\) can activate either a specific phosphatase or a specific phosphotransferase.

Spector et al. (1980) have just demonstrated that the \(\beta\)-subunit (\(M_r 53,000\)) of \(Na^+/K^+-ATPase\) in ascites tumour cells is phosphorylated on a single tyrosine residue by a membrane-bound kinase. This leads to a decrease in the efficiency of the pump, causing more molecules of ATP to be hydrolysed to maintain a particular rate of ion transport. As \(Na^+/K^+-ATPase\) provides a futile cycle, then this decrease in efficiency will lead to an increase in ATP utilization. The
net effect of this will be to increase both glycolysis and heat production, observations that are often made of transformed cells. As it is rather uncommon to find phosphotyrosine residues in proteins, then such modifications may well be related to processes that lead to cell transformation. Indeed a number of transformation proteins - the Harvey murine sarcoma virus p21sre protein (Shih et al., 1980; Willingham et al., 1980), polyoma middle T antigen (Ito et al., 1977), Abelson MuLV p120 protein (Witte et al., 1980), and the simian virus 40 (SV40) p90 protein (Tijan & Robbins, 1979; Griffin et al., 1979) - exhibit similar properties. They are all associated with the cell plasma membrane, are exposed at its cytosol surface, are autophosphorylated, and express kinase or phosphotransferase activity. Furthermore, a transformation-dependent protein kinase (Mr 18 000) is induced (Summerhayes & Chen, 1980) in epithelial cells exposed to the carcinogen 7,12-dimethylbenz[a]anthracene. This is also phosphorylated, although it is not known whether it is membrane-bound. Indeed, the levels of plasma-membrane cyclic AMP-independent protein kinase activity appears to be higher in proliferating cells than in resting ones, whereas the opposite is true of the cyclic AMP-dependent kinase activity (Branton, 1980). Thus investigations on the nature and targets of cyclic AMP-independent kinases, especially those modifying tyrosine residues, should prove rewarding in understanding cell growth, proliferation, and transformation.

EGF

Mouse EGF is a single polypeptide (Mr 6 000) containing three intrachain disulphide bridges that are essential for activity, whereas human EGF appears to consist of two non-identical polypeptide chains linked by a disulphide bridge (Carpenter & Cohen, 1979). These mitogenic peptides bind to a single class of receptor glycoproteins on the cell plasma membrane, whereupon they stimulate cell growth (Hollenberg & Cuatrecasas, 1975; Carpenter et al., 1975). EGF receptors are free to diffuse over the surface of the cell, but upon occupancy they rapidly form clusters and are subsequently internalized by receptor-mediated endocytosis (Schechter et al., 1978; Schlessinger et al., 1978). The formation of endocytotic vesicles is believed to require Ca2+ and to involve the activity of a transglutaminase enzyme (Davies et al., 1980) and the cell cytoskeleton (Salisbury et al., 1980). An analogue (CNBr-EGF) of EGF, made by cleavage with cyanogen bromide, although able to bind with reduced affinity to EGF receptors, is unable to cause their internalization and is devoid of biological activity. However, by cross-linking CNBr-EGF with anti-EGF antibodies, receptor aggregation can be induced and biological activity restored. Thus the cross-linking of EGF receptors may be connected with cell activation (Schechter et al., 1979).

EGF causes the rapid, cyclic-nucleotide-independent phosphorylation of a number of membrane proteins when added to purified plasma membranes from an epidermal carcinoma cell line (King et al., 1980). Both ATP and GTP can act as the phosphate donor in a system which attains maximal levels at 120 nM EGF, requiring Mn2+ or Mg2+ to optimize the activity. The major species phosphorylated
has molecular weights ($M_r$) of 170 000, 150 000, 80 000, and 22 500, with less reproducible effects on some other components ($M_r$ 60 000, 55 000, 30-40 000). The two most heavily phosphorylated species ($M_r$ 170 000 and 150 000) are glycoproteins and, like the EGF-stimulated kinase, are also integral proteins. The kinase can, however, act on soluble proteins such as histones and ribonuclease.

Very recently Ushiro and Cohen (1980) have shown that the EGF-stimulated kinase specifically phosphorylates protein tyrosine residues. Furthermore the major target for phosphorylation is believed to be the EGF receptor itself (King et al., 1980; Ushiro & Cohen, 1980). This bears analogy with pp$60^{src}$; however, in this instance the EGF receptor does not itself express a protein kinase activity (Carpenter et al., 1979; King et al., 1980). As the mobility of the EGF receptor and its ability to cluster in the presence of hormone are crucial to its biological activity, it is likely that the EGF receptor and the kinase are normally independent entities, free to migrate in the bilayer. Addition of EGF might then trigger their association and the activation of the kinase as suggested by the Mobile Receptor and Collision Coupling Models (Fig. 2) which are obeyed by glucagon or $\beta$-adrenergic receptors when activating adenylate cyclase (see e.g. Houslay et al., 1977, 1980; Schramm et al., 1977; Tolkovsky & Levitzki, 1978; Martin et al., 1979). In view of this it would be of interest to examine the effects of CNBr-EGF on protein phosphorylation.

**Fig. 2. Receptor-target protein interaction.** This model suggests that both the receptor (R) and the kinase catalytic unit (C) are integral membrane proteins able to undergo free lateral diffusion in the plane of the bilayer. When effector (e) occupies the receptor, this complex interacts with the kinase, activating it and forming a transmembrane complex (Mobile Receptor Model). The kinase may in some instances be released in an activated (C*) state from this complex (Collision Coupling Model).
EGF stimulates glycolysis and the transport of a variety of solutes into the cell (Carpenter & Cohen, 1979). These may well be effected by the phosphorylation of plasma-membrane proteins. Indeed, the phosphorylation of a component (Mr 55 000) of similar size to the β-subunit of the Na+/K+-ATPase has been noted. It is also possible that the internalization events which lead to the downregulation of EGF receptors (Catt et al., 1979) depend upon phosphorylation. This could trigger a Ca2+-flux essential for the activation of either transglutaminase (Davies et al., 1980) or myosin light-chain kinase (Nishikawa et al., 1980), or it may directly influence cytoskeleton-associated internalization events (Salisbury et al., 1980), perhaps by phosphorylating myosin. In this respect it is rather interesting that the phosphorylation of a small, membrane-associated protein (Mr 22 500) is stimulated by EGF.

As to why EGF triggers the endocytosis of its receptors, one can speculate that the trigger may be related to the phosphorylation of the receptor. This could explain why transformation by RNA sarcoma viruses, which leads to the expression of a plasma-membrane protein kinase phosphorylating tyrosine residues (pp60src), actually eliminates or greatly reduces the amount of EGF receptor in the membrane (Todaro et al., 1977). Indeed the downregulation of β-receptors is associated with the appearance of two specific phosphoproteins (Chuang & Costa, 1979), one of which is similar in size to a subunit of the β-receptor itself (Stagni et al., 1977; Strosberg et al., 1980). Furthermore, pp60src, which is autophosphorylated, has been identified in small amounts within the cell (Willingham et al., 1979). This might well reflect its association with endocytotic vesicles that are either destined for degradation in the lysosomes (Anderson et al., 1977) or involved in membrane recycling (Stanley et al., 1980).

The ability of EGF receptors, then, to interact with and stimulate a cyclic AMP-independent protein kinase which specifically phosphorylates tyrosine residues may well be exploited by a variety of agents. Examples of these are tumour promoters, such as phorbol esters and diterpenes, and also growth-stimulating peptides released from various sarcoma cell lines, all of which bind to EGF receptors (Todaro et al., 1980). This evidence again suggests that kinases connected with the phosphorylation of tyrosine residues on target proteins are connected with processes modulating cell growth and proliferation.

Insulin

This polypeptide hormone plays a key role in regulating blood-glucose levels and metabolic events within the cell. In this respect its central targets for action are the liver, fat, and muscle. However, most cells possess insulin receptors which may reflect the importance of this hormone in maintaining the growth of cells in culture, in stimulating tissue regeneration, and in promoting normal growth in mammals.

Insulin binds with affinity to receptor glycoproteins exposed at the external surface of the plasma membrane (Cuatrecasas, 1974). Solubilized receptors have an overall molecular weight of about 300 000 (Cuatrecasas, 1972; Pilch & Czech, 1980) and consist of
non-identical subunits held together by disulphide bridges. This complex appears to consist of two identical glycosylated subunits ($M_r$ 125-135,000) together with another glycosylated component ($M_r$ 90,000), which itself may consist of two identical subunits (Harmon et al., 1980; Heinrich et al., 1980; Jacobs et al., 1980; Pilch & Czech, 1979, 1980; Yeung et al., 1980; Yip et al., 1980). However, there are indications that two other components ($M_r$ 56,000 and 34,000) may also be associated with, or form part of, the receptor (Lang et al., 1980).

As with EGF, insulin induces its mobile receptors (Schechter et al., 1978) to cluster and be internalized (Schlessinger et al., 1978), although the rates of internalization vary with cell type (see Catt et al., 1979). This clustering of insulin receptors may well be of importance in mediating the action of insulin, as it has been shown that bivalent anti-insulin-receptor antibodies can mimic both the short-term antilipolytic action of insulin on adipocytes (Kahn et al., 1978) and its long-term effects on adipocyte lipoprotein lipase (Van Obberghen et al., 1979). In contrast, univalent fragments of these antibodies, whilst still binding to the receptor, were ineffective unless cross-linked together to form a bivalent reagent capable of aggregating the receptors.

The action of insulin on the phosphorylation of adipocyte plasma-membrane proteins has been studied by using either isolated membranes or whole cells that were subsequently fractionated. These studies have undoubtedly been confused owing to difficulties in obtaining pure plasma-membrane preparations and, in whole-cell studies, by the time taken to resolve the component membranes. However, a recently devised method (Belsham et al., 1980) for preparing subcellular fractions looks very promising provided that lower EGTA concentrations are used in order to avoid stripping off peripheral proteins.

Avruch et al. (1976) demonstrated that in intact adipocytes, insulin decreased the cyclic AMP-mediated phosphorylation of one peptide ($M_r$ 26,000). However, in the presence of adrenaline and presumably elevated intracellular cyclic AMP levels, it triggered the phosphorylation of two other plasma-membrane peptides ($M_r$ 62,000 and 79,000). More recently Belsham et al. (1980) have added either insulin or adrenaline alone to fat cells and observed increases in the phosphorylation of a single component ($M_r$ 61,000). This has also been noted by Benjamin and Clayton (1978), who also observed that adrenaline decreased the phosphorylation of this band, whereas Belsham et al., (1980) found that adrenaline increased its phosphorylation. However, Benjamin and Clayton (1978) had not isolated a pure plasma-membrane fraction, so it is possible that these workers are looking at different bands. The addition of insulin alone to isolated adipocyte plasma membranes (Seals et al., 1979a) has also been shown to cause a small cyclic AMP-independent decrease in the phosphorylation of a $M_r$-120,000 component.

Insulin also affects the phosphorylation of other proteins; for example it inhibits the adrenaline-stimulated phosphorylation of a peptide ($M_r$ 69,000) in the endoplasmic reticulum. However, insulin or adrenaline, either together or alone, both stimulate the phosphorylation (Benjamin & Clayton, 1978; Hughes et al., 1980; Avruch et al.,
of a soluble peptide ($M_r$ 123-130 000) which has been shown to be ATP-citrate lyase (Alexander et al., 1979). In other studies (Benjamin & Singer 1974, 1975), insulin apparently enhanced the phosphorylation of two proteins ($M_r$ 140 000 and 50 000), whilst it inhibited the cyclic AMP-dependent phosphorylation of another ($M_r$ 60 000). However, the localization of these peptides was unfortunately not ascertained. Insulin also stimulates the phosphorylation (Hughes et al., 1980) of another protein which may well be the ribosomal S6 protein (Smith et al., 1979). Indeed, the phosphorylation of ribosomal S6 protein is also enhanced by glucagon (Gressner & Wool, 1976).

One of the most interesting effects of insulin on the phosphorylation of adipocyte proteins is that it causes the dephosphorylation of the $\alpha$-subunit ($M_r$ 42 000) of mitochondrial pyruvate dehydrogenase (PDH) (Seals et al., 1979a,b; Hughes et al., 1980). This effect can be seen in intact cells and in a mixture of mitochondria and plasma membranes, but not in a purified mitochondrial fraction (Seals et al., 1979b). It has been suggested that this dephosphorylation, which leads to the activation of PDH, is achieved by insulin through the release of a 'peptide-like' second messenger from the plasma membrane (Jarett & Seals, 1979; Larner et al., 1979; Seals & Jarett, 1980). This is apparently produced by stimulation of an arginine-specific intrinsic plasma-membrane protease, and may be derived from the insulin receptor itself (Seals & Czech, 1980). The aggregation of insulin receptors could well be of importance in this event as the action of insulin in activating PDH in this fashion is mimicked by anti-insulin-receptor antibodies and concanavalin A acting on adipocyte plasma membranes (Seals & Jarett, 1980). These effects are extremely interesting, although it must be kept in mind that the mitochondria used in these studies were undoubtedly uncoupled. Furthermore, the stimulation of PDH was relatively small (25%), especially as ATP was present, which itself resulted in up to a 75% inhibition of the basal activity (Seals & Jarett, 1980). This factor does, however, appear to activate a phosphoprotein phosphatase and inhibit a cyclic AMP-dependent protein kinase (Larner et al., 1979), implying that it could well reflect the means by which insulin controls phosphorylation-dephosphorylation reactions within the cell. Indeed insulin can in vivo decrease the phosphorylation of protein phosphatase-1 inhibitor-1 (Foulkes et al., 1980), presumably either by activating a protein phosphatase or by inhibiting a protein kinase. The net effect of this is that the dephosphorylation of inhibitor 1 leads to the activation of protein phosphatase 1, which can then act on both soluble and membrane-bound phosphoproteins. Such events could well complicate comparisons between studies using purified membranes and studies using whole cells where the membranes are isolated after insulin challenge.

Purified rat-liver plasma membranes have been used to investigate the effects of insulin on protein phosphorylation (Marchmont & Houslay, 1980c). Here, insulin blocked the phosphorylation of two integral membrane proteins, I 1 ($M_r$ 140 000) and I 2 ($M_r$ 80 000), by inhibiting a cyclic AMP-dependent protein kinase. At the same time, it triggered the cyclic AMP-dependent phosphorylation of three
peripheral membrane proteins, P1 (Mr 52 000), P2 (Mr 28 000), and P3 (Mr 14 000). The $K_a$ for insulin in promoting both of these processes was about $2 \times 10^{-10}$ M, which reflects the high affinity component for insulin binding specifically to its receptor (Cuatrecasas, 1974). However, the $K_a$ values for the cyclic AMP dependence of these two processes were significantly different, being $1.6 \times 10^{-6}$ M for its effect on the peripheral proteins and $2.5 \times 10^{-6}$ M for its effect on the integral proteins. This implies that different cyclic AMP-dependent protein kinases are involved. The magnitude of the $K_a$ values for the cyclic AMP-dependence of these events indicates that resting levels of cyclic AMP (0.3-0.5 μM) in the hepatocyte (Exton et al., 1973; Smith et al., 1978) would be too low either to allow the integral proteins to be phosphorylated or for insulin to trigger the phosphorylation of the peripheral proteins. However, if the intracellular cyclic AMP concentration were to rise, as it does to 2-4 μM after exposure to glucagon or adrenaline (Jefferson et al., 1968; Blackmore et al., 1979), then this would be sufficient to allow these reactions to occur. Presumably the effects of insulin on these phosphorylation processes reflect in part the ability of insulin to antagonize the action of glucagon. These results bear comparison with those of Avruch and coworkers (1976), who noted that in adipocytes insulin could either antagonize the effects of noradrenaline-stimulated phosphorylation or promote, in the presence of noradrenaline, the phosphorylation of certain plasma-membrane proteins. Disparate effects of insulin on protein phosphorylation are likely to be encountered, dependent upon the levels of cyclic AMP within the cell preparations and whether or not peripheral proteins have been removed during the preparation of the membranes.

The P₁ component has been identified as a peripheral plasma-membrane cyclic AMP phosphodiesterase, whose activity at physiological concentrations of cyclic AMP is doubled upon its phosphorylation (Marchmont & Houslay, 1980a,b). Upon activation this enzyme incorporates 1 mol of alkali-labile phosphate into each mole (Mr 52 000) of protein. This phosphate can be removed using either a purified alkaline phosphatase or the protein phosphatase activity in rat-liver cytosol, whereupon the activity of the enzyme reverts to its original level. The most significant kinetic parameter altered by phosphorylation (Fig. 3) is the Hill coefficient, which is reduced from 0.6 to 0.45. The net effect of this is to provide a greater activation at physiological (low) substrate concentrations, and indeed theoretical studies have shown that intracellular cyclic AMP levels are extremely sensitive to small changes in the Hill coefficients of cyclic AMP phosphodiesterase (Erneux et al., 1980). The activation of this enzyme, which can only occur in the hepatocyte after intracellular cyclic AMP levels have been elevated above basal, may, at least in part, explain why insulin does not affect basal cyclic AMP levels, yet can antagonize the effect of glucagon on raising cyclic AMP levels.

The release of the peripheral cyclic AMP phosphodiesterase from the membrane is usually achieved by increasing the ionic strength (Marchmont & Houslay, 1979) of the medium, but release is also extremely sensitive to the presence of EGTA (Fig. 4). Thus methods of preparing plasma membranes which involve chelating agents could well remove peripheral proteins.
Fig. 3. Cyclic AMP phosphodiesterase activity in intact rat-liver plasma membranes. Lineweaver-Burk plots of the cyclic AMP phosphodiesterase activity (pmol min⁻¹ mg⁻¹) in native rat-liver plasma membranes and those pretreated with insulin (10 nM) together with ATP (3 μM) and cyclic AMP (0.1 mM). This plot represents the sum of the activities of the integral enzyme (high $K_m$) and the insulin-activated peripheral enzyme (low $K_m$).

Fig. 4. Release of the peripheral cyclic AMP phosphodiesterase from rat-liver plasma membranes. Effect of increasing ionic strength on the release of enzyme (see Marchmont & Houslay, 1979) (●) and the potentiation of release by 2 mM EGTA (▲) and inhibition of release by 2 mM Ca²⁺ (○).
Insulin might be expected to effect the phosphorylation of these membrane proteins in a number of ways: i) the insulin receptor might have an associated kinase activity; ii) the receptor could interact with a membrane-bound kinase through lateral collisions (Fig. 2); iii) the receptor could alter the conformation of the target proteins and hence their susceptibility to phosphorylation; iv) the receptor could cause the production of a 'peptide-like' factor (Jarett & Seals, 1979; Larner et al., 1979). It is rather interesting then that the peripheral cyclic AMP phosphodiesterase can be shown to bind with high affinity ($K_D$ of 24 nM) to a single class of protein sites on the plasma membrane at a density of about 2 pmol/mg membrane protein. This is a rather similar number to the amount of insulin receptors present in these membranes (Cuatrecasas, 1974), and so it is tempting to suggest that the peripheral proteins (P1,2,3) bind either to the insulin receptor or to a kinase that can interact with it.

Despite their potential importance little is known about membrane-bound protein kinases. In the erythrocyte (Rubin, 1979), the membrane-bound type-I cyclic AMP-dependent protein kinase is an asymmetric tetramer ($R_C_4$) attached to the membrane through its regulatory (R) subunit. This enables its catalytic subunits (C) to exchange with those in the cytosol. However, it is highly likely that a variety of other situations can occur (see Fig. 5). Indeed both the R and C subunits of the cyclic AMP-dependent kinase that phosphorylates P1 in the liver appear to be integral proteins, as they do in myoblast plasma membranes (Scott & Dousa, 1978). It is important to appreciate the structure of such kinases in order to

Fig. 5. Organization of cyclic AMP-dependent protein kinase in the membrane. Cyclic AMP-dependent protein kinases consist of regulatory (R) subunits ($\Delta$) and catalytic (C) subunits (O). These, in different tissues, may both be integral proteins (a); all be peripheral proteins (b); be R integral and C peripheral (c); be C integral and R peripheral (d). The top of the bilayer schematic faces the cell exterior, and the bottom, the cell cytosol.
assess likely targets, to see whether exchange between soluble components is possible, and for kinetic reasons. For Swillens et al. (1974) have pointed out that the $K_a$ for activation or phosphorylation by cyclic AMP-dependent protein kinases is related to the $K_{eq}$ for the dissociation of the $R_nC_n$ complex (inactive) into its component monomeric units (active). Thus $K_a$ is dependent upon the kinase concentration, and the form of this dependence is affected by the number of components ($n$) in the complex.

Insulin, then, may well be able to affect a variety of cellular processes by virtue of its ability to control both cyclic AMP-dependent and -independent phosphorylation and dephosphorylation reactions. This could explain why, if cellular ATP levels fall below their normal levels, insulin fails to stimulate glucose transport (Siegel & Olefsky, 1980) and fails to activate a particulate cyclic AMP phosphodiesterase (Zinman & Hollenberg, 1974; Kono et al., 1977) in intact adipocytes. In fact there are indications that insulin may cause the phosphorylation of the glucose carrier (Halperin et al., 1978). Furthermore it has been claimed that both Na+/K+-ATPase (Luly et al., 1972) and an amino-acid-transporting protein (Nilssen-Hamilton & Hamilton, 1979) can be inhibited by cyclic AMP-dependent phosphorylation. Thus insulin may exert an activating effect (Czech, 1977) on such systems either by blocking their phosphorylation or by stimulating their dephosphorylation. In this respect the dependence of certain of the actions of insulin on elevated intracellular cyclic AMP levels might also explain why both insulin and glucagon are necessary for liver regeneration (Bucher & Swaffield, 1976). The identity of the target proteins, other than Pl, affected in the liver plasma membrane has yet to be resolved. They may well be proteins that reflect the effect of insulin on Na+/K+ exchange (Moore et al., 1979) or transport (Luly et al., 1972), glucose transport, Ca²⁺-movement, and amino-acid transport (see Czech, 1977), or they may be components of the cell cytoskeleton. As yet there is no evidence to suggest that a component of the insulin receptor itself is phosphorylated. However, if, as suggested, phosphorylation can act as a signal for the receptor to enter the endocytotic pathway, then the phosphorylation of a tightly bound peripheral protein may well suffice.

Clearly insulin, EGF, and pp60⁶⁶⁶ have the potential for modulating a variety of cellular processes by virtue of their ability to control phosphorylation and dephosphorylation reactions.

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References
