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REVIEW ARTICLE

Role of the phosphorylation of red blood cell membrane proteins

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INTRODUCTION

The red blood cell membrane is composed of a lipid bilayer and two types of proteins: the integral proteins, which extend all the way across the lipid bilayer, and the proteins which do not penetrate the lipid bilayer and for this reason are called external proteins. The most important external proteins make up the membrane skeleton on which depend the membrane stability and the shape and deformability of the red cell.

The red cell membrane skeleton is a submembranous network composed of three main proteins: spectrin, actin and protein 4.1 and several minor components: protein 4.9, which is an actin-capping protein, adducin, which is a calmodulin-binding protein, and at least one of the components of protein band 7. Under electron microscopy this skeleton unit appears as a primary hexagonal lattice of spectrin tetramer fibres (Liu *et al.*, 1987) with junctional 'nodes' of actin–protein 4.9–protein 4.1 and adducin.

The membrane skeleton is linked to the other membrane components, proteins and lipids, at several interaction sites. The β chain of spectrin is bound to protein 2.1 (ankyrin) which itself is linked to the cytosolic fragment of protein band 3, the anion channel. Protein 4.1 is linked to protein 3 and to glycophorins through a process necessitating the presence of phosphatidylinositol 4- or 4,5-phosphate (Anderson & Lovrien, 1984; Anderson & Marchesi, 1985; Ferrell & Huestis, 1984). Furthermore spectrin and protein 4.1 interact with the inner leaflet of the lipid bilayer, probably through phosphatidylserine (Cohen *et al.*, 1986; Rybicki *et al.*, 1988).

Membrane equilibrium depends, on the one hand, on the quantitative and qualitative structural integrity of the skeleton proteins and, on the other hand, on normal molecular interactions between the skeleton components themselves and the other parts of the membrane. It also indirectly depends on the links between the internal portion of integral proteins and cytosolic components such as haemoglobin and enzymes.

Protein phosphorylation–dephosphorylation is one of the physiological processes possibly controlling the membrane stability. In fact, recent data indicate that phosphorylation is involved in molecular interactions at several levels and thereby contributes to the integrity of the general membrane structure.

I present here a survey of the current knowledge about the potential role of phosphorous exchanges in red cell membrane stability. This review will be limited to the mature circulating mammalian red cell and concerns itself mainly with the possible action of protein phosphorylation upon molecular interactions involved in red cell shape and deformability. Indeed the evaluation of the phosphorylation processes during the phases of erythroblast division and maturation and their role in the molecular interactions involved in membrane assembly are still unknown.

The human red cell contains a number of well-characterized membranous and cytosolic protein kinases and several phosphoprotein phosphatases: two cyclic AMP-dependent protein kinases, one of type I located in the membrane (Fairbanks & Avruch, 1974; Boivin & Galand, 1978), the other, type II, in the cytosol; two cyclic-nucleotide-independent casein kinases: the membranous one is a serine-protein kinase (casein kinase I) named spectrin kinase and the other, present in the cytosol, of type II, phosphorylates serine and threonine (Boivin & Galand, 1980; Boivin *et al.*, 1980; Avruch & Fairbanks, 1974); a protein kinase C, translocated from the cytosol to the membrane under the influence of diacylglycerol or phorbol esters (Johnson *et al.*, 1982; Ling & Sapirstein, 1984; Cohen & Foley, 1986*a*; Faquin *et al.*, 1986); a calcium/calmodulin-dependent protein kinase; a membrane-bound tyrosine kinase and at least two other protein kinases which copurify with protein 4.1 and protein 4.9 respectively (Cohen & Foley, 1986*a*,*b*).

Several neutral phosphoprotein phosphatases active towards phosphoserine and phosphothreonine and at least one acidic phosphotyrosine phosphatase from the cytosol have been characterized (Graham *et al.*, 1974; Clari & Moret, 1981, 1982; Usui *et al.*, 1983; Boivin & Galand, 1986; Boivin *et al.*, 1987; Kiener *et al.*, 1987). A very low phosphoprotein phosphatase activity is present in ghosts, perhaps translocated from the cytosol with a loss of its activity because of binding, as observed for many cytosolic enzymes once they are membrane-bound. Furthermore, a membrane-bound phosphotyrosyl phosphatase was recently extracted from human erythrocytes (Clari *et al.*, 1987).

PHOSPHORYLATION OF THE MEMBRANE SKELETON

Phosphorylation of spectrin

Cyclic AMP-independent phosphorylation of the β chain of spectrin. When whole erythrocytes are incubated with glucose (which allows the regeneration of ATP), and ^{32}P-labelled phosphate (*in vivo* conditions) only the β chain of spectrin is phosphorylated; the same occurs when ghosts are incubated with [γ-^{32}P]ATP; cyclic AMP does not modify the spectrin phosphorylation.

Harris & Lux (1980) have shown by tryptic and chymotryptic digestion that the β chain has seven phosphates, four of which are exchangeable: the sites, com-

Abbreviation used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

posed of three serine and one threonine residues, are located near the *C*-terminal and close to the head of the β chain.

Purified casein kinases from membrane and cytosol are both able to phosphorylate *in vitro* the β chain of purified spectrin with [γ-^{32}P]ATP as a phosphoryl donor (Hosey & Tao, 1976; Hosey & Tao, 1977*a*,*b*; Plut *et al.*, 1978; Boivin & Galand, 1980; Boivin *et al.*, 1980; Simkowski & Tao, 1980). The membrane kinase has a higher affinity for spectrin than does the cytosol kinase (Boivin *et al.*, 1980). It does not use [^{32}P]GTP as phosphoryl donor, as opposed to the cytosol enzyme (Boivin *et al.*, 1980). In our laboratory, we phosphorylated purified spectrin dimers with either membrane or cytosol casein kinase. Then we submitted spectrin to tryptic digestion, separated the resulting peptides by h.p.l.c. and compared the phosphorylated peptide diagrams. Some peptides were similar whatever the kinase; others were different and specific for each kinase. Identification of the phosphorylated amino acid(s) in each phosphorylated peptide was performed showing that, as expected, only phosphoserine was present after membrane kinase action, and phosphoserine + phosphothreonine after the use of cytosol kinase. It was also observed that the phosphothreonine to phosphoserine ratio was increased when [γ-^{32}P]GTP was used instead of [γ-^{32}P]ATP. Further experiments were carried out with phosphorylation (under *in vivo* conditions): spectrin was purified and analysed after its phosphorylation in intact red cells. Phosphorylated peptides specific for both casein kinases were detected and the presence of phosphothreonine was observed. These results demonstrated that physiologically the spectrin β chain is not only phosphorylated by the membrane casein kinase (which was thus erroneously called spectrin kinase) but also by the cytosol kinase which phosphorylated the threonine residues, maybe from GTP rather than ATP. This dual phosphorylation should be considered to suggest a hypothesis on the role of spectrin β chain phosphorylation, especially in the light of the possible casein kinase II activation in response to membrane signal transduction, as recently demonstrated for insulin and epidermal growth factor in cultured cells (Sommercorn *et al.*, 1987).

Anyway, the stoichiometry of spectrin β chain phosphorylation is between 0.1 and 0.3 mol of phosphate/mol of spectrin, and this is not modified by dephosphorylating spectrin before phosphorylation.

Possible roles for the cyclic AMP-independent phosphorylation of spectrin. Greenquist & Shohet (1975) have shown that under *in vivo* conditions spectrin phosphorylation depends on the ATP level; during incubation under conditions of erythrocyte metabolism arrest, i.e. without glucose, the decrease in phosphorylation is parallel to the decrease in red blood cell ATP.

These data gave Singer *et al.* (Birchmeier & Singer, 1977; Sheetz & Singer, 1977) a good argument in favour of their hypothesis that phosphorylation plays a role in the shape and the deformability of red blood cells: a decrease in ATP is simultaneous with the appearance of echinocytosis, and the reversibility of both is correlated with the reversibility of the decrease in spectrin phosphorylation. This hypothesis was contradicted by Anderson & Tyler (1980) who showed that shape abnormalities induced by ATP depletion preceded the decrease in spectrin phosphorylation; thus the question is still up for debate, the more so since Pinder *et al.* (1977) invoked the role of phosphorylation in the formation of the spectrin–actin–protein 4.1 complex. They used the following argument: the incubation of normal ghosts with ATP and purified membrane casein kinase results in a gelification which does not occur in the absence of either ATP or protein kinase. However this important observation lacks a satisfactory explanation, as Brenner & Korn (1979) think that phosphorylation is not involved in spectrin–actin interactions.

It is now considered that the phosphorylation of the β chain has no effect upon the interactions between spectrin dimers, i.e. the tetramerization process. It does not seem to play a role in the spectrin–protein 4.1 interactions, although according to Eder *et al.* (1986), phosphorylated spectrin might have a slightly higher affinity for protein 4.1 than unphosphorylated spectrin. It is not involved in spectrin–ankyrin interactions whether in ghosts or with the isolated proteins (Lu *et al.*, 1985).

In summary, the role of the phosphorylation of the spectrin β chain is as yet unknown.

Cyclic AMP-dependent phosphorylation of spectrin. Several authors (Greenquist & Shohet, 1975; Lecomte *et al.*, 1982; Lutz, 1984) observed that the cyclic AMP-dependent protein kinase was able to phosphorylate both spectrin chains. We also observed a cyclic AMP-dependent α chain phosphorylation under particular conditions: this occurred in water-soluble proteins but neither in intact red blood cells nor in isolated ghosts (Boivin *et al.*, 1981). These observations *in vitro* are in contradiction with those made *in vivo* where, as stated above, only the β chain is phosphorylated. This contradiction lead Maretski & Lutz (1986) to put forward the hypothesis that *in vivo* there is an inhibition of the α chain phosphorylation. The same authors observed that calmodulin inhibited dimer autophosphorylation on the β chain in the absence of cyclic AMP and on the α chain when the cyclic nucleotide is present. This inhibition was calcium-dependent and under optimal conditions reached 65% for the β chain, with a high calmodulin excess and a molar calmodulin: spectrin ratio of 32:1. However, the inhibition reached 30% for the α chain with a calmodulin: spectrin ratio of only 2:1. One must note that the inhibition is only partial and that α chains are obviously much less phosphorylated than the β chains, as seen on autoradiographs.

More recently Lutz *et al.* (1986) succeeded in demonstrating a cyclic AMP-dependent α chain phosphorylation in whole red cells if and when red blood cells are overloaded with cyclic AMP. The important fact is that this phosphorylation alters the properties of spectrin which thereby becomes inextractable and adheres to inside-out vesicles.

These results must be considered together with current knowledge regarding recently defined direct interactions between spectrin and calmodulin (Agre *et al.*, 1983; Anderson & Morrow, 1987). The use of a photoactivated calmodulin derivative permitted a demonstration of an interaction of calmodulin with the spectrin β chain, ankyrin, Ca^{2+}/Mg^{2+}-ATPase, a 105–120 kDa doublet and a 57 kDa protein (Anderson & Morrow, 1987).

Calmodulin inhibits the binding of actin to spectrin even if protein 4.1 is present, whatever the Ca^{2+} concentration. It is postulated that calmodulin regulates the actin-to-spectrin binding antagonistically to the stimu-

lation caused by protein 4.1. However, the affinity of calmodulin for spectrin (4.6×10^4 M^{-1} for the tetramer; Husain *et al.*, 1984) seems to be much lower than that of protein 4.1.

There is an apparent correlation between the above observations tending to show that calmodulin may cause some destabilization of the skeleton through its inhibition of actin to spectrin binding and those of Lutz suggesting that the calmodulin inhibition of cyclic AMP-dependent protein kinase would decrease the level of inextractable spectrin. More work is necessary to know whether cyclic AMP-dependent phosphorylation of the α chain does not exist and what its role is.

Is there a phosphorylation of spectrin on tyrosine? Red cell spectrin belongs to a superfamily of proteins sharing partial structural and immunological homology. The most important of these proteins is brain fodrin, which can be phosphorylated *in vitro* by a spleen tyrosine kinase. Both α and β chains are phosphorylated with a higher phosphorylation rate for α than for β chains; three phosphorylation sites seem to be present in the α chain and only one in the β chain (Wang *et al.*, 1988).

We have extracted and partially purified a tyrosine kinase from the red cell membrane: it does not phosphorylate spectrin (Boivin *et al.*, 1986); however, we cannot exclude that other tyrosine kinase activities present in the red cell membrane or cytosol could phosphorylate spectrin.

Dephosphorylation of spectrin. Phosphorylated spectrin can be 'dephosphorylated' by either non-specific alkaline phosphatases or by red cell cytosol protein phosphatases (Graham *et al.*, 1976; Usui *et al.*, 1983; Clari & Moret, 1982). Up to now, no spectrin phosphatase has been identified in the red cell membrane.

Phosphorylation of protein 4.1

Protein 4.1 is a phosphoprotein with a molecular mass of about 80 kDa. It is one of the major elements of the membrane skeleton. It has a higher-affinity binding to the β chain of spectrin and its presence stimulates the binding of actin to spectrin, allowing the formation of a spectrin–actin–protein 4.1 ternary complex which is the basis of the membrane skeleton. Moreover, protein 4.1 is bound to protein 3 (Pasternack *et al.*, 1985) and glycophorin according to a process necessitating the presence of phosphatidylinositol 4- or 4,5-phosphate (Anderson & Marchesi, 1985). Its role in the stabilization of the membrane skeleton is shown by pathological observations: the congenital lack of protein 4.1 is responsible for constitutional elliptocytosis and a severe hyperhaemolysis when this lack is complete.

Under experimental conditions using whole red blood cells incubated with [^{32}P]P_i, protein 4.1 seems to be a substrate for three or possibly four different protein kinases: a cyclic AMP-dependent protein kinase, a protein kinase C, a Ca^{2+}/calmodulin-dependent protein kinase, and a Ca^{2+}/cyclic AMP-independent kinase associated with protein 4.1 (Cohen & Foley, 1986*a*,*b*). Furthermore, purified protein 4.1 is a substrate *in vitro* for cyclic nucleotide-independent membranous and cytosolic casein kinases extracted from red blood cells (Eder *et al.*, 1986).

When red blood cells are incubated under *in vivo* conditions, in the presence of [^{32}P]P_i and 2 mM-dibutyryl cyclic AMP, the main phosphorylated proteins are ankyrin and proteins 4.1 and 4.9. After limited chymotryptic digestion protein 4.1 is cleaved into four main domains with molecular masses of 30, 16, 10 and 24 kDa respectively from the *N*- to the *C*-terminal end (Leto & Marchesi, 1984). Horne *et al.* (1985) have shown that the cyclic AMP-dependent phosphorylation site was located on the 10 kDa peptidic domain. The same domain carries the interaction site with spectrin, which suggests that protein 4.1 phosphorylation could modulate spectrin–protein 4.1 interactions.

The phospholipid- and calcium-dependent protein kinases C are present in most cells and play a role in the activity of membrane receptors. Their activity is stimulated by diacylglycerol which considerably decreases the need for Ca^{2+}. In red blood cells, as well as in other cells, diacylglycerol is produced by the cleavage of phosphatidylinositol 4- and mainly 4,5-phosphate by phospholipase C, which gives inositol trisphosphate and diacylglycerol. The erythrocyte membrane contains the enzymic machinery necessary for the metabolism of phosphatidylinositol phosphates, and phospholipase C.

The effect of diacylglycerol upon protein kinase C is mimicked by tumorigenic phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), when bound to a membrane receptor which probably is the protein kinase C itself. The phosphorylation of certain erythrocytic membrane proteins by a protein kinase C-like activity was initially demonstrated by Ling & Sapirstein (1984). These authors incubated rabbit red blood cells with [^{32}P]P_i in order to produce [^{32}P]ATP, and then with a TPA solution for a few minutes. SDS/polyacrylamide-gel electrophoresis and autoradiographs of phosphorylated proteins showed that in the presence of TPA there is a significant increase in protein 4.1 phosphorylation. When applied to human red cells this method allowed the recognition of the TPA-dependent phosphorylation of proteins 4.1 and 4.9 and a 100–110 kDa protein doublet which is part of the cytoskeleton and binds calmodulin (Johnson *et al.*, 1982; Ling & Sapirstein, 1984; Cohen & Foley, 1986*a*; Faquin *et al.*, 1986).

On protein 4.1 the phosphorylation site for protein kinase C is different from the site for the cyclic AMP-dependent protein kinase; it is located on the 16 kDa peptide domain obtained from a chymotryptic digestion. Phosphorylated amino acids are mainly serine and to a lesser extent threonine (Johnson *et al.*, 1982). In red blood cells, as well as in other cells, TPA provokes the translocation of the cytosolic protein kinase C to the membrane which contains the protein substrates.

Applying the experimental conditions of Cohen & Foley (1986*a*,*b*), the stoichiometry of the protein kinase C phosphorylation reaction would be 0.3 mol of P_i incorporated/mol of protein 4.1.

The phosphorylation of protein 4.1 by a Ca^{2+}/calmodulin-dependent protein kinase was demonstrated in ghosts and inside-out vesicles. After incubation with 10 μM-Ca^{2+} and [γ-^{32}P]ATP, there is high level of protein 4.1 phosphorylation in both of them. Calmodulin is involved in this phosphorylation since it is inhibited by trifluoperazine, an inhibitor of calmodulin-dependent reactions. The stoichiometry is 0.2 mol of P_i incorporated/mol of protein 4.1.

The last protein 4.1 phosphorylation reaction is due to a protein kinase which is copurified with protein 4.1 itself (Cohen & Foley, 1986*b*). When incubated with

[γ-^{32}P]ATP, purified 4.1 is readily phosphorylated. The enzyme which is purified with protein 4.1 can also phosphorylate histone and spectrin; it is cyclic AMP- and Ca^{2+}-independent. Its precise nature is still unknown.

In whole red blood cells protein 4.1 is only weakly phosphorylated in the absence of cyclic AMP, TPA or Ca^{2+} stimulation; however, once purified, it can be phosphorylated by membrane or cytosol cyclic AMP-independent casein kinases (Eder *et al.*, 1986). Both enzymes, with an identical mode of action on protein 4.1 and probably the same site, incorporate 2 mol of P_i/mol of protein 4.1. The phosphorylated amino acids are threonine and serine in a 2:1 ratio. The domain(s) carrying the phosphorylation site(s) have not been determined; a comparison with cyclic AMP-, TPA- and Ca^{2+}/calmodulin-dependent physiological phosphorylations is therefore difficult.

Role of protein 4.1 phosphorylation. In the light of recent work, protein 4.1 phosphorylation appears to be a way to regulate the spectrin–actin–protein 4.1 complex formation.

Cohen & Foley (1986*a*,*b*) dephosphorylated protein 4.1 with a nonspecific alkaline phosphatase. They observed an increase in the binding of spectrin to actin in the presence of dephosphorylated protein 4.1 as compared to what is obtained when protein 4.1 is not treated with a phosphatase. The decrease in the affinity for spectrin when protein 4.1 is phosphorylated was also observed by Eder *et al.* (1986); protein 4.1 phosphorylated by membrane or cytosolic casein kinases has an affinity for spectrin five times lower than the unphosphorylated protein (the K_d goes from 2×10^{-6} M to 9.4×10^{-6} M). Recently Ling *et al.* (1987) confirmed that phosphorylation of protein 4.1 by either cyclic AMP-dependent or Ca^{2+}/phospholipid-dependent protein kinase reduces the binding to spectrin by 50–90% and also reduces by 60–80% the ability of protein 4.1 to promote spectrin binding to actin.

Therefore it seems that the protein 4.1 phosphorylation–dephosphorylation process is a modulating mechanism for the formation of the spectrin–4.1–actin ternary complex. It remains to be determined which enzymes and sites are involved in the regulating mechanism *in vivo*.

Whatever they may be, protein 4.1 phosphorylation would result in some loosening of the skeleton lattice, by decreasing ternary complex formation; conversely a dephosphorylation by as yet unknown erythrocytic phosphatase(s) would induce a stiffening of the skeleton. It is highly probable that these phenomena would play a role in erythrocyte deformability. Protein 4.1 phosphorylation might also affect the binding to glycophorin and protein 3, thereby acting again on the structure and physical properties of the membrane skeleton.

Phosphorylation of actin

Actin can be phosphorylated by cyclic AMP-dependent protein kinases in various cellular types, particularly in muscle cells (Pratje & Heilmeyer, 1972; Grazi & Magri, 1979; Hofstein *et al.*, 1980; Steinberg, 1980; Walsh *et al.*, 1981). In the red blood cell membrane, after electrophoresis of phosphorylated proteins it is difficult to distinguish clearly between protein 4.9 and actin phosphorylation. Actin is present in the proteins solubilized at low ionic strength along with the other elements of the ternary complex and the cyclic AMP-dependent protein kinase. If [γ-^{32}P]ATP and cyclic AMP are added to the skeletal protein solution, actin is phosphorylated. Conversely, purified erythrocytic actin G is not phosphorylated when incubated with [γ-^{32}P]ATP, cyclic AMP and the cyclic AMP-dependent protein kinase (Boivin *et al.*, 1981). It would thus appear that the presence of other elements of the skeleton is necessary for actin phosphorylation; they might be needed to induce a conformation presenting the proper phosphorylation sites.

The problem is not entirely solved and the role of actin phosphorylation is still unknown.

Phosphorylation of ankyrin

In whole red cells or ghosts the cyclic AMP-dependent protein kinase phosphorylates ankyrin, one of its major substrates. Protein kinase C also phosphorylates ankyrin to a small extent. The affinity of spectrin-depleted inside-out vesicles for spectrin is not modified when ankyrin is phosphorylated by cyclic AMP-dependent protein kinase (Lecomte *et al.*, 1982*b*).

Purified ankyrin can be phosphorylated by cyclic AMP-independent membrane or cytosolic casein kinases (Lu *et al.*, 1985). Both these kinases act in identical ways and the stoichiometry is 7 mol of phosphate/mol of ankyrin with each one.

Tetrameric spectrin binds to twice as much ankyrin as does the dimer and the affinity of the tetramer for ankyrin is greater than that of the dimer with a K_d of 1×10^{-7} M and 1×10^{-6} M respectively (Weaver *et al.*, 1984). As studied by ^{125}I labelling, the affinity of phosphorylated ankyrin is greatly decreased for tetrameric spectrin but not for the dimer: the K_d for the tetramer goes from 2.7×10^{-7} to 1.2×10^{-6} M (Lu *et al.*, 1985). The analysis of peptides obtained from the fractionated tryptic digestion of pure ankyrin (Wallin *et al.*, 1984; Weaver *et al.*, 1984) showed that the physiological phosphorylation site was within a 32 kDa peptide which also contains the site of interaction with spectrin (Weaver *et al.*, 1984). But the casein kinase phosphorylation sites have not been identified.

Soong *et al.* (1987) studied the interaction of ankyrin with the 43 kDa internal fragment of band 3 *in vitro*. They observed the formation of two types of complex 43 kDa–ankyrin: one with 1.7 mol of 43 kDa/mol of ankyrin, the other with only 0.43 mol of 43 kDa/mol of ankyrin. Above all, they observed that ankyrin phosphorylation by casein kinases decreased by half the amount of 43 kDa bound to ankyrin: 0.8 and 0.28 mol of 43 kDa/mol of ankyrin for each complex respectively.

One might suggest that ankyrin phosphorylation, which decreases its affinity for both spectrin and the band 3 internal fragment, reduces interactions between the membrane skeleton and the rest of the membrane, thereby loosening the skeletal structure. Dephosphorylation might produce the reverse.

However one must be careful in drawing conclusions from experiments done with cyclic AMP-independent casein kinases under conditions which do not seem physiological. It is necessary to demonstrate that the sites phosphorylated by casein kinases are the physiological ones.

However it is interesting to note from the above results that the phosphorylations of protein 4.1 and ankyrin by membrane and cytosolic casein kinases seem to have a similar effect of weakening the skeleton and its bonds to the rest of the membrane.

Phosphorylation of adducin (Gardner & Bennett, 1986, 1987; Mische *et al.*, 1987)

Adducin is one of the most recently identified component of the red blood cell skeleton. It is present with spectrin, actin and protein 4.1 in a low-ionic-strength extract of ghosts where it appears as a doublet of minor proteins of about 100/110 kDa. We observed that these minor proteins were phosphorylated by a cyclic AMP-dependent protein kinase present in hydrosoluble proteins and they were more easily detected by autoradiography than by Coomassie Blue staining (Lecomte *et al.*, 1982*a*).

Several authors showed that this doublet was also phosphorylated by protein kinase C (Palfrey & Waseem, 1985; Cohen & Foley, 1986*a*,*b*; Ling *et al.*, 1986) and Ca^{2+}/calmodulin-dependent protein kinase (Cohen & Foley, 1986*a*). Adducin is a calmodulin-binding protein of 200 kDa; it is a heterodimer with two subunits α and β of 103–110 kDa and 97–100 kDa (Gardner & Bennett, 1987). The lower subunit binds to calmodulin. The number of heterodimer copies was estimated to be about 30000 per red cell. Adducin binds to actin and bundles actin filaments by forming crossbridges. Furthermore it promotes the binding of spectrin to actin independently from protein 4.1. Formation of the spectrin–actin–adducin ternary complex is down-regulated by calmodulin and calcium (Mische *et al.*, 1987).

Adducin is phosphorylated in intact red blood cells incubated with TPA, in ghosts and in solution of proteins solubilized at low ionic strength when purified protein kinase C is present. Each α and β subunit accepts 3 mol of phosphate/mol of protein. As far as specific activity is concerned, phosphorylation by protein kinase C is 18 times more intense than for protein 4.1.

Phosphorylation of protein 4.9

The molecular mass of protein 4.9 is 46–50 kDa, slightly higher than that of actin; this explains its position after SDS/polyacrylamide-gel electrophoresis. Protein 4.9 is part of the membrane skeleton. Although its role is not, as yet, completely defined, it seems to be the limiting factor for actin polymerization. It might perhaps also interact with spectrin.

In whole red blood cells as well as in ghosts, protein 4.9 is phosphorylated by the cyclic AMP-dependent membrane protein kinase (Fairbanks & Avruch, 1974; Hosey & Tao, 1977*a*,*b*), by the TPA-stimulated protein kinase C (Ferrell & Huestis, 1984) and perhaps by the cytosolic casein kinase (Plut *et al.*, 1978).

Horne *et al.* (1985) have shown that in the presence of dibutyryl cyclic AMP and TPA, there was an 8-fold and 3-fold increase respectively in the phosphorylation of protein 4.9, in inverse proportions to what occurs for the calmodulin binding protein (adducin).

Both types of protein kinases seem to share certain phosphorylation sites: other sites are specific for one or the other. As opposed to adducin and protein 4.1, protein 4.9 is not phosphorylated by the Ca^{2+}/calmodulin-dependent kinase (Cohen & Foley, 1986*a*,*b*).

Recently Husain & Branton (1987) separated protein 4.9 from a protein kinase activity which is able to phosphorylate protein 4.9 itself. They observed that protein 4.9 phosphorylation by this protein kinase completely abolishes protein 4.9–actin binding activity. Conversely, phosphorylation *in vitro* of protein 4.9 by protein kinase C does not affect this bundling activity. Indeed, it seems that protein 4.9 phosphorylation is involved in the limitation of actin polymerization and its interaction with spectrin.

Other skeletal proteins

On one- or two-dimensional SDS/polyacrylamide-gel electrophoresis protein 7 can be broken into several components. At least some of them would be part of the membrane skeleton in as much as they are found in ghost residues after Triton extraction (Lande *et al.*, 1982). This protein might be phosphorylated (Harrel & Morrison, 1979) on at least some of its components (Johnson *et al.*, 1982).

PHOSPHORYLATION OF INTEGRAL PROTEINS

Integral proteins extend all the way across the membrane lipid bilayer. They are linked to the skeleton through their internal cytosolic fragment at several points: on the β chain of spectrin through ankyrin, itself bound to band 3; on protein 4.1, through its bonds to glycophorin (this binding is phosphatidylinositol 4,5-bisphosphate-dependent); protein 4.1 is also bound to band 3; there are perhaps other still unknown anchorage points.

Phosphorylation of protein 3

Protein 3 amounts to about 25–30% of membrane proteins. In intact red blood cells as well as in ghosts its phosphorylation is cyclic AMP-independent. At least three protein kinases may be involved: the membrane casein kinase, as demonstrated by the phosphorylation of ghosts incubated with [^{32}P]ATP, the cytosolic casein kinase and a tyrosine kinase. Protein 3 is made of two parts easily separated by proteolytic digestion: one is an external transmembrane 55 kDa component which is the anion channel, the other, with a molecular mass of 43 kDa, is the component through which protein 3 is in contact with the membrane skeleton and cytosolic components. This 43 kDa segment bears the binding site to ankyrin, and, near its *N*-terminal end, binding sites to haemoglobin and the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase, aldolase, phosphofructokinase and phosphoglycerate kinase (reviewed by Low, 1986).

The internal segment bears several phosphorylation sites on serine, threonine and tyrosine residues (Dekowski *et al.*, 1983; Drickamer, 1976). The characteristics and the action of the phosphorylation by purified membrane and cytosolic casein kinases were studied on the isolated 43 kDa fragment (Soong *et al.*, 1987). Both these casein kinases phosphorylate the 43 kDa fragment identically, essentially on serine and threonine residues, with the same stoichiometry of 1 mol of phosphate/mol of 43 kDa. Their effect is not additive. According to our own experience, the cytosolic casein kinase we purified has a low affinity for the 43 kDa fragment as compared to the membrane kinase. It is possible that Soong *et al.* (1987) purified different cytosol kinases.

In any case the phosphorylation of the 43 kDa fragment does not change its affinity for ankyrin while, as we saw above, the phosphorylation of ankyrin decreases its binding capacity for the 43 kDa fragment.

Dekowski *et al.* (1983) demonstrated the presence in the red blood cell membrane of a tyrosine kinase which

phosphorylates the internal segment of band 3. This enzyme was purified and characterized to some extent (Boivin *et al.*, 1986; Mohamed & Steck, 1986; Phan-Dinh-Tuy *et al.*, 1985).

The phosphorylation site is Tyr-8 very close to the *N*-terminal end of the 43 kDa molecule. The stoichiometry of phosphorylation is very low: as have Mohamed & Steck (1986), we observed that with the purified red blood cell tyrosine kinase and the 43 kDa fragment only 1% of the 43 kDa molecules could be phosphorylated *in vitro*.

With an exogenous tyrosine kinase extracted from beef thymus Low *et al.* (1987) obtained a phosphorylation of the isolated 43 kDa fragment of 1.8 mol of phosphate/mol of 43 kDa. They immobilized the phosphorylated and unphosphorylated 43 kDa fragments on Affi-Gel and studied interactions with glycolysis enzymes. It is known that glyceraldehyde 3-phosphate dehydrogenase, aldolase, phosphofructokinase, phosphoglycerate kinase and haemoglobin are bound to the 43 kDa fragment through a purely electrostatic mechanism: the enzymes are effectively bound to the phosphorylated immobilized 43 kDa molecule and to it when 'dephosphorylated' by a prostatic acid phosphotyrosine phosphatase. However when the 43 kDa is phosphorylated the binding is reduced to 50% through a decrease in the number of binding site, whereas the affinity is unchanged. When the enzymes are bound to the 43 kDa fragment their activity is lost or profoundly altered: it is thus suggested that the Tyr-8 phosphorylation could modulate glycolysis by releasing bound enzymes; the dephosphorylation, apparently by a cytosolic acid phosphatase, which we have shown to be a tyrosine phosphatase (Boivin & Galand, 1986), would have the reverse effect. Inasmuch as glycolysis is involved in the shape and deformability of red blood cells through ATP synthesis, one may think that its modulation by band 3 phosphorylation has thus an effect on these properties. However one must note that blocking of the 43 kDa fragment-enzyme binding was incomplete and that the tyrosine kinase used by Low *et al.* (1987) was not extracted from red cell.

Phosphorylation of glycophorins

The main membrane glycophorin is glycophorin A (glycoprotein α); on SDS/polyacrylamide gels it migrates as an α_2 dimer near and more or less continuously with zone 3 and as a 38 kDa α_1 monomer between proteins 4 and 5.

In intact red blood cells, as well as in ghosts, glycophorin A is phosphorylated. However the spot visible on autoradiographs after electrophoresis is due not only to the α_2 protein but also to the glycophorin-bound polyphosphoinositides (Shapiro & Marchesi, 1977). The phosphorylating enzyme is not clearly identified: a rabbit red blood cell cyclic nucleotide-independent protein kinase (Hosey & Tao, 1977*b*) different from the human membrane casein kinase, is active on human glycophorin; human cytosolic casein kinase is also active (Simkowski & Tao, 1980). About 80% of the radioactivity is due to phosphoserine and 20% to phosphothreonine (Sheetz & Singer, 1977). As shown by limited tryptic digestion the phosphorylation sites are near the *C*-terminal, i.e. the protein segment which is in contact with the cytosol (Sheetz & Singer, 1977). As we have seen above, regarding the phosphorylation of other proteins, especially that of protein 3 tyrosine, only a very small number of glycophorin A molecules can be phosphorylated; the molar ratio of $^{32}PO_4$ to glycophorin is about 1 to 100 (Sheetz & Singer, 1977). In spite of the very low proportion of molecules than can be phosphorylated, some authors estimate that 70% of the radioactivity in the protein 3 region could be due to phosphorylated glycophorin A dimers (Dzandu *et al.*, 1985).

Among other membrane glycoproteins, glycophorin B (glycoprotein δ) is phosphorylated in intact red blood cells and ghosts whereas glycophorin C (glycoprotein β) is not phosphorylated or to a very low extent (Johnson *et al.*, 1982).

The role of the phosphorylation of glycophorins is yet to be established. If one bears in mind, on the one hand, that the bonds between glycophorin A phosphoinositides and protein 4.1 may be involved in the control of membrane physical properties, and, on the other hand, that glycophorin C is also bound to protein 4.1 (for this it was called glycoconnectin), one may suspect that the phosphorylation of glycophorins affects these bonds and therefore the integrity of membrane structures.

CONCLUSION

When the phosphorylation of membrane proteins was discovered it was hoped that they might be the modulating elements of the shape and deformability of erythrocytes. The enthusiasm of the first years was soon to disappear to the point that these phosphorylations were seen only as signs of cellular events occurring during the multiplication and maturation of erythroblasts. Recent data put the initial hypotheses in the limelight again, showing that the phosphorylation process is involved in molecular interactions at several levels and thereby contributes to the integrity of the general membrane structure. Many points still remain unclear. One can wonder what is the relevance of experiments carried out *in vitro* with purified proteins and enzymes. It is possible to conclude from their results that the events *in vivo* are identical? The properties of purified proteins may be different from those within the complex membrane structures. Because of a higher or lesser degree of denaturation one may reveal a site which does not exist in the membrane or is normally masked by molecular interactions. Can one extrapolate the results to isoforms of erythrocytic proteins found in other types of cells and generalize the data obtained from the erythrocyte model to the physical properties of all cell membranes? One may hope that answers to these questions may come to light in the near future.

REFERENCES

Agre, P., Gardner, K. & Bennett, V. (1983) J. Biol. Chem. **258**, 6258–6265

Anderson, J. P. & Morrow, J. S. (1987) J. Biol. Chem. **262**, 6365–6372

Anderson, J. M. & Tyler, J. M. (1980) J. Biol. Chem. **255**, 1259–1265

Anderson, R. A. & Lovrien, R. E. (1984) Nature (London) **307**, 655–658

Anderson, R. A. & Marchesi, V. T. (1985) Nature (London) **358**, 295–298

Avruch, J. & Fairbanks, G. (1974) Biochemistry **13**, 5507–5513

Birchmeier, W. & Singer, S. J. (1977) J. Cell. Biol. **73**, 647–659

Boivin, P. & Galand, C. (1978) Biochem. Biophys. Res. Commun. **81**, 473–480

Boivin, P. & Galand, C. (1980) Biochem. Biophys. Res. Commun. **93**, 24–28
Boivin, P. & Galand, C. (1986) Biochem. Biophys. Res. Commun. **134**, 557–564
Boivin, P., Garbarz, M., Dhermy, D. & Galand, C. (1981). Biochim. Biophys. Acta **647**, 1–6
Boivin, P., Garbarz, M. & Galand, C. (1980). Int. J. Biochem. **12**, 445–449
Boivin, P., Galand, C. & Bertrand, O. (1986). Biochim. Biophys. Acta **860**, 243–252
Boivin, P., Galand, C. & Bertrand, O. (1987) Int. J. Biochem. **19**, 613–618
Brenner, S. L. & Korn, E. D. (1979) J. Biol. Chem. **254**, 8620–8627
Clari, G. & Moret, V. (1981) Biochem. Int. **3**, 433–440
Clari, G. & Moret, V. (1982) Biochem. Int. **4**, 585–593
Clari, G., Brunati, A. M. & Moret, V. (1987) Biochem. Biophys. Res. Commun. **142**, 587–594
Cohen, A. M., Liu, S. C., Derick, L. H. & Palek, J. (1986) Blood **68**, 920–926
Cohen, C. M. & Foley, S. F. (1986*a*). J. Biol. Chem. **261**, 7701–7709
Cohen, C. M. & Foley, S. F. (1986*b*) in Membrane Skeletons and Cytoskeletal–Membrane Associations (Bennett, V., *et al.*, eds.), pp. 211–222, R. Liss, New York
Dekowski, S. A., Ribicki, A. & Drikamer, K. (1983) J. Biol. Chem. **258**, 2750–2753
Drickamer, L. K. (1976) J. Biol. Chem. **251**, 5115–5123
Dzandu, J. K., Deh, M. E. & Kiener, P. (1985) Biochem. Biophys. Res. Commun. **127**, 878–884
Eder, P. S., Soong, C. J. & Tao, M. (1986) Biochemistry **25**, 1764–1770
Fairbanks, G. & Avruch, J. (1974) Biochemistry **13**, 5514–5521
Faquin, W. C., Chahwala, S. B., Cantley, L. C. & Branton, D. (1986) Biochim. Biophys. Acta **887**, 142–149
Ferrell, J. E. & Huestis, W. H. (1984). J. Cell, Biol. **98**, 1992–1998
Gardner, K. & Bennett, V. (1986) J. Biol. Chem. **261**, 1339–1348
Gardner, K. & Bennett, V. (1987) J. Cell. Biochem. Suppl. **11B**, 132 (abstr.)
Graham, C., Avruch, J. & Fairbanks, G. (1976) Biochem. Biophys. Res. Commun. **72**, 701–708
Grazi, E. & Magri, E. (1979) FEBS Lett. **104**, 284–286
Greenquist, A. C. & Shohet, S. B. (1975) in Erythrocyte Structure and Function (Brewer, C. J., ed.), pp. 515–530, Alan R. Liss, New York
Harrel, D. & Morrison, P. (1979) Arch. Biochem. Biophys. **193**, 158–168
Harris, H. W. & Lux, S. E. (1980) J. Biol. Chem. **255**, 11512–11520
Hofstein, R., Hershkowitz, M., Gozes, I. & Samuel, D. (1980) Biochim. Biophys Acta **624**, 158–162
Horne, W. C., Leto, T. L. & Marchesi, V. T. (1985) J. Biol. Chem. **260**, 9073–9076
Hosey, M. M. & Tao, M. (1976) Biochemistry **15**, 1561–1568
Hosey, M. M. & Tao, M. (1977*a*) Biochemistry **16**, 4578–4583
Hosey, M. M. & Tao, M. (1977*b*) J. Biol. Chem. **252**, 102–109
Husain, A. & Branton, D. (1987) J. Cell Biol. **105**, 39a
Husain, A., Howlett, G. J. & Sawyer, W. H. (1984) Biochem. Biophys. Res. Commun. **122**, 1194–1200
Johnson, R. M., McGowan, M. W., Morse, P. D. & Dzandu, J. K. (1982). Biochemistry **21**, 3599–3604
Kiener, P. A., Carroll, D., Roth, B. J. & Westhead, E. W. (1987) J. Biol. Chem. **262**, 2016–2024
Lande, W. M., Thiemann, P. V. W. & Mentzer, W. C. (1982) J. Clin. Invest. **70**, 1273–1280
Lecomte, M. C., Galand, C. & Boivin, P. (1982*a*). Nouv. Rev. Fr. Hematol. **24**, 349–358
Lecomte, M. C., Gautero, H. & Boivin, P. (1982*b*) Biochem. Int. **5**, 793–798
Leto, T. L. & Marchesi, V. T. (1984) J. Biol. Chem. **259**, 4603–4608
Ling, E. & Sapirstein, V. (1984) Biophys. Res. Commun. **120**, 291–298
Ling, E., Gardner, K. & Bennett, V. (1986) J. Biol. Chem. **261**, 13875–13878
Ling, E., Danilov, Y. N. & Cohen, C. M. (1987) J. Cell Biol. **105**, 39a
Liu, S. C., Derick, L. H. & Palek, S. (1987) J. Cell Biol. **104**, 527–536
Low, P. S. (1986) Biochim. Biophys. Acta **864**, 145–147
Low, P. S., Allen, D. P., Zioncheck, T. F., Chari, P., Willardson, B. M., Geahlen, R. L. & Harrison, M. L. (1987) J. Biol. Chem. **262**, 4592–4596
Lu, P. W., Soong, C. J. & Tao, M. (1985) J. Biol. Chem. **260**, 14958–14964
Lutz, H. U. (1984) FEBS Lett. **169**, 323–329
Lutz, H. U., Stringaro-Wipf, G. & Maretzki, D. (1986) J. Cardiovasc. Pharmacol. **8** (Suppl. **8**), 576–579
Maretski, D. & Lutz, H. U. (1986) Arch. Biochem. Biophys. **246**, 469–477
Mische, S. M., Mooseker, M. S. & Morrow, J. S. (1987) J. Cell. Biochem. **154**, 2837–2845
Mohamed, A. H. & Steck, T. L. (1986) J. Biol. Chem. **261**, 2804–2809
Palfrey, H. C. & Waseem, A. (1985) J. Biol. Chem. **260**, 16021–16029
Pasternack, G. R., Anderson, R. A., Leto, T. L. & Marchesi, V. T. (1985) J. Biol. Chem. **260**, 3673–3683
Phan-Dinh-Tuy, F., Henry, J. & Kahn, A. (1985) Biochem. Biophys. Res. Commun. **126**, 304–312
Pinder, J. C., Bray, D. & Gratzer, W. B. (1977) Nature (London) **270**, 752–754
Plut, D. A., Hosey, M. M. & Tao, M. (1978) Eur. J. Biochem. **82**, 333–338
Pratje, E. & Heilmeyer, L. M. G. (1972) FEBS Lett. **27**, 89–93
Rybicki, A. C., Heath, R., Lubin, B. & Schwartz, R. S. (1988) J. Clin. Invest. **81**, 255–260
Shapiro, D. G. & Marchesi, V. T. (1977) J. Biol. Chem. **252**, 508–517
Sheetz, M. P. & Singer, S. J. (1977) J. Cell Biol. **73**, 638–646
Simkowski, K. W. & Tao, M. (1980) J. Biol. Chem. **255**, 6456–6461
Sommercorn, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. G. (1987). Proc. Natl. Acad. Sci. U.S.A. **84**, 8834–8838
Soong, C. J., Lu, P. W. & Tao, M. (1987) Arch. Biochem. Biophys. **254**, 509–517
Steinberg, R. H. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 910–914
Tao, M., Conway, R., Cheta, S., Chang, H. C. & Yan, T. F. (1981) Cold Spring Harbor Conf. Cell. Prolif. **8**, 1301–1312
Usui, H., Kinohara, N., Yoshikawa, K., Imazu, M., Imaoka, T. & Takeda, M. (1983). J. Biol. Chem. **258**, 10455–10463
Wallin, R., Culp, E. N., Coleman, D. B. & Goodman, S. R. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 4095–4099
Walsh, M. P., Hinkins, S. & Hartshone, D. J. (1981) Biochem. Biophys. Res. Commun. **102**, 149–157
Wang, C., Kong, S. K. & Wang, J. H. (1988) Biochemistry **27**, 1254–1260
Weaver, D. C., Pasternack, G. R. & Marchesi, V. T. (1984) J. Biol. Chem. **259**, 6170–6175
Wyatt, J. L., Greenquist, A. C. & Shohet, S. B. (1977) Biochem. Biophys. Res. Commun. **79**, 1279–1285