The Cytoskeleton and the Regulation of Gluconeogenesis: A Hypothesis

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Previous data from our laboratory indicated a role for the cytoskeleton in the hormonal stimulation of gluconeogenesis. To gain further insight into the role of the cytoskeleton in the regulation of gluconeogenesis, we performed further experiments to examine the possibility that the cytoskeleton is involved in the glucagon-mediated changes in calcium distribution. Perfused livers or isolated liver cells were pretreated with either cytochalasin B or colchicine, agents that disrupt microfilaments and microtubules, respectively. Pretreatment with either agent significantly decreased the glucagon-evoked efflux of labeled calcium. Pretreatment with colchicine was more effective in blocking the influx of calcium into the cells than pretreatment with cytochalasin B. These drugs also prevented the characteristic increase in O_2 uptake, which is usually observed after glucagon administration. Thus, an intact cytoskeleton seems to be a prerequisite for the glucagon-evoked changes in calcium distribution to occur. These changes in calcium fluxes were shown previously to be an essential link in the chain of events leading to the metabolic effects of glucagon. Based on these results and on data obtained by others, a hypothesis is presented here. The hypothesis presumes that the cytoskeleton plays a crucial role in the regulation of gluconeogenesis. The hypothesis further assumes that the cytoskeleton influences gluconeogenesis in 3 ways: (1) by influencing the process of calcium signaling; (2) by changing the rate of enzymatic reactions through association and dissociation of enzymes with the cytoskeleton; and (3) by altering the position of intracellular organelles and the movements of molecules. Each of these points is discussed separately. It is known that the intracellular environment exists as a dense mesh in dynamic motion. According to our hypothesis, hormonal stimulation changes this environment by affecting the ionic composition in the cytosol and the structure of the cytoskeleton. Motion and conformational changes by the cytoskeleton play crucial regulatory functions, influencing metabolic processes.

GLUCONEOGENESIS IS the process by which the liver replenishes blood glucose as needed in cases such as fasting or in a low-carbohydrate diet. Gluconeogensis is hormonally regulated; the physiologic stimulus for increased glucose production is an increase in the level of glucagon. Insulin suppresses the process. The pathway of gluconeogenesis is usually described in textbooks as consisting of enzymes present and interacting in the aqueous environment of the cytosol. We suggest that the regulation of gluconeogenesis involves the interaction between the enzymes of the pathway and the cytoskeleton.

One of the early studies pointing to the involvement of the cytoskeleton in the hormonal regulation of metabolism is the report by Tomomura et al,[1] which suggests a role for the cytoskeleton in the stimulation of glycogenolysis by glucagon. In that study, administration of either colchicine or cytochalasins prevented the glucagon-stimulation of glycogenolysis, even though neither cyclic adenosine monophosphate (cAMP) formation nor the activation of protein kinase A (PKA) were inhibited. More recently, it was reported that in response to glucose administration, glycogen synthetase moves from the cytosol to the actin-rich area below the plasma membrane.[2] The gluconeogenic pathway is far more complex than the glycogenolytic pathway. The enzymes of the pathway are situated in 3 different cellular compartments. Thus, the first and flux-generating enzyme of the pathway, pyruvate carboxylase, is intramitochondrial. The second specific enzyme of the pathway, phospho-enolpyruvate carboxykinase, is cytosolic or intramitochondrial, or both, according to the species. The last enzyme of the pathway, glucose-6-phosphatase, is anchored in the endoplasmic reticulum. The compartmentalization of the pathway offers regulatory points.

The pathway of gluconeogenesis has been described in textbooks and in numerous review articles.[3-5] Part of the pathway shares enzymes with the glycolytic pathway and is the reversal of it. The enzymes of the glycolytic pathway bind reversibly to cytoskeletal elements. This association and dissociation of the enzymes to the cytoskeleton plays an important role in the regulation of the rate of glycolysis.[6-8] The function of this clustering of enzymes is often described as "channeling," namely the positioning of sequential enzymes in the pathway in a fashion that facilitates the passage of substrates between enzymes and thereby facilitates the enzymatic reaction.[9] Because some of the enzymes in the gluconeogenic pathway are shared with the glycolytic pathway, the well-proven association of the glycolytic enzymes with the cytoskeleton is an indicator pointing to a possible role for the cytoskeleton in the regulation of gluconeogenesis.

The association of enzymes with cytoskeletal elements is not restricted to carbohydrate metabolism. For instance, the transport of amino acids into the liver was shown to involve microtubules.[10] The induction of tyrosine aminotransferase, a related phenomenon, was shown to depend on the organization of the cytoskeleton.[11] According to a recent study, the enzymes involved in fatty acid translocation and ketogenesis are also associated with the cytoskeleton in the liver.[12] Thus, the association between enzymes and the cytoskeleton may be widespread.

An important aspect of the regulation of gluconeogenesis and the topic of our hypothesis is the hormonal stimulation of the pathway. In the liver, the physiologic stimulator of glucone-

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ogenesis is glucagon (reviewed in Kraus-Friedman[4]). Glucagon acts by activating adenylyl cyclase and generating cAMP.[13] It was shown in a number of cell types that cAMP can alter the structure of the cytoskeleton. For instance, the secretory response evoked by the administration of isoproterenol to parotid acinar cells is associated with changes in actin labeling.[14] In the rat parotid gland, the β-adrenergic stimulation of calcium fluxes depends on an intact microfilamental system.[15] Vasopressin in the kidney, where it acts by elevating cAMP levels, depolimerizes actin.[16] In rat bladder carcinoma cells, it was shown that elevation of cAMP levels is associated with changes in the organization of F-actin, and these changes were prevented by the prior administration of PKA inhibitors.[17] Thus, in different cell types, the actions of cAMP were shown to be both dependent on and influencing the organization of the cytoskeleton.

The exact mechanism by which cAMP affects cytoskeletal elements is not known. It is generally assumed that, with some exceptions such as the cyclic nucleotide gated channels, which do not require phoshporylation for activity, cAMP exerts its influence by activating PKA. Indeed, there are reports that indicate an interaction between PKA and the cytoskeleton. For instance, in the above study,[17] inhibitors of PKA activity prevented the cAMP-evoked changes in the cytoskeleton. In a recent study, the role of PKA anchoring during oogenesis in Drosophila was investigated by analyzing DAKAP200, a protein that contains a PKA-anchoring domain. According to this study, the PKA-anchoring domain may be responsible for regulating the subcortical cytoskeleton. The results provided evidence that PKA localization is important and required for the morphogenesis of specific cytoskeletal structures.[18] Whether or not this study in Drosophila is also relevant to mammalian systems remains to be seen. In another study, the phosphorylation of microtubule-associated protein 2(MAP2), a neuronal phosphoprotein, by PKA was investigated. PKA activity resulted in the disruption the MAP2-microtubule interaction in HeLa cells and promoted the translocation of MAP2 to peripheral membranes enriched in actin.[19] Thus, it is possible that the activation of PKA is involved in the effects of cAMP on the cytoskeleton. Also, a small, guanosine triphosphate (GTP)-binding protein, Rho, is indicated to play a role in the organization of the cytoskeleton.[20,21] Whether this protein has a function in the action of glucagon on the cytoskeleton is not known. An additional possibility is that the changes in the cytoskeleton are secondary to the changes in calcium concentration, which are associated with the effects of glucagon.

In the liver, the stimulatory effect of glucagon on gluconeogenesis is associated with major changes in ion fluxes, including calcium influx, release, and the subsequent increase in cytosolic-free calcium levels. As described in detail in a previous report, this redistribution of calcium and the ensuing increase in cytosolic-free calcium levels are an essential and integral part of the mechanism by which gluconeogenesis is stimulated.[22] It was suggested in that report that the stimulation of gluconeogenesis by calcium is accomplished in 2 ways: (1) by increasing the activities of the Krebs cycle and oxygen consumption, thereby providing adenosine triphosphate (ATP) and reducing equivalents to the process and (2) by influencing the activities of key enzymes. In an earlier study, we found that disruption of the cortical F-actin layer resulted in the inhibition of glucagon-stimulated gluconeogenesis,[23] and it seemed possible that the inhibition was caused by a defect in the calcium response. We set out to examine this possibility, and we present the data here. The data support the notion that disruption of the cytoskeleton impairs calcium response. The inhibition of the hormonally-evoked calcium fluxes results in the inhibition of the metabolic effects.[4,22]

Inspired by these findings and by the many relevant reports in the literature (unfortunately only a fraction can be cited), a hypothesis is presented here. The hypothesis suggests that the cytoskeleton plays a major role in the hormonal regulation of gluconeogenesis. It is further assumed that the cytoskeleton is involved in the regulation of the pathway in 3 ways: (1) by affecting calcium signaling; (2) by affecting the association/dissociation of enzymes to the cytoskeleton; and (3) by facilitating the movement of intracellular organelles and individual molecules. Thus, this hypothesis relates to cellular events that occur after the cells are stimulated by a hormone. It does not deal with secretory processes, such as hormone secretion. Hormone secretion has been shown to be influenced by the cytoskeleton.[24] The hypothesis deals only with the acute regulation of gluconeogenesis. Chronic stimulation, which involves enzyme induction, is not discussed here, because it entails different regulatory mechanisms.

This hypothesis is presented in the hope that it will generate further discussion and result in follow-up studies. Last, but not least, we hope that this article will contribute to the long-awaited revision of the way in which metabolic regulation is presented in the textbooks. As pointed out recently by Ovadi and Srere,[8] ". . .current texts of general biochemistry still view metabolism occurring by a series of independent enzymes dispersed in a uniform aqueous environment. This notion has been shown to be deeply flawed by both experimental and theoretical considerations. . .".

CALCIUM SIGNALING

Calcium emerged as a regulatory and integrating element in cells with the appearance of the eucaryotic organism.[25] One of the characteristics of eucaryotic cells, which distinguishes them from procaryotic cells, is the presence of a dense, internal filamentous structure, the cytoskeleton. References for a few review articles dealing with the cytoskeleton are included here.[26-30] Calcium interacts with the cytoskeleton in numerous ways. One of the recently discussed and experimentally probed interactions between calcium and the cytoskeleton relates to a possible role for the cytoskeleton in agonist-induced calcium signaling. Glucagon, the hormone in charge of the stimulation of gluconeogenesis, as well as cAMP, the mediator of the effects of glucagon, were shown to alter calcium fluxes in the liver. Thus, stimulation by glucagon was shown to be followed by the influx of cations, including calcium.[31-34] This influx is followed by the release of calcium from an intracellular store, and as the result of these changes, an increase in cytosolic-free calcium follows.[35] However, the entry of calcium is not a prerequisite for the release, because release was shown to occur even in the complete absence of extracellular calcium.[31] With respect to glucagon, there is a clear consensus as to the se-

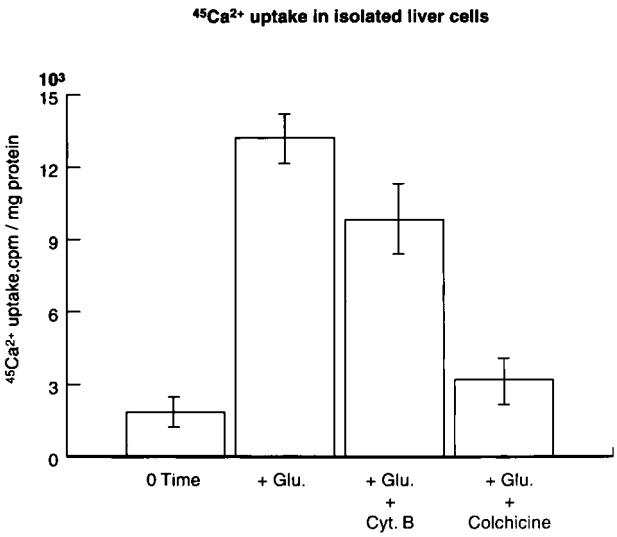

Fig 1. The effects of glucagon (10^{-9} mol/L) on the uptake of calcium into the liver in the presence and absence of either cytochalasin B (5 μm) or colchicine (10 μm). The experimental design and the method used are described in the Methods.

quence of ion fluxes. These changes in ion fluxes are an integral part of the mechanism by which glucagon acts, because blocking these changes results in the inhibition of the metabolic effects of glucagon, while eliciting such changes in ion fluxes are sufficient to stimulate gluconeogenesis.[22]

Relevant to our hypothesis were the observations made in our previous study, which showed that cytochalasin D treatment of perfused livers, which disrupted the cortical actin layer below the plasma membrane, inhibited the glucagon-stimulation of increase in glucose output.[23] The mechanism underlying this inhibitory effect was not clarified in the previous study. However, because cytochalasins interact specifically with actin, we assumed that the inhibition is due to the disruption of the cortical actin structure.[23,36] Because the changes in calcium fluxes are an integral part of the gluconeogenic response, it seemed possible that the disruption of the cytoskeleton interferes with the initial redistribution of ions. This possibility was especially likely, because various other calcium movements in the liver had already been shown to be influenced by the cytoskeleton.[37-42] It seemed, therefore, imperative to clarify this question. We performed such experiments, and the results are presented here. First, the data are shown, followed by a discussion of the relevance of the data to the hypothesis.

METHODS

Measurement of $^{45}Ca^{2+}$ Uptake

Isolated liver cells were prepared from fed rats using the collagenase technique with some modifications as in previous studies.[43] The cells were suspended in Krebs-Ringer bicarbonate buffer (KRB) containing 3% bovine albumin, Cohn fraction V, and 20 mmol/L glucose, with or without drugs. The cells were incubated in Erlenmeyers placed in shaking water bath kept at 33°C, gassed with a mixture of 95% O_2 and 5% CO_2 for 90 minutes. $^{45}Ca^{2+}$ uptake was measured with the millipore method described by Keppens et al[32] with or without drugs. For obtaining the control values, 5 aliquots, sampled every 30 seconds, were taken. Subsequently, glucagon was added, and 5 more aliquots were taken for the experimental values. The 5 measurements were averaged. A minimum of 3 to 4 such experiments were performed for each condition.

Measurement of $^{45}Ca^{2+}$ Release

Livers from fed rats were perfused with KRB containing 3% albumin, Cohn fraction V, and 0.1 μCi $^{45}Ca^{2+}$, and the experiments performed as described previously.[44] In the experiments in which the effects of the drugs were tested, they were added at the start of the perfusion and were present during the entire 90 minutes of perfusion.

Measurement of Gluconeogenesis

Gluconeogenesis was measured using cells isolated from overnight fasted rats, as previously described.[44,45] The cells were incubated in KRB containing 3% albumin, Cohn fraction V, 5 mmol/L l-alanine, 10 mmol/L lactate, and 1 mmol/L pyruvate. Samples for glucose determinations were taken after 60 and 120 minutes of incubation. Glucose was determined with Sigma Diagnostic Infinity Glucose Reagent (procedure #17-UV).

Protein determination was performed using the method of Bradford.[46]

Oxygen uptake in perfused livers was performed as described previously.[47] The drugs were perfused for 60 minutes before the addition of glucagon.

RESULTS

The data presented in Figs 1 and 2 show that disruption of the cytoskeleton prevents the usual responses to glucagon.

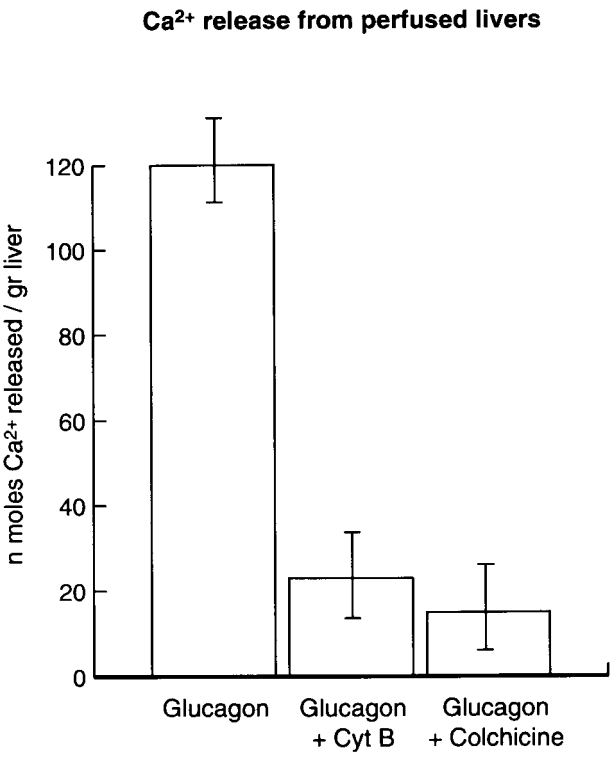

Fig 2. The glucagon-stimulation (10^{-9} mol/L) of the release of labeled calcium from the liver in the presence or absence of either cytochalasin B (5 μm) or colchicine (10 μm). The experimental design and the method used is described in the Methods.

Thus, the glucagon stimulation of the uptake of $^{45}Ca^{2+}$ was inhibited by colchicine and reduced by cytochalasin B (Fig 1). The glucagon stimulation of the release of preloaded $^{45}Ca^{2+}$ was equally reduced by treatment with either of the drugs.

Both colchicine and cytochalasins inhibited the stimulatory effect of glucagon on O_2 uptake, (values in μatom O_2/min/g liver): control, 1.80 $\pm$ 0.13; glucagon, 2.17 $\pm$ 0.18; glucagon with colchicine, 1.95 $\pm$ 0.15; glucagon with cytochalasin D, 1.81 $\pm$ 0.12.

We previously reported that cytochalasin D inhibited the gluconeogenic response to glucagon in perfused livers.[23] Here, we tried to measure the effects of colchicine. The rates of glucose production were the following (in micrograms glucose/milligrams protein): control, 4 $\pm$ 0.3 and glucagon, 24 $\pm$ 1.8. However, colchicine by itself (without cells), gave a strong reaction with the color reagent. The color reaction with colchicine alone (blank), was 3 times stronger than the maximum values obtained with glucagon. Thus, we could not use the usual colorimetric method to measure glucose production in the presence of colchicine. Because colchicine does interfere with the glucagon-evoked fluxes of calcium and O_2 uptake, it is also likely to inhibit gluconeogenesis.

DISCUSSION

These data, together with our previous results, and in combination with the many relevant studies reported in the literature, form the basis of our hypothesis. The results shown in Fig 1 and 2 show that an intact cytoskeleton is required for a normal calcium response to glucagon. When the cytoskeleton is disrupted either with cytochalasins or colchicine, both the influx and the release of calcium are reduced, if not totally inhibited. It is worthwhile to point out here that the cytoskeleton is a web, a network in which a connection between the different types of filaments exist.[28] Thus, while in the above experiments the initial points of the lesions are well defined, namely cytochalasins affect the microfilaments while colchicine affects the microtubules, it is likely that the cytoskeleton as a whole is affected.[25] The individual steps in the calcium response to glucagon are discussed in detail below.

As previously mentioned, glucagon administration is followed by an immediate influx of calcium.[32-34,48] Furthermore, the administration of glucagon, catecholamines, cyclic-guanosine monophosphate (cGMP) and cAMP to perfused livers is also followed by an immediate uptake of other cations, such as Na^+ and K^+.[31] Combining these observations and extrapolating from them, we assumed that glucagon activates a multication channel. However, the identity of the channel was not known. Indeed, while there are quite a few studies on hormone-induced calcium fluxes in the liver, little is known about the channels responsible for the fluxes. The liver does not seem to posses voltage-gated channels.[49] Based on the assumption that the channel activated is likely to be a channel, which is not specific to calcium only, we checked for the message in the liver for cyclic nucleotide-gated channels (CNGCs). This family of ion channels carries both mono and divalent cations. Some of these channels show clear preference for calcium.[50-52] We demonstrated that the message for a CNGC is present both in the rat and in fetal human livers.[53-55] Using specific antibodies developed against such a channel, we demonstrated, using Western blot analysis, that the channel protein is present in isolated rat liver plasma membrane fraction.[53] Indeed, CNGCs were localized to the plasma membrane in every cell type in which they were studied.[56] Because the sequence analysis of the polymerase chain reaction (PCR) products obtained in these studies indicated that the hepatic channel is almost identical to the rod channel, it is likely that it carries similar current. We performed 1 successful experiment, which showed cAMP activation (opening) of a channel in isolated liver cells (unpublished results). The presence of a CNGC in the liver is compatible with the observations on the nature of the ion fluxes. Thus, it is likely that this CNGC is the channel activated by glucagon administration. The data presented in Fig 1 implies that the activity of the channel responsible for the inflow is influenced by the state of the cytoskeleton. However, we are not aware of studies showing a connection between cytoskeletal elements and CNGCs.

While there is no experimental evidence that shows a connection between CNGCs and the cytoskeleton, such connections between other types of channels and the cytoskeleton are well documented. For instance, the activity of the L-type cardiac channel was shown to be controlled by the cytoskeleton.[57-59] The actin cytoskeleton plays a crucial role in the regulation of the cystic fibrosis transmembrane conductance regulator ion channel.[60] Epithelial sodium channels are also regulated by actin filaments.[61] Microtubules were shown to affect the activity of smooth muscle calcium channels.[62] These are just a few, randomly selected examples of interactions between ion channels and the cytoskeleton. Examination of the literature shows that an association between ion channels and cytoskeletal elements is more the rule than the exception.[42,63] Clearly, the elucidation of the glucagon stimulation of the calcium influx mechanism in the liver requires more study.

Figure 2 shows the effects of cytochalasin B and colchicine on the release of $^{45}Ca^{2+}$ from the liver. The results show that both treatments resulted in a decrease in the release of $^{45}Ca^{2+}$. However, the experimental design does not allow one to distinguish between an effect on the loading of the calcium into the store or on its release. Either 1 or both could have been affected by the treatments. In either case, the results show a connection between calcium handling by the liver cell in response to glucagon and the cytoskeleton. Again, one has to point out that, as is the case with the influx of calcium, the mechanism by which glucagon evokes the release of calcium from the liver is not clear. The addition of cAMP to isolated, calcium-loaded microsomal vesicles does not cause a release of stored calcium (unpublished data from our laboratory). The addition of GTP does evoke calcium release by inhibiting the Ca^{2+} adenosine triphosphatase (ATPase).[64] Indeed, the only effective way of releasing stored calcium from such a preparation in our laboratory was by the addition of heavy metals, which affect sulfhydryl (SH) groups.[65] SH groups were shown to play a crucial role in calcium release and also in skeletal muscle.[66,67] Thus, critical SH groups seem to play a role in the process of calcium release.

Relevant to the question of the mechanism of calcium release are our studies, which showed the presence of high-affinity binding sites for ryanodine in the hepatic microsomal frac-

tion.[68] Ryanodine is a plant alkaloid, a very polar molecule that binds to many sites. Ryanodine was used successfully to mark a calcium release channel in muscle cells.[69] In the muscle, the ryanodine receptor (RyR) was shown to possess critical SH groups involved in calcium release.[66] Several subtypes of RyR have been identified. We have suggested in a previous report that the RyR in the liver might be a calcium channel that is influenced and gated, at least partially, by forces exerted by the cytoskeleton, leading to its opening.[39]

The finding that high-affinity binding sites for ryanodine are present in the hepatic microsomal fraction is somewhat controversial. This is because the nonspecific binding of labeled ryanodine in the microsomal fraction is very high, and because in studies in which the distribution of the message for the known isoforms of RyRs were probed in different organs, none was found in the liver.[70] Yet, there are indications for similarities between the known RyRs and the findings in the liver. The skeletal RyR always copurifies with a binding protein for the immunosuppressant drug, FK506.[71] In the liver, addition of FK506 to the binding assay medium greatly reduced ryanodine binding.[72] We also found that the addition of ryanodine to liver cells changed calcium distribution and changed metabolism in a fashion that was compatible with increases in intracellular-free calcium levels.[43,45]

Other investigators[73] have reported that ryanodine releases calcium from isolated, hepatic microsomal fraction. In another interesting study, the importance of the RyR in nonmuscle cells was explored by creating a deficiency in all of the 3 known RyR genes in mutant mice.[74] They found that mice lacking subtypes RyR1 and RyR2 developed hypertrophy of the liver and accumulated excessive amounts of glycogen granules. It is likely that the excessive amount of glycogen results from a problem with glycogen breakdown caused by problems in calcium mobilization, glycogen breakdown being a calcium-dependent process. Another study reported that the state of the liver influences the affinity of ryanodine for the RyR, because partial hepatectomy increased the dissociation constant (Kd) for ryanodine.[75] These findings support the notion that a RyR is present in the liver, and it is playing a role in calcium distribution. The hepatic RyR is different from all of the well-characterized 3 subtypes of RyRs. Even though the density of the receptor is low in the liver, it still might be possible to purify the receptor and to characterize it. According to the estimates of Martinez-Merlos et al,[76] rat and guinea pig livers contain 0.35 pmol/RyR/mg protein. This amount seems to be sufficient for purifying the receptor.

As mentioned above, in our laboratory, the most effective way of releasing calcium from microsomal vesicles was by the addition of heavy metals.[65] The effect of heavy metals is due partially to the inhibition of the Ca^{2+}-ATPase and to a large extent to the binding of the metals to critical SH groups. Relevant to these observations is a more recent study in which thiol oxidation was followed in liver microsomes.[77] The investigators concluded that a ryanodine-sensitive channel might be activated by hepatotoxins, probably due to the oxidation of thiol groups. The effects of hepatotoxins were extensively studied in Orrenius' group, and oxidation-dependent actin crosslinking, sensitive to thiol reduction in the liver, was demonstrated.[78] The answer to the question of whether the hepatic RyR-like protein also possesses SH groups that serve regulatory functions in the gating of the channel, cannot be answered presently and awaits the purification of the protein. Based on the present state of knowledge, our hypothesis assumes that calcium release is accomplished partially by a change in the channel structure, which is transduced by the cytoskeleton, and partially by affecting critical SH groups. These 2 effects might be linked.

An additional connection between the cytoskeleton and the release of calcium from intracellular storage relates to the concept of "capacitative calcium entry." This concept was suggested to explain the observation that emptying the store, for instance by the use of thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, results in the influx of calcium by opening a so-called "store activated" channel.[79] While this observation has been reproduced in several laboratories, the mechanism by which the 2 processes, calcium release from the endoplasmic reticulum and the opening of a channel located at the plasma membrane, are coupled is not known and is currently an area of intensive research.[80] We, and others, suggested that the mechanism of coupling resides in the cytoskeleton. Namely, emptying of the calcium store causes a conformational change that is transduced by the cytoskeleton from the endoplasmic reticulum to the plasma membrane channel. This results in a channel opening.[81-84] Moreover, according to a recent study, store-operated calcium entry (namely entry following calcium release) in pulmonary artery endothelial cells occurs through a CNGC, activated by the release.[85] These data support the notion that the plasma membrane CNGC, identified as being present in the liver, might mediate not only the entry of calcium following appropriate hormonal stimulation, but also the entry of calcium following store emptying.[53-55] The data also point to the possibility that CNGCs connect with the cytoskeleton.

An interesting question is the sequence of events in this process. Does glucagon, by increasing cyclic AMP levels, elicit the influx of calcium first, which then affects cytoskeletal elements, or is it the other way around? It seems likely that both options function, depending on the conditions.

Following the glucagon-evoked influx and release of calcium from intracellular stores, free cytosolic calcium level increases.[35] Subsequently, calcium binds to calcium binding proteins, among others, regucalcin, which seems to be specific to the liver.[86,87] The effects of the increase in cytosolic calcium levels are attributed to the binding of calcium to various target proteins. These effects, in respect to glucagon effects on gluconeogenesis, were discussed in detail in previous reports.[4,22]

Summary of Calcium Signaling

Data from our own laboratory and that of others show that glucagon administration causes an immediate uptake of Ca^{2+} and other cations. This is followed by the release of calcium from intracellular stores, leading to an increase in cytosolic-free calcium levels. These changes in calcium distribution require an intact cytoskeleton. Because the disruption of the cytoskeleton also inhibits the metabolic responses to glucagon, we propose that the inhibition of the stimulation of gluconeogenesis by agents that disrupt the cytoskeleton is due, at least partially, to the inhibition of the initial changes in the ion fluxes.

ASSOCIATION AND DISSOCIATION OF ENZYMES WITH THE CYTOSKELETON

Glycolytic enzymes and the pathway are described in textbooks as soluble enzymes interacting in a uniform, aqueous environment. Yet, it has been demonstrated in different cell types that while part of the enzymes in the pathway are present in soluble form, a fraction of the enzymes are bound to structural elements, mostly to the cytoskeleton, and also to other types of membranes.[6,88] The association of the glycolytic enzymes to thin filaments in the muscle and to microfilaments, mostly in other cell types, has a profound effect on the rate of the enzymatic reactions. The attachment leads to altered conformational characteristics of the enzymes.[7,9,89,90] Skeletal and cardiac muscle enzymes are the most studied models. In skeletal muscle, over 50% of aldolase, 25% of phosphofructokinase, and over 40% of glyceraldehyde phosphate dehyrogenase were found to be bound.[88] It is well demonstrated in muscle cells that binding of the enzymes results in a more effective interaction between the enzymes of the pathway and in an increase in the rate of glycolysis.[8,9] It is not surprising therefore, that insulin, a hormone that stimulates glycolysis, was shown to promote the binding of the glycolytic enzymes to the cytoskeleton.[91] This is 1 way by which insulin affects glucose utilization. On the other hand, an increase in cytosolic-free calcium levels was shown to result in the dissociation between the enzymes and the cytoskeleton. This results in the decrease in the rate of glycolysis.[7,92] Extrapolating from these findings, one could assume that hormones that increase free-cytosolic calcium levels, such as glucagon, would cause the dissociation of the glycolytic enzymes from the cytoskeleton, thereby decreasing the rate of glycolysis.

While the association of glycolytic enzymes with the cytoskeleton in muscle and other cell types has been extensively studied, very few studies have been conducted on this topic using liver cells. It seems that in the liver, too, glycolytic enzymes are present partially in soluble and partially in bound forms. There is a report by Masters[88] in which he discusses this topic and states: "Our group first obtained evidence for an assembly of glycolytic enzymes in liver cytoplasm some years ago, but noted that this complexing occurs only in the presence of contractile components." The reference given refers to these data as unpublished results.[6] In another study by Foemmel et al,[93] immunologic localization techniques were used to probe the possible association of fructose 1,6-bisphosphatase aldolase with cellular structures. Using this in situ approach, they found that the enzyme is associated with the endoplasmic reticulum. However, examination of the data shows that one cannot distinguish between the possibility that the enzyme is associated with the endoplasmic reticulum or with cytoskeletal elements connected with the endoplasmic reticulum.

Another hepatic enzyme studied in this respect is pyruvate kinase (PK). Of the 4 known isoforms of this enzyme, 2 isoforms were shown to be present in the liver: PK-B4 is present in parenchymal cells, while PK-C4 is present in nonparenchymal cells.[94] In this study, not only the association of PK-B4 and PK-C4 with structural components was tested, but also their pattern of binding during development. In adult mouse livers, around 5% of PK-B4 is found in bound form. While it was not directly tested, PK was presumed to be attached to microfilaments.

It is worthwhile to mention that brain- and muscle-specific isoforms of PK were shown to influence microtubular structure. More precisely, PK was shown to destabilize microtubules. Based on this finding, it was suggested that in addition to its accepted function, PK also serves as a modulator of microtubule dynamics.[95] Whether the hepatic isoforms exhibit similar characteristics is not known.

Two additional studies were performed using nonmammalian organisms. In the liver of a teleost fish, the glycolytic enzymes were found to be partitioned between soluble and bound forms.[96]

In another study using the hepatopancreas of a snail, Otala lactea, the method of differential centrifugation combined with the use of drugs targeting the components of the cytoskeleton were used to pinpoint the exact sites of enzyme binding.[97] The findings were rather unexpected: in these cells, the different enzymes responded very differently to the added drugs. These results were interpreted as an indication that the enzymes of the glycolytic pathway might be bound to different structural components.

Based on the existing studies performed with the liver, which show an association between glycolytic enzymes and the cytoskeleton, our hypothesis presumes that an association between the hepatic enzymes and cytoskeletal elements is present in the liver. It further assumes that some of the characteristics of such association, well established in other cell types, such as muscle, apply also to this type of association in the liver. Thus, it is extrapolated from studies with muscle and other cell types that in the liver, too, an increase in cytosolic-free calcium would cause a detachment of the enzymes from the cytoskeleton. Because glucagon increases cytosolic-free calcium levels, this would result in the dissociation of the bound enzymes and, thereby, in lower rates of glycolysis. Glycolysis is inhibited by glucagon, an effect that is calcium-dependent.[98-101] The inhibition of glycolysis is likely to have a stimulatory effect on the rate of gluconeogenesis. This point is discussed in more detail in the Conclusions. While gluconeogenesis is used as the example, glucagon affects many metabolic processes in the liver; for instance, it stimulates lipolysis in a calcium-dependent manner.[102] Thus, it is likely that the dissociation of other enzymes from bound to unbound form influences the whole spectrum of glucagon actions on the liver. This argument is supported by reports that glucagon has a profound effect on the structure of the cytoskeleton. For instance, it was shown by Kojima et al[103] that following the administration of glucagon to cultured hepatocytes, an actin filament network, forming dome-like structures, is generated. These dome-like actin filament structures exhibited sensitivity to cytochalasins. It was previously mentioned that cAMP has a well-demonstrated effect in many cell types on the organization of the cytoskeleton, and the possible mechanisms by which glucagon or cAMP might affect these changes were discussed above.

Insulin promotes the association of the glycolytic enzymes with the cytoskeleton, an effect that contributes to the stimulation of glycolysis by insulin.[91] Insulin administration profoundly influences the structure of the cytoskeleton in most cell types. Polymerization of actin occurs within seconds to minutes

after insulin administration.[104] The stimulation of glycolysis by insulin is likely to contribute to the inhibitory effect of insulin in gluconeogenesis.

Summary of Association and Dissociation of Enzymes to the Cytoskeleton

The emphasis in this part was on glycolysis, because this pathway is intimately connected with the pathway of gluconeogenesis. However, the existence of glycogen particles containing all of the enzymes involved in glycogen metabolism was demonstrated a long time ago.[105] More recently, it was shown that lipid metabolism is also influenced by the cytoskeleton.[12] Thus, the structural organization of related enzymes by binding to the cytoskeleton seems to be widespread. The rate of metabolic processes are altered by the attachment and organization of enzymes. Our hypothesis proposes that the effect of glucagon on gluconeogenesis is influenced by the impact of glucagon on the structure of the cytoskeleton. Glucagon either directly, or indirectly by increasing calcium levels, is likely to cause the dissociation of glycolytic enzymes from the cytoskeleton. This would result in a decrease of the rate of glycolysis and in the increase of the rate of gluconeogenesis.

RECRUITMENT AND TRANSLOCATION OF ENZYMES

In addition to forming a web in which the different cytoskeletal components are interconnected,[28] the cytoskeleton also forms a network connecting the various intracellular organelles. This characteristic of the cytoskeleton enables it to "sense" and to coordinate cellular activities. I wish to compare the function of the cytoskeleton in individual cells to the nervous and endocrine systems in multicellular organisms. A few randomly selected examples are quoted to illustrate this point. Thus, in the liver beneath the plasma membrane, there is a cortical actin layer.[36] It is well demonstrated that cytoskeletal elements interact with the lipids in the plasma membrane.[106] This means that during signaling events, which involve proteins in or at the plasma membrane, it is likely that cytoskeletal elements would be modified. A good example for such interaction is provided by the activation of the phosphoinositide pathway.[107,108]

Inside the cell, the cytoskeleton has links to the endoplasmic reticulum.[109] Specific subdomains of the endoplasmic reticulum, the transitional ER, are involved in the generation of the Golgi cisternae. These subdomains redistribute rapidly when microtubules are depolymerized.[110] The Golgi complex connects not only with microtubules, but also with microfilaments.[111,112] Microtubules and microfilaments have been reported to form attachments to mitochondria and to lysosomes.[113-117] It was suggested that mitochondria and microtubules interact by binding microtubule-associated proteins to porin-containing domains of the mitochondrial outer membrane.[118] This might be relevant to the next point, the attachment of hexokinase to the mitochondria. Also, mitochondria relocate in the cytosol, changing position according to different metabolic states.

There is extensive literature on the structure of the cytoskeleton and the connection between the cytoskeleton and different intracellular organelles, which cannot be reviewed here. The few examples given must suffice to illustrate the interconnectedness of the cytoskeleton and intracellular organelles. This form of organization enables the intracellular movements of organelles according to needs, the reorganization of the intracellular environment to meet demands, and the movement of individual molecules. Three cases, relevant to gluconeogenesis, are briefly described: the intracellular movements of the glucose transporter, GLUT2, and the association and compartmentalization of 2 enzymes, hexokinase and glucokinase.

The rate and direction of glucose movement across the liver cell plasma membrane is a crucial determinant of carbohydrate metabolism. The mechanism of glucose release is facilitated in the liver by the glucose transporter, GLUT2, while the uptake of glucose into muscle or adipose tissue is facilitated by GLUT 4. Most of the studies on the characterization of glucose transporters were performed with GLUT4. Thus, a brief discussion of GLUT4 is necessary. Insulin, which stimulates the uptake of glucose, does so by recruiting GLUT4 from an intracellular site to the plasma membrane.[119] It was shown recently that the recruitment of GLUT4 almost fully accounts for the increase in the transport of glucose observed after the administration of insulin.[120] The ability of insulin to recruit GLUT4 from the intracellular pool to the plasma membrane depends on and is the result of the insulin-stimulated reorganization of the cytoskeleton.[121,122]

In the liver, GLUT2 is involved in the release of glucose, the last step in the pathway of gluconeogenesis. However, there are data, which indicate that glucose release can also occur by an alternative, membrane traffic-based pathway.[123] Nevertheless, in GLUT2-null livers, mobilization of glycogen seems to be seriously impaired. Indeed, GLUT2-null mice were shown to die within the first 3 weeks of being born, indicating that GLUT2 deficiency is a lethal defect.[124] The expression of GLUT2 is hormonally regulated; insulin, which decreases gluconeogenesis, suppresses GLUT2 expression.[125] Glucagon and thyroid hormone increase GLUT2 expression.[126-128] Because of the limited number of studies performed with the hepatic transporter, one can only extrapolate from studies with GLUT4 and assume that similar mechanisms exist in the liver. Recent studies with GLUT4 demonstrated the importance of the cytoskeleton for the recruitment of GLUT4.[129-131] Similarities between the liver and the transport of GLUT4 in adipocytes were pointed out in a study by Lange et al.[132] In their study, it was reported that insulin in the liver evokes major changes in the surface morphology of the liver by altering the structure of the microvilli. Similar effects of insulin were noted in adipocytes, in which GLUT4 was shown to be present in the microvilli. Thus, it might be that the recruitment of GLUT2 in situations associated with increased glucose eflux also involves the cytoskeleton. As pointed out above, both glucagon and insulin administration to the liver result in major changes in the structure of the cytoskeleton.

After the uptake of glucose into the liver, glucose phosphorylation becomes the rate-limiting step in its further metabolism. The enzymes involved in the phosphorylation belong to the hexokinase family of enzymes. Hexokinase IV, also called glucokinase, is specific to the liver. The liver contains mostly glucokinase and less than 10% of other kinds of hexokinases. These enzymes are present in the liver both in soluble and bound forms. Hexokinase is an actin binding protein,[133] which is activated by binding to the mitochondria.[134] The binding and

dissociation of hexokinase to and from the mitochondria plays an important role in the utilization of glucose.[135,136] Thus, in tumor cells, which have high rates of glycolysis, hexokinase has a high rate of binding to the mitochondria.[137] This could result in massive increases in enzyme activity; in 1 study, the increase was 8-fold.[138] Hexokinase was cloned and overexpressed in active form in *Escherichia coli*. The overexpressed enzyme readily bound to rat liver mitochondria.[139] Hexokinase binds to specialized domains to the outer membrane of the mitochondria, which also forms cross-bridges to cytoskeletal elements.[118] As pointed out above, connection and communication between the cytoskeleton and mitochondria is well documented. Insulin stimulates the binding of hexokinase to the mitochondria. Indeed, it was suggested that this binding is a crucial element in the mode of action of insulin.[135,136] By affecting the structure of the cytoskeleton in the liver, glucagon and insulin are likely to influence the degree of hexokinase binding to liver mitochondria. The binding of hexokinase results in a higher rate of enzyme activity.

The second example is the case of hexokinase IV, commonly called glucokinase. Glucokinase is specific to the liver and the endocrine pancreas. Glucokinase is the rate-limiting enzyme for glucose utilization[140]; in the human liver, it accounts for about 95% of the glucose phosphorylation.[141] At low glucose concentrations, glucokinase is found inside the nucleus, bound to a regulatory protein. At a high glucose concentration, the enzyme is recruited from the nucleus to the cytoplasm.[142,143] There are data suggestive of the possibility that in the cytoplasm, glucokinase is bound to microfilaments.[144] However, Adams et al[134] found no bound glucokinase in the cytosol. Thus, whether the cytosolic glucokinase is bound or free is unresolved. Glucagon and epidermal growth factor inhibit the expression of glucokinase, while insulin stimulates it.[145] The activity of glucokinase is a decisive factor in hepatic carbohydrate metabolism. The compartmentalization of this enzyme in the nucleus, its recruitment to the cytosol, and its possible binding to microfilaments indicate a possible role for the cytoskeleton in the regulation of the activity of this enzyme.

Summary of Recruitment and Translocation of Enzymes

Earlier in this report, 2 points were emphasized: (1) that the cytoskeleton connects with every intracellular organelle and (2) because of this, the cytoskeleton is involved in the recruitment and translocation of molecules. In the liver, the enzymes involved in the phosphorylation of glucose and the mechanism of glucose release by GLUT2, processes that are known to influence gluconeogenesis, were discussed.

UNKNOWNS AND DATA THAT CONTRADICT THE HYPOTHESIS

There are many unknowns in the presented hypothesis, and we are aware of experiments that raise questions about the validity of some of the details proposed. Starting with the unknowns, a major question is the mechanism by which the cytoskeleton is altered. Is it due directly to cAMP or to the activation of PKA? Or, is it due to changes following the activation of the CNGC? Also, while the CNGC channel was shown to be present in the liver in 2 independent studies, the actual activation of the channel was shown only in 1 (unpublished experiment by the author). Similarly, the calcium release channel protein present in the calcium storage site need to be identified and characterized. Clearly, an increase in cytosolic calcium levels also would exert an influence on the structure of cytoskeletal elements. It is suggested here that when the parenchymal cells are in the gluconeogenic mode, the glycolytic enzymes dissociate from the cytoskeleton. Very little work has been published on this topic in the liver. Thus, sorting out the sequence of events, the causes and effects, would be a challenging, but important endeavor.

We have suggested that the cytoskeleton couples the calcium store with the plasma membrane and mediates "capacitative calcium entry." Different views are held by others. Ribeiro et al[146] reported that at least in NIH 3T3 cells, the cytoskeleton is

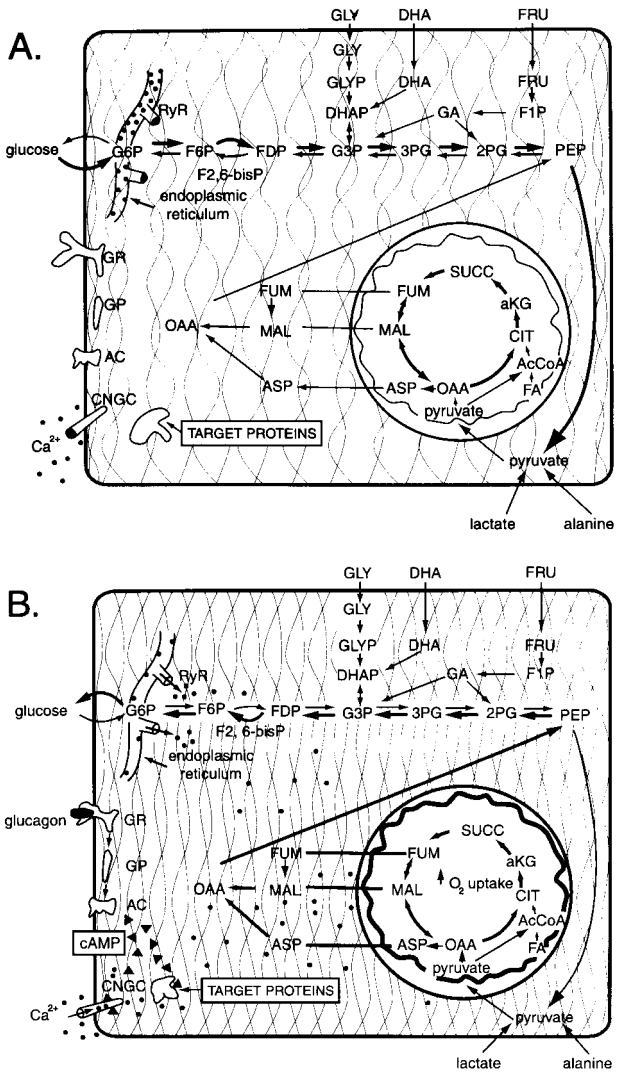

Fig 3. Schematic representation of the proposed hypothesis. In (A), the liver takes up glucose and metabolizes it. In (B), the liver is under the influence of glucagon. It produces glucose and releases it from the liver. Detailed explanations for each panel are described in the Conclusions. (Data from Kraus-Friedmann.[4])

required for agonist-stimulated calcium signaling, but not for capacitative calcium entry. Dealing with a different type of stimulation, nevertheless relevant to our hypothesis, is the suggestion by Patterson et al[147] that capacitative calcium entry operates as a secretion type coupling model, namely direct coupling between the ER and plasma membrane calcium channels.

Our hypothesis does not conform to the currently accepted views on the regulation of gluconeogenesis. According to the accepted view, gluconeogenesis is regulated by glucagon exclusively by the inhibition of futile cycling, which is accomplished by the generation of fructose 2,6-bisphosphate.[5] While there is no clear consensus on the degree of futile cycling, it is unlikely to account fully for the stimulation of gluconeogenesis.[22] Moreover, as reviewed in Kraus-Friedman[4] and Kraus-Friedman and Feng,[22] the initiation of a change in calcium distribution, for instance by cGMP, or hormones acting exclusively by elevating cytosolic-free calcium levels, such as vasopressin, is sufficient by itself to stimulate gluconeogenesis.

CONCLUSIONS

Based on existing data and extrapolating from them, we present a hypothesis, which attributes a major role to the cytoskeleton in the regulation of gluconeogenesis. The hypothesis is presented in a schematic fashion in Fig 3. In Fig 3A, the parenchymal cell is presented in the glucose utilization mode. The liver is in that mode after a carbohydrate-containing meal, and it is under the influence of insulin. The liver cell takes up glucose and phosphorylates, partially by hexokinase, which is attached to the mitochondria, and mostly by glucokinase, which is recruited from the nucleus to the cytoplasm, where it might be attached to the microfilaments. Subsequent steps in its metabolism are carried out by the enzymes of the glycolytic pathway, which at this mode, are attached to the cytoskeleton. The cytosolic calcium level is at the resting, low level. The cytoskeleton, represented by the background mesh, is in a state that reflects this mode. It is further assumed that in this mode, the rate of glucose production by the pathway of gluconeogenesis is very low.

In Fig 3B, the parenchymal cell is presented in the gluconeogenic mode, stimulated by glucagon. The liver is in this mode when available carbohydrate levels are either low because of diet, exercise, or fasting or are perceived as low because of defective responses, as in diabetes. Glucagon binds to the glucagon receptor, and this binding initiates a series of reactions resulting in an increase in the cAMP level. CAMP binds to target proteins. Thus, among others, cAMP binds to the CNGC present in the plasma membrane. This results in the opening of the channel and the influxes of calcium and monovalent cations, such as K^+. Simultaneously, the structure of the cytoskeletal web changes. This change is transmitted to the calcium channel present in the endoplasmic reticulum, where, as a consequence of these changes, the calcium-release channel opens and calcium is released. There are some older data, which indicated that mitochondria might also constitute a calcium storage site from which calcium is released.[148] In recent years, the role of mitochondria in the calcium-mediated signal transduction has been under re-evaluation.[149] Subsequent to these changes in ion fluxes, cytosolic-free calcium level increases. Calcium binds to target proteins. Calcium also enters the mitochondria. This results in an increased uptake of O_2 and in the stimulation of all the mitochondrial processes associated with the stimulation of gluconeogenesis.[150] As the level of calcium increases, the enzymes of the glycolytic pathway dissociate from the cytoskeleton, as does glucokinase. These changes contribute to the inhibition of glycolysis. GLUT2 is inserted into the plasma membrane. The parenchymal cell now is in the mode of producing and releasing glucose in sufficient amounts to prevent blood glucose from falling.

The experiments presented here and the data in the literature support a role for calcium in the regulation of gluconeogenesis. The suggested role for the cytoskeleton expands this view by proposing a mechanistic framework that might offer an explanation to some of the currently unexplained observations.

NOTE IN PROOF

Since the submission of the manuscript, Wu S, Sangerman J, Li M, et al reported in J Cell Biol 154(6):1225-1234, 2001 that in an endothelial cell type, the physical coupling between calcium release and calcium entry is dependent on the integrity of the spectrin component of the cytoskeleton.

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