Rabbit erythrocytes of progressively increasing age were isolated using an avidin-biotin affinity technique and the activity of protein kinases and other enzymes was analyzed in cytosols and membranes from the isolated cells. The activities of cytosolic protein kinase C (PKC), cAMP-dependent kinase (PKA), and casein kinase type I and II (CKI and II) were all found to undergo an age-dependent decrease of twofold to fourfold over the 8-week lifespan of the cells. Membrane-associated tyrosine kinase showed little or no decrease, but membrane-associated CKI showed a dramatic eightfold decrease over the 8-week period. By contrast, various cytosolic enzymes, including lactate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, and acid phosphatase, showed no change in activity over the same time period. Density-separated human erythrocytes showed qualitatively similar decreases in cytosolic protein kinase activities in the densest fractions, which contain the oldest cells. Our results show that aging erythrocytes undergo progressive loss of protein kinases that may adversely affect various cellular processes. The age-dependent loss of kinase activity reported here is one of the most striking manifestations of erythrocyte senescence yet to be reported.

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Isolation of senescent rabbit erythrocytes. Rabbit erythrocytes were biotinylated in vivo essentially as described by Dale et al.\textsuperscript{19,22} Briefly, rabbits were made anemic by subcutaneous injection with phenylhydrazine on days $-13,-12,$ and $-11$ of the experiment and erythrocytes were labeled with biotin in vivo essentially as described by Dale and Daniels.\textsuperscript{23} Blood was withdrawn 3 hours after the injection (week 0 time point) and once every 7 days for 8 weeks. The volume withdrawn was increased from 4 mL on day 0 to 15 mL on day 56. The blood was washed twice with phosphate-buffered saline (PBS), and the buffy coat was carefully removed. The cells were resuspended to a hematocrit of approximately 10% in PBS and passed through a matrix of a-cellulose and Sepacel type 50 (4/1 ratio, w/v) to remove leukocytes. Biotinylated erythrocytes were isolated from such suspensions essentially as described,\textsuperscript{24} with minor modifications. Approximately 70 to 90 mL of packed erythrocytes were recovered at each time point. The recovered erythrocytes were lysed in 20 vol of ice-cold 25 mMol/L Tris-HCl, pH 7.4, 5 mMol/L dithiothreitol (DTT), 2 mMol/L EGTA, 2 mMol/L EDTA, 20 μg/mL phenylmethyl sulfonyl fluoride (PMSF), 2 μg/mL leupeptin, and 2 μg/mL pepstatin A (lysis buffer). The lysate was centrifuged in a DuPont-Sorvall SS34 rotor (DuPont, Wilmington, DE) at 20,000 rpm for 1 hour. After 5 washes with 0.1% Tween-20 in PBS, followed by two washes with 1 mL of 5 mMol/L Na<sub>2</sub>HPO<sub>4</sub> (pH 8.0), 0.5 mMol/L EGTA, 20 μg/mL PMSF, and 2 μg/mL leupeptin and immediately frozen at $-70^\circ$C.

Kinase assays. PKC assay of erythrocyte cytosol was performed according to Ai and Cohen,\textsuperscript{25} with minor modifications. PKA assay of erythrocyte cytosol was performed according to Danilov et al.\textsuperscript{26} PKA activity is reported as the difference in kinase activity between the samples with and without cAMP. Total cytosolic casein kinase (CK) activity was assayed by incubating 100 μg of protein from erythrocyte cytosol in a 50 μL reaction mixture containing 45 mMol/L Tris-HCl, pH 7.5, 5.6 mMol/L MgCl<sub>2</sub>, 150 mMol/L KCl, and 200 μmol/L [γ-<sup>32</sup>P]-ATP (200 μCl/mL) with or without (as background) 2 mMol/L casein at 37°C for 10 minutes. The reaction was terminated by adding 0.5 mL ice-cold 10% trichloroacetic acid (TCA) and the samples were left on ice for 10 minutes followed by filtration through a 2.4-cm diameter Whatman GF/C filter. The filter was washed with 10 mL 10% TCA and air-dried. The 32P content of the filter was determined by liquid scintillation counting. Heparin inhibitable CK activity, which reflects only CKII activity,\textsuperscript{27,28} was measured in the presence of 2 μg/mL heparin and was expressed as the difference of the 32P incorporation in the absence and presence of heparin. CKI-7 inhibitable activity, which reflects only CKI activity,\textsuperscript{27} was measured as described above as the difference between total activity and activity in the presence of the specific inhibitor CKI-7 present at a final concentration of 100 μmol/L. All cytosolic kinase assays were performed in triplicate at each time point and the means ± SD are shown.

Erythrocyte membrane-associated tyrosine kinase activity was assayed according to Schieven et al.\textsuperscript{15} using Tyr<sup>489</sup>Glu<sup>50</sup> as a substrate. Erythrocyte membrane-associated CKI activity was measured in membranes as described by Manno et al.\textsuperscript{16} with minor modifications. Briefly, 15 μg of ghost membranes was incubated at 0°C for 10 minutes with 20 mMol/L Tris-HCl (pH 7.5), 0.5 mMol/L MgATP containing [γ-<sup>32</sup>P]-ATP (6 to $8 \times 10^6$ cpn) in a final volume of 100 μL. The reaction was terminated by adding electrophoresis sample buffer. Radiolabeled proteins were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide 9% according to Laemmli.\textsuperscript{17} The gels were stained for proteins with Coomassie brilliant blue and were dried between two sheets of dialysis membrane. Phosphorylated membrane proteins were identified by autoradiography. The radioactivity of specific proteins was determined by excising corresponding bands from dried gels and counting in a liquid scintillation counter.

Assay of other erythrocyte cytosolic enzymes. Acid phosphatase activity was measured by incubating 300 μg of cytosolic protein in 50 mMol/L Tris-HCl (pH 5.2), 20 mMol/L MgCl<sub>2</sub> with 20 mMol/L p-nitrophenylphosphate at 37°C. Phosphatase activity, measured as the release of p-nitrophenol from p-nitrophenylphosphate, was monitored spectrophotometrically at 410 nm.\textsuperscript{29} Samples without cytosol were used as controls whose activities were subtracted from those of the experimental samples. Activities of pyruvate kinase, phosphoglycerate kinase, and lactate dehydrogenase were measured as described in Stefler.\textsuperscript{30}

Separation of human erythrocytes on Stractan density gradients. Blood was collected in lithium heparin vacutainer tubes from healthy normal individuals. The red blood cells were washed three times in PBS and filtered through cellulose as described above to remove white blood cells. Packed washed erythrocytes were resuspended to 50% hematocrit in PBS and 1.5 mL was layered on the top of a gradient of Stractan prepared as described by Corash.\textsuperscript{31} Briefly, 1.5 mL aliquots of isoosmotic Stractan solutions of densities 1.08, 1.085, 1.091, 1.099, 1.106, and 1.113 were layered on a cushion of 1.120 g/mL Stractan in 13-mL ultra-clear Beckman centrifuge tubes. The samples were centrifuged in a Beckman L5-75 ultracentrifuge (Beckman Instruments, Fullerton, CA) in an SW 41 Ti rotor for 30 minutes at 30,000 rpm at 4°C. Individual fractions at the gradient interfaces were harvested using a Pasteur pipette and washed three times with PBS. Cells from individual fractions were lysed in 20 mL of ice-cold lysis buffer (5 mMol/L sodium phosphate, pH 8.0, containing 20 μg/mL PMSF and 2 μg/mL leupeptin). The lysates were centrifuged at 18,000g for 15 minutes, and the supernatants were removed and stored in small aliquots at $-70^\circ$C before analysis of kinase activities.

Immunoblot analysis using antiphosphoserine antibodies. After electrophoresis, proteins were transferred onto a nitrocellulose membrane overnight in a Bio-Rad apparatus (Bio-Rad, Richmond, CA). The membranes were incubated with 5% bovine serum albumin (BSA) in PBS at room temperature for 1 to 2 hours, followed by washing at least for 5 times with 0.1% Tween-20 in PBS. Blots were then incubated with monoclonal antiphosphoserine antibodies diluted 1:5,000 in PBS containing 5% BSA at room temperature for 1 hour. After 5 washes with 0.1% Tween-20 in PBS, followed by incubation with goat antimouse IgG conjugated with peroxidase (1:10,000 dilution in PBS) at room temperature for 45 to 60 minutes, the blots were finally washed four times in 0.1% Tween-20 in PBS. The blots were developed by using the substrate solution of a chemiluminescence reagent kit (DuPont, NEN, Boston, MA) followed by exposure to hyperfilm-MP (Amersham, Arlington Heights, IL).

Other methods. Protein concentrations were determined by the method of Bradford.\textsuperscript{32} Reticulocyte counts were performed after staining of cells with new methylene blue by standard procedures. Ten fields of 100 cells each were counted and averaged at each time point shown.

RESULTS

Rabbit erythrocytes were covalently biotinylated by intra-venous injection of n-hydroxysuccinimidio-biotin in vivo on day 0 and isolated on avidin-coated petri dishes over an 8-week period. Because the rabbits had been made anemic in the prior week, the majority of their circulating erythrocytes on day 0 were expected to be young cells. Thus, the biotin is expected to label an age cohort of cells that could then
KINASE ACTIVITIES IN SENESCENT ERYTHROCYTES

Fig 1. Cytosolic kinase activities in age-defined rabbit erythrocytes. Rabbit erythrocytes were isolated at the indicated times after biotinylation, and equivalent amounts of cytosolic protein were assayed for kinase activities as described in the Materials and Methods. Each set of data is from a single rabbit, and each point represents the mean of triplicate measurements of the sample. Standard deviations are shown except when they are smaller than the data points. In all cases, (0) represent kinase activities in the cytosol of erythrocytes taken at week 8 but not subjected to avidin affinity selection and therefore represent a control value for unfractionated circulating erythrocytes of mixed age. (A) Cytosolic PKC and reticulocyte count (L). (B) Cytosolic PKA. (C) Cytosolic CKI. (D) Cytosolic CKII.

be separated from cells produced subsequent to day 0 (and would therefore not be biotinylated) by affinity selection on avidin-coated petri dishes.

Figure 1A through D shows the results of protein kinase assays on cytosols from rabbit erythrocytes taken at the indicated weeks after in vivo biotinylation and isolated on avidin-coated petri dishes. The figures show that each of the kinases tested decreased dramatically over the course of the 8-week life expectancy of the rabbit erythrocytes. The data shown in Fig 1 are from a single rabbit in each case. The complete experiment (in vivo biotinylation, red blood cell isolation, and kinase assay) was performed four times, using two rabbits each time, for a total of eight rabbits. In each case results similar or identical to those shown in Fig 1 were obtained. Table 1 shows a summary of the data, indicating the reduction in kinase activity at week 8 relative either to biotinylated cells taken on day 0 (the day of biotinylation) or to unfractionated cells (not subjected to avidin selection) taken on week 8 that represent a population of heterogeneous age.

In all cases, the kinase activities of biotinylated cells were higher on day 0 than at any subsequent time point, including the unfractionated week 8 sample. This could be due to genuinely higher enzyme activities in the youngest erythrocytes or to the presence of residual reticulocytes, both of which would likely be present in higher than normal amounts on day 0 due to the phenylhydrazine treatment. We measured the reticulocyte content of avidin-selected cells taken from a single rabbit and used in the experiments shown in Figs 1A and C and 4A. The results are plotted in Fig 1A and show that, although there was a decrease in the reticulocyte content between day 0 and the end of week 1, the reticulocyte count changed little between weeks 1 and 8. Thus, although a decrease in reticulocytes might account for the decrease in kinase activities between day 0 and the end of week 1, it cannot account for the significant decrease in kinase activities beyond week 1.

By the end of week 1 or 2, the kinase activities of the age-selected biotinylated cells were generally equivalent to the activities of unfractionated cells taken on week 8. This unfractionated week 8 sample reflects the enzymatic activities in a population of circulating erythrocytes. The cytosolic kinases lost the majority of their activities between weeks 0 and 4, but this trend was most striking with PKA and the casein kinases, in which no further reductions were seen beyond week 4. It should be noted that the kinase activity in unfractionated cells is a function of the percentage of cells of a given age present in the circulation at any time weighted by the kinase activity of those cells (strictly speaking, it is the integral over cell age of the number of cells of a given age times the kinase activity per cell at each age). Thus, the activity of a given kinase in unfractionated cells will be dependent on the shape of the kinase versus age curve.

To determine whether human erythrocytes have a similar loss of kinase activities with age, we separated human cells on a Stractan density gradient. This technique is less selective than the biotinylation approach, but it is generally held that the most dense erythrocytes isolated on Stractan gradients are enriched in the oldest cells, although the exact degree of enrichment is controversial. Figure 2A, B, and
Table 1. Reduction in Cytosolic Protein Kinase Activities and Membrane-Associated Kinase Activities of Senescent Erythrocytes

<table>
<thead>
<tr>
<th>Cytosolic protein kinase activities</th>
<th>Rabbit Erythrocytes</th>
<th>Human Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Fold Reduction by Week 8 Relative to Day 0 Cells</td>
<td>Average Fold Reduction by Week 8 Relative to Unfractionated Cells</td>
<td>Average Fold Reduction in Most Dense Erythrocytes Relative to Unfractionated Erythrocytes</td>
</tr>
<tr>
<td>PKC</td>
<td>2.4 (1.8-4.2)</td>
<td>2.6 (2.0-4.3)</td>
</tr>
<tr>
<td>PKA</td>
<td>2.4 (1.6-4.5)</td>
<td>1.8 (1.5-2.1)</td>
</tr>
<tr>
<td>CKI (CKI-7-inhibitable)</td>
<td>4.2 (3.5-5.0)</td>
<td>1.7 (1.5-1.9)</td>
</tr>
<tr>
<td>CKII (heparin-inhibitable)</td>
<td>4.5 (3.7-6.2)</td>
<td>2.1 (1.61-3.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Membrane-associated kinase activities</th>
<th>Rabbit Erythrocytes</th>
<th>Human Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine kinase</td>
<td>1.7 (1.4-1.8)</td>
<td>1.2 (0-1.4)</td>
</tr>
<tr>
<td>CKI</td>
<td>6.6 (7.0-10.2)</td>
<td>2.45 (2.4-2.5)</td>
</tr>
</tbody>
</table>

Rabbit erythrocytes (columns 1 and 2): Biotinylated rabbit erythrocytes were isolated according to age and enzyme activities assayed as described in the Materials and Methods. The values shown are the means of between 2 and 6 independent determinations on erythrocyte samples taken from a total of eight rabbits. The range of the values is shown in parentheses. "Week 8 unfractionated cells" refers to cytosol from erythrocytes taken at week 8 (the last blood withdrawal) and not subjected to affinity adsorption on avidin plates. Human erythrocytes (column 3): Human erythrocytes were fractionated according to density on a Stractan gradient and cytosolic protein kinase activities were assayed as described in the Materials and Methods. The values shown are the means of between 2 and 4 independent determinations performed on separate blood samples. The range of the values is shown in parentheses.

Abbreviation: ND, not determined.

Figure 2. Cytosolic protein kinase activities in density fractionated human erythrocytes. Human erythrocytes were separated by density on discontinuous Stractan gradients as described in the Materials and Methods. Equivalent amounts of cytosolic protein from each of the 6 fractions were assayed for the indicated enzyme activities as described in the Materials and Methods. The symbol (○) above fraction 6 represent in each case the corresponding kinase activities in the cytosol of unfractionated erythrocytes. The values shown are the means of triplicate determinations on a single sample of blood. Standard deviations are shown except when they are smaller than the data points. The same experiment was repeated two other times with different blood samples and gave qualitatively similar results. (A) Cytosolic PKC. (B) Cytosolic PKA. (C) Cytosolic CKII.

Figure 3 shows that cytosolic kinase activities decreased as erythrocyte density increased for the three kinases tested. In each case, the highest kinase activities were found in the least dense fraction (fraction 1) and the lowest activities in the most dense fraction (fraction 6), which contained 5% of the cells in the gradient. Table 1 presents a tabulation of the reduction in kinase activities in fraction 6 (most dense) relative to unfractionated erythrocytes and shows that PKA had the smallest decrease in activity, whereas PKC had the largest.

To determine whether other erythrocyte enzymes showed the same age-dependent decrease in activity as protein kinases, we analyzed the activities of cytosolic lactate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, and acid phosphatase in rabbit erythrocyte cytosols. Figure 3 shows that the activities of lactate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, and acid phosphatase remained constant throughout the lifespan of the cells when compared either with day 0 cells or to unfractionated cells.

We also measured two membrane-associated protein ki-
Fig 3. Activities of cytosolic enzymes in age-defined rabbit erythrocytes. Rabbit erythrocytes were isolated at the indicated times after biotinylation and the activities of the indicated enzymes were assayed as described in the Materials and Methods. In all cases, (A) and (V) refer to erythrocytes isolated at the indicated times and fractionated on avidin plates. Data are shown for two separate rabbits, rabbit 1 (A, V) and rabbit 2 (V, V) at the week 8.5 position are the activities of erythrocytes taken on week 8 not subjected to avidin selection and therefore represent a control value for circulating erythrocytes of mixed age. Each point represents the mean of duplicate or triplicate determinations and the standard deviations (triplicate measurements) or ranges (duplicate measurements) are shown except when they are smaller than the data points.

Fig 4. Membrane-associated protein kinase activities in age-defined rabbit erythrocytes. Rabbit erythrocytes were isolated at the indicated times after biotinylation and membranes were prepared as described in the Materials and Methods. Equivalent amounts of membrane protein were assayed for kinase activities at each time point as described in the Materials and Methods. Each set of data is from a single rabbit. In all cases, the open symbols at week 8 represent kinase activities of membranes from erythrocytes taken at week 8 but not subjected to avidin affinity selection and therefore represent a control value for unfractionated circulating erythrocytes of mixed age. (A) Membrane-associated casein kinase I. Data are shown for two rabbits (rabbit 1 [A, V] and rabbit 2 (V, V)). Each data point is from a single determination. (B) Membrane-associated tyrosine kinase activity. Data are shown for a single rabbit. Data from the second rabbit analyzed for membrane-associated tyrosine kinase activity was similar to that for the rabbit shown (Table 1). Each point represents the mean of duplicate determinations and the ranges are shown except when they are smaller than the data points.
nase activities, tyrosine kinase and casein kinase I. Figure 4 shows that, whereas tyrosine kinase activity decreased only 1.7-fold between day 0 and week 8, casein kinase 1 activity declined an average of 8.6-fold during the same time period (Table 1). This was the largest reduction seen in any of the enzyme activities measured. Moreover, the kinetics of membrane-associated CKI disappearance was quite distinct from that of the cytosolic kinases, showing a precipitous decrease after week 4, a time by which the cytosolic kinases had already lost most or all of their activity.

Membrane-associated CKI is one of the major kinases responsible for phosphorylating membrane proteins in the human erythrocyte.15,16 We therefore asked whether the dramatic age-dependent reduction in the activity of this enzyme would be reflected in the level of phosphorylation of rabbit erythrocyte membrane proteins. Figure 5 shows the results of a Western blot of membranes from age-defined rabbit erythrocytes after electrophoresis and reaction with anti-phosphoserine antibodies. Figure 5 shows that the level of endogenous phosphoserine of three major phosphorylated proteins, spectrin, band 3, and band 4.1, remains relatively constant over the lifespan of the erythrocytes.

Discussion

Our results provide evidence for an age-dependent loss of specific cytosolic and membrane-associated protein kinase activities in ageing rabbit erythrocytes. The loss of these kinase activities contrasted markedly with the stability of the intracellular enzymes lactate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, and acid phosphatase. This indicates that the decrease in kinase activities was not a general phenomenon common to all erythrocyte intracellular enzymes.

Biotinylated rabbit erythrocytes have been used to study the age-dependence of a variety of enzymes. Suzuki and Dale18 showed that 9 enzymes of the glycolytic pathway as well as intracellular ATP, 2,3-diphosphoglycerate, and glutathione had no significant age-dependent decrease during the lifespan of rabbit erythrocytes. In a related study, Zimran et al14 showed a biphasic decrease in several enzymes. Generally, enzyme activity decreased rapidly during the first 7 days followed by a much slower and, in most cases, insignificant decrease thereafter. These results could be interpreted to suggest that there is an initial rapid loss of the activity of various enzymes during the maturation of the reticulocyte or young erythrocyte, followed by essentially stable activity thereafter. In only one reported case, that of adenosine 5'-monophosphate deaminase, has there been shown to be a lower enzymatic activity in biotin/avidin selected senescent cells than in unfractionated cells.11

Although Fig 2 suggests that senescent human erythrocytes may also experience age-dependent losses of cytosolic protein kinases, the results must be interpreted with caution. It has been repeatedly noted that even the most dense fractions of human erythrocytes consist only partly of genuinely senescent cells. Moreover, it is likely that a large proportion of density-dependent differences in human erythrocyte enzymatic activities is due to the partitioning of reticulocytes, which are found in the lightest fractions and tend to have high metabolic and other enzymatic activities (see Clark1 and Zimran et al13). Thus, although the data of Fig 2 are consistent with an age-dependent loss from human erythrocytes of the kinases shown, they do not prove it.

Although the mechanism leading to the specific loss of protein kinases reported here is unknown, we previously put forth a hypothesis that could explain the age-dependent decrease in PKC activity.23 We showed that activation of PKC in human erythrocytes by phorbol 12-myristate 13-acetate (PMA) results in the translocation of the enzyme to the plasma membrane followed by its proteolytic cleavage. From these results we speculated that, if erythrocyte PKC were repeatedly activated in the circulation, the fraction of PKC that translocated to the membrane during each activation event would be proteolyzed and lost. Thus, repeated activation of PKC would be predicted to lead to a progressive and irreversible decrease in cytosolic PKC activity, because erythrocytes have no capacity to synthesize new enzyme.

Although PMA is a potent activator of erythrocyte PKC, it is unknown whether there are physiologic circumstances that might result in the activation of this enzyme in the erythrocyte. One possible pathway for erythrocyte PKC activation involves transient influxes of Ca2+, possibly induced by shear stress or other circumstances. Raval and Allan37 showed that elevation of intracellular Ca2+ in human erythrocytes by Ca2+ ionophore leads to activation of PKC, and others38,39 showed that physiologic shear forces experienced by the erythrocyte in the circulation can induce transient elevations in erythrocyte cytosolic Ca2+. Moreover, sicker erythrocytes, which may experience greater than normal Ca2+ leakage due to the repeated membrane stress of sickling,40-42 were shown to have decreased cytosolic PKC content,41 possibly due to accelerated depletion of PKC resulting from hyper-activation. This information suggests that shear stress or membrane deformation, resulting in Ca2+ influx, could lead to progressive depletion of erythrocyte PKC and could account for the age-related decrease in activity seen here.

Whereas shear-stress induced Ca2+ influx may account for the age-dependent loss of PKC, it is unclear what could account for the losses of both PKA and CKI and II. Treatment of erythrocytes in vitro with PMA for up to 24 hours, which we have previously shown to completely eliminate human erythrocyte PKC activity,25 had no effect on the activities of erythrocyte PKA or CK (Z. Ai and C.M. Cohen, unpublished data). Thus, depletion of PKC is not directly responsible for the reduction in the activities of the other kinases over short times. However, it is possible that, over longer time periods in vivo, a deficit in PKC might affect the activities of these kinases. For example, a direct involvement of PKC with casein kinase is suggested by studies that show that PKC phosphorylates the β subunit of CKII (heparin-inhibitable CK), leading to enhanced enzyme activity.27 Other studies have suggested an involvement of PKC with PKA via phosphorylation of adenylly cyclase.28,29 Determination of whether these effects occur in the erythrocyte or are involved in the age-dependent decrease of the enzymes seen here must await further studies.

Are the alterations in kinase activities reported here simply...
kinase activities in senescent erythrocytes

Fig 5. Phosphoserine Western blot analysis of rabbit erythrocyte membranes. Rabbit erythrocytes were isolated at the indicated times after biotinylation and membranes were prepared as described in the Materials and Methods. Equivalent amounts of membrane protein were electrophoresed, transferred to nitrocellulose, and analyzed by Western blotting for phosphoserine as described in the Materials and Methods. (A) Western blot using antiphosphoserine antibodies. The lane marked U contains membranes from erythrocytes taken at week 8 but not subjected to avidin affinity selection, which therefore represent a control value for circulating erythrocytes of mixed age. (B) Results of scanning densitometry of Western blot in (A). The integrated band intensities for spectrin (○, ■), band 3 (□, □), and band 4.1 (▲, ▲) proteins are shown. In each case, the open symbols represent the values from the lane marked U in (A) containing erythrocytes of mixed age taken at week 8. Similar results were obtained from an identical experiment performed on one other rabbit.

manifestations of the aging process or are they involved in the mechanism of senescent cell destruction? Rabbit erythrocytes survive an average of 57 days in the circulation. It has been shown that approximately 28% of cells (0.5% per day) are lost by so-called random destruction, with the remainder expected to survive for an average of 57 days. If the decrease in kinase activities shown here is causally related to cell destruction, it might be expected that the kinase activities would show the same kinetics as that for cell destruction. This is clearly not the case with PKA or CKII, which decrease rapidly up to 4 weeks and remain stable thereafter. Although the activity of PKC continued to decrease throughout the lifespan of the cells remaining in the circulation, it is clear that the bulk of the decrease occurred during the first 4 weeks. The only kinase that showed a time-dependent decrease at all reminiscent of erythrocyte survival kinetics was membrane-associated CKI.

Membrane-associated CKI is likely responsible for most of the steady-state phosphorylation of erythrocyte membrane proteins in vivo. Phosphorylation by CKI has been shown to affect the associations of membrane proteins in vitro as well as the mechanical properties of erythrocyte membranes. Phosphorylation of band 4.1 by partially purified membrane-associated CK (largely CKI) reduces its affinity for spectrin.
in vitro, and phosphorylation of ankyrin reduces its binding to both spectrin and band 3. Moreover, phosphorylation of spectrin by membrane-associated CKI has been shown to correlate with decreased membrane stability as measured by the ektacytometer. Thus, it is possible that the level of membrane-associated CK activity has some effect on cellular mechanical properties in vivo. There have been no direct measurements of the mechanical stability of senescent rabbit erythrocytes. Although it has been shown that such membranes have normal elasticity, this is a different biophysical parameter that may reflect different membrane molecular interactions.

Despite the possible role that membrane-associated CKI may play in controlling membrane mechanical properties, our phosphoserine Western blot showed that there was no consistent alteration in the level of phosphoserine of three major rabbit erythrocyte membrane proteins with cell age. This is an unexpected observation given the dramatic age-dependent decrease in membrane associated CKI as well as the other kinases. Possible explanations for this finding include a coordinate reduction in membrane-active phosphatase activity (not measured here) or the possibility that only a small amount of kinase activity is required to maintain optimal steady-state levels of phosphorylation. Whatever the explanation, the results suggest that phosphorylation-mediated changes in the major membrane proteins are unlikely to causally contribute to the senescence process.

Finally, it is possible that decreasing protein kinase activity is detrimental to erythrocyte components other than the membrane skeleton. For example, PKC has been reported to regulate the activity of the erythrocyte Ca2+-ATPase and the Na+-H+ exchange pathway, and it is certain that PKA, CKI, and CKII phosphorylate a variety of other substrates in the erythrocyte. The determination of the precise role, if any, for protein kinases in the senescence process will require additional studies. However, decreasing kinase activities are, at this point, one of the most striking alterations observed in senescent erythrocytes.

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KINASE ACTIVITIES IN SENESCENT ERYTHROCYTES


Specific loss of protein kinase activities in senescent erythrocytes

HK Jindal, Z Ai, P Gascard, C Horton and CM Cohen

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