Published in final edited form as:
Adv Enzymol Relat Areas Mol Biol. 2011 ; 78: 385–414.

TRANSGLUTAMINASE-MEDIATED REMODELING OF THE HUMAN ERYTHROCYTE MEMBRANE SKELETON: RELEVANCE FOR ERYTHROCYTE DISEASES WITH SHORTENED CELL LIFESPAN

Laszlo Lorand, **S. N. Prasanna Murthy**, **Anwar A. Khan**, **Weihua Xue**, **Oksana Lockridge**, and **Athar H. Chishti**

I. INTRODUCTION

The human red blood cell transglutaminase (hRBC TG2) was the first in this family of enzymes for which an important role in cell–matrix interaction was found by demonstrating that the protein—when released from cells—could form an extremely tight complex with human fibronectin (FN). The binding, with a stoichiometry of 2TG2:FN (i.e., 1TG2 per constituent chain of FN), is independent of the catalytic activity of TG2 and occurs in the absence as well as in the presence of Ca^{2+} ions [1–3]. Residues 81–106 of TG2, located at the extended hairpin between antiparallel β strands 5 and 6 of the first domain of the protein, seem to be essential for binding to FN; mutations of Asp94 and Asp97 to Ala reduce the binding affinity of TG2 to FN significantly. A synthetic peptide, corresponding to the sequence 88WTATVVDQQDCTLSLQLTT106 in TG2, inhibits the TG2–FN interaction, and also TG2-dependent cell adhesion and spreading [4]. The complementary binding sites of FN are located in a 42-kDa collagen-binding domain of the protein, comprising motifs I_6-II_1-II_2-I_7-I_8-I_9. This fragment shows as high an affinity for TG2 as the individual parent FN chains themselves [5]; furthermore, the 42-kDa fragment of FN can neutralize the functions of TG2 on cell surfaces [6]. Binding to TG2 is so specific that an affinity column made by coupling the 42-kDa fragment of FN to a gel matrix can be used for isolating hRBC TG2 to the highest purity with a single passage of hemoglobin-depleted erythrocyte lysate [5] (Figure 1A). This procedure was employed for purifying the TG2 protein on which nucleotide-binding studies were carried out [7], and on which the large conformational change—attendant to binding GTP—could be demonstrated by transition from a slow-moving, extended structure to a faster moving, compact configuration in nondenaturing electrophoresis [8] (Figure 1B).

TG2s of different species vary in sensitivities to inhibition by GTP, but hRBC TG2 binds tightly to the nucleotide (measured by a fluorescently labeled analog), with an association constant of $4 \times 10^7\ M^{-1}$ [7]. Even in the highly purified form, this TG2 seems to exist preponderantly in the closed compact, inactive configuration of the enzyme, corresponding to the electrophoretically fast-moving GDP-bound form (Figure 1B).

It is perhaps more relevant to the present discussion that human red cells provided the paradigm for showing that TG2—though inactive in the intracellular milieu—becomes rapidly converted by entry of Ca^{2+} to an active transamidase, producing profound alterations in the structural organization and physical properties of the cell [10–14]. It is remarkable that the changes brought about by treating normal hRBCs with Ca^{2+} plus ionophore closely parallel those seen in some erythrocyte diseases in which the lifespans of the cells are appreciably shortened. Therefore, the sequence of events in the hRBC diseases, and also in the experimental model with Ca^{2+} overload, may be illustrated by Scheme 1.

In the resting cell, TG2 is kept in the inactive, latent form by virtue of its tight binding to GTP. However, the entry of Ca^{2+} ions removes the inhibition by GTP and allows expression of transamidating activity. The enzyme catalyzes the cross-linking of protein substrates (P_1, P_2, P_3, …, P_n) by covalent γ:ε isopeptide bridges and the concomitant formation of large-molecular-weight polymeric structures in the cell membrane. This activity causes a stiffening of the membrane and irreversible fixation of cell shape [15], which are thought to promote the premature removal of the affected hRBCs from the circulation. Moreover, competitive and noncompetitive inhibitors of TG2 prevent the protein cross-linking by the enzyme (i.e., the formation of abnormal protein polymers) and also block the physical consequences of membrane stiffening and fixation of cell shape.

It is important to bear in mind that the polymeric structures created by TG2 action are not conventional molecular aggregates; they cannot be separated into their original building blocks by protein-solubilizing agents (such as urea, weak acids and alkalis, ionic or nonionic detergents, including sodium dodecylsulfate, SDS) or their combinations. The portions of the polymers soluble in a mixture of SDS and a reducing agent, such as dithiothreitol (DTT), contain a fraction of the N^{ε} (γ-glutaminyl)lysine-bonded constituent chain framework—essentially the backbone of a branched structure with ill-defined geometry—onto which other polypeptides may be attached in the cell, for example, by disulfide interchain linkages.

II. Hb KOLN DISEASE, A PROTOTYPE OF ERYTHROCYTE DISORDERS CHARACTERIZED BY THE PRESENCE OF γ:ε-BONDED, ABNORMAL MEMBRANE SKELETAL POLYMERS

The primary defect in Hb Koln disease is a Val98Met mutation in the β subunits of hemoglobin near the heme pocket. This creates molecular instability, placing Hb Koln into the family of “unstable hemoglobin diseases,” characterized by hemolytic anemia. The trait, unlike sickle cell disease, does not seem to be inherited; each case is thought to arise from fresh (parental gonadal?) mutation. Extensive metabolic, deformability, and survival studies have been carried out on Hb Koln erythrocytes [16, 17]. Among the many biochemical, morphological, and functional changes that have been reported, including a marked loss of cellular potassium, intracellular dehydration, low ATP concentration (around 66% of normal), decreased osmotic fragility, and decreased cell deformability are noteworthy. Because of the large reduction of intracellular ATP, it may be assumed that the TG2-inhibitory concentration of GTP would also be appreciably lower than normal; hence, expression of transamidating activity in the Hb Koln cells would be expected to occur with smaller augmentation of internal concentration of Ca^{2+} ions than in normal red cells. Membrane rigidity probably contributes to the enhanced splenic entrapment of Hb Koln erythrocytes and accounts for their drastically reduced lifespan in the circulation, which is only about one-fourth of normal. There is improvement of red cell lifespan after splenectomy, from about 31 days to 47 days.

Because membrane rigidity appears to be responsible for the premature death of erythrocytes in Hb Koln disease, our research focused on the biochemistry of the membrane compartment of this abnormal cell [18]. Erythrocyte ghosts, isolated from a patient with Hb Koln anemia, were uniformly of higher density than normal (1.18 g/mL versus 1.16 g/mL; Figure 2A). In another case, the density distribution of erythrocyte ghosts showed a dual profile, with a main peak at near the normal density of 1.15 g/mL and a smaller peak at 1.21 g/mL.

Since the metabolically impaired Hb Koln cells probably allowed entry of Ca^{2+} ions, which activated the resident TG2, we undertook a search for high-molecular-weight polymeric

membrane skeletal products covalently bonded by N^{ε} (γ-glutaminyl)lysine side chain bridges, the "footprints" of TG2 activity. Three polymeric fractions were obtained from the following: (1) the 0.1 N NaOH extract of erythrocyte ghosts, comprising peripherally associated membrane proteins; (2) the 1% SDS–10% DTT extractable portion of the membrane, which contained the intrinsic membrane proteins; and (3) the remaining insoluble pellet. The preparations were subjected to complete enzymatic digestion, and quantitative analysis for N^{ε} (γ-glutaminyl)lysine isopeptide was performed. All three fractions contained substantial amounts of the isopeptide, but the highest cross-link content was found in the SDS–DTT-insoluble pellet with a frequency of 1 mole per 120,000 g of protein (Figure 2B). However, because some protease-resistant core remained unprocessed from the material, this is probably a significant underestimation of the true frequency of cross-links in this fraction of Hb Koln membranes. Using similar techniques, no detectable amounts of polymers or N^{ε} (γ-glutaminyl)lysine peptides were obtained from a comparable pool of normal erythrocyte membranes. In regard to its biological significance, we should recall that similar frequencies of N^{ε} (γ-glutaminyl)lysine cross-links, introduced by factor XIIIa into fibrin, would result in close to a fivefold increase in the elastic storage modulus (i.e., stiffness) of the clot network [19], and their absence could cause potentially life-threatening hemorrhage [20]. Hence, it is justified to conclude that TG2 activity in Hb Koln erythrocytes contributes significantly to the stiffening of membrane skeletal structure by catalyzing the formation of N^{ε} (γ-glutaminyl)lysine protein-to-protein side chain bridges.

Observations very similar to those described for the Hb Koln case were made with the membrane preparation from a sickle cell patient [21]; it still remains to be investigated whether the γ:ε-bonded polymers were derived exclusively from irreversibly sickled cell population. Sickle cell is yet another disease in which red cell survival is markedly reduced on account of increased membrane rigidity and change in cell shape. The finding supports the notion that TG2-mediated formation of abnormal membrane protein polymers may be a common finding in anemias of different etiologies and that it might actually account for the premature death of red cells in such diseases.

III. THE Ca^{2+}-ENRICHED HUMAN RED BLOOD CELL AS MODEL FOR THE PHYSICAL AND BIOCHEMICAL ABNORMALITIES OBSERVED IN ERYTHROCYTE DISEASES WITH SHORTENED CELL LIFESPAN

A. MORPHOLOGICAL AND STRUCTURAL CHANGES IN HUMAN ERYTHROCYTES, MEDIATED BY TRANSGLUTAMINASE 2 (TG2), UPON INFLUX OF Ca^{2+} IONS: PERMANENT FIXATION OF ABNORMAL CELL SHAPE AND LOSS OF MEMBRANE PLASTICITY

Treatment of hRBCs with Ca^{2+} plus ionophore (e.g., A23187 or ionomycin) causes a rapid change in shape from discocyte to spheroechinocyte [15], i.e., spheres with sea urchin-like surface spicules (Figure 3), seen also in some anemias [16].

The crenated cells become somewhat dehydrated in spite of the presence of potassium in the medium for lowering the efflux of water. Following short periods of Ca^{2+} loading (around 30 min), replacement of the incubation medium with one containing bovine serum albumin (BSA) and the Ca^{2+} chelator ethylenediaminetetraacetate (EDTA) allows the majority of the cells to revert to the normal biconcave shapes. However, following longer periods of Ca^{2+} exposure, reversibility is lost and the cells retain their abnormal shapes. It is this irreversible fixation of shape that is caused by the covalent remodeling of the membrane skeleton by TG2, the slower kinetic step in the process of Ca^{2+}-induced shape change. The experimental evidence is based on findings with competitive and noncompetitive inhibitors of TG2, which specifically block protein cross-linking by the enzyme. For example, following a 2 h of exposure of hRBCs to Ca^{2+} plus ionophore, only 23% of the spheroechinocytes reverted

back to discocytes upon removal of cellular Ca^{2+}; however, in the presence of 20 mM cystamine during Ca^{2+} treatment, reversibility to discocytes rose to 70% [15]. As a primary amine substrate of TG2, cystamine is a good inhibitor of protein cross-linking in erythrocytes [13]; however, it is known to act not only as a competitive inhibitor, but also by directly interfering with TG2 function [22] through formation of mixed disulfides with sulfhydryl groups, including that of the active center CysH residue when the enzyme is in the open configuration.

Primary amine substrates of TG2 serve as competitive inhibitors of protein cross-linking by virtue of the fact that they themselves become incorporated into the enzyme-reactive γ-glutamyl sites of acceptor proteins (Figure 4). Conversely, suitable Gln-containing short peptide substrates of the enzymes inhibit cross-linking reactions by blocking the ε-lysyl functionalities in donor proteins. Thus, with appropriate tags (isotope, fluorescent, and others), small substrates of transglutaminase can be employed for marking the potential cross-linking sites of proteins in biological systems in an enzyme-specific manner; this approach was first exploited to good advantage for probing the cross-linking sites of human fibrin by factor XIIIa [24]. However, in human red cells, only labeled primary amines were used with success, which allowed identification of some of the intracellular, potential acceptor proteins of cross-linking [13].

Concomitantly with the enzymatic remodeling of membrane skeletal structures by TG2, a loss of membrane deformability sets in [15]. The term is defined as the capacity for passive change of cellular configuration in response to shear forces. This depends on the viscoelastic properties of the membrane, the viscosity of the cytoplasm, and the shape of the cell. The biconcave disc shape of the erythrocyte has excess area compared with the minimum needed for enveloping the cell volume; thus, given its membrane elastic properties and the rather low viscosity of intracellular contents, passive change of shape occurs at relatively small forces. Factors that reduce the surface area, i.e., make the cell more spherical, have a pronounced effect on cellular deformability, particularly if deformation occurs at a rapid rate; increase in cytoplasmic viscosity or reduction of membrane elastic properties also reduces cellular deformability. The elastic shear modulus is estimated from the lengths of small extensions of membranes aspirated into micropipettes under conditions of low negative pressure. This procedure was used to evaluate the role played by TG2 in modifying loss of membrane deformability in hRBCs. After treatment with Ca^{2+} ions and ionophore, followed by removal of internal Ca^{2+} by BSA/EDTA, the red cells showed a significantly reduced membrane extension in comparison with both controls (incubated with Mg^{2+} ions and ionophore) and cells that were exposed to Ca^{2+} plus ionophore in the presence of histamine [15].

In view of these observations, the important issue was to identify the protein-remodeling reactions that appear to be the proximal cause of profound changes in the properties of the cell membrane in Ca^{2+}-enriched hRBCs.

B. POSTTRANSLATIONAL BIOCHEMICAL MODIFICATIONS OF PROTEINS TRIGGERED BY THE INFLUX OF Ca^{2+} IONS: PROTEOLYTIC CLEAVAGE OF TRANSMEMBRANE PROTEINS AND THE TG2-CATALYZED CROSS-LINKING OF MEMBRANE SKELETAL PROTEINS

1. Proteolytic Degradation—Though the focus of the present review is on protein cross-linking reactions by transamidation, as catalyzed by TG2 in erythrocyte diseases and in the Ca^{2+}-enriched cell model, it needs to be mentioned that elevation of intracellular Ca^{2+} also activates membrane and cytosolic proteases [25]. Whereas transamidation is the prime response of hRBCs to the influx of Ca^{2+}, proteolysis predominates in rat red cells, affecting mostly the band 4.1, the band 3 anion transporter, and the band 2.1 (ankyrin) protein substrates. Incidentally, this may explain the far greater ease of cell-to-cell fusion of rat

erythrocyte membranes compared with human, which has been attributed to a much higher ratio of protease to endogenous protease inhibitor (i.e., calpain to calpastatin) in the rodent [26, 27]. As with the rat, mouse red cells also respond to the influx of Ca^{2+} by activating calpain, which causes protein degradation [28].

Two transmembrane proteins, anion transporter band 3 and glycophorin, are the major targets of proteolysis in Ca^{2+}-enriched human erythrocytes, and more than one protease may be involved in the process. Nevertheless, pepstatin alone can inhibit the degradation of both membrane proteins (see Figure 5). It is also noteworthy that the Ca^{2+}-dependent proteolytic phenomenon in hRBCs—though readily observable in freshly drawn cells—can no longer be elicited after a few days of blood bank storage [29] in the conventional preservative of CPDA-1 (a mixture of citric acid, sodium citrate, sodium biphosphate, dextrose, and adenine).

2. TG2-Catalyzed Cross-Linking of Skeletal Proteins by Transamidation—In addition to small primary amine substrates of TG2 (histamine, aminoacetonitrile, and cystamine) that can act as inhibitors of protein cross-linking [13] in hRBCs exposed to an overload of Ca^{2+} (see reaction 2 in Figure 4), other TG2 inhibitors can also effectively block membrane skeletal alterations in these cells. Noteworthy among the findings is inhibition by 2-[3-(diallylamino)propionyl]benzothiophene or DAPBT [30]. The compound, originally synthesized as a “nontoxic” and noncompetitive inhibitor for coagulation factor XIIIa, was found to inhibit also the transamidase activity of the hRBC TG2. When tested on fresh red cells, it blocked both the protein-to-protein cross-linking reaction of TG2 and the proteolytic degradation of band 3 and glycophorin (Figure 6).

IV. THE POLYDISPERSE NATURE OF HIGH-MOLECULAR-WEIGHT, γ:ε-BONDED MEMBRANE SKELETAL POLYMERS GENERATED IN HUMAN ERYTHROCYTES WITH Ca^{2+} OVERLOAD

The SDS–DTT-soluble high-molecular-weight (>10^6 Da) products of protein cross-linking, found in the membranes of the Hb Koln and sickle cell patient’s erythrocytes, and also in the Ca^{2+}/ionophore-treated cell model, can be readily detected on one-dimensional polyacrylamide gels. Since they are too large to enter such gels, they are visualized on top of the separating and stacking gels (marked X1 and X2 in Figures 7 and 8) on the SDS-PAGE profiles of membrane proteins. A significant fraction remains with the cell membrane even after extraction with weak alkali (0.1 N NaOH), which removes peripherally associated proteins. The largest of these structures can be isolated by exclusion chromatography from the *a*lkali-*s*tripped *g*hosts (ASG, Figure 7A, X2) and also from the *a*lkali *e*xtract of membranes of Ca^{2+}-treated cells (AE, Figure 7B, X2). SDS electrophoresis in 2% agarose (a medium in which they can migrate; Figure 7C) shows them to be comprised of polypeptide chain assemblies estimated to range in size between 3 and 6 × 10^6 Da for those from the stripped ghosts (X2ASG) and 1 to 3 × 10^6 Da for those from the alkali extract (X2AE). Nevertheless, the diffuse staining pattern reflects the polydisperse nature of these products and contrasts with the distinct banding profile of nonreduced fibrin—cross-linked by coagulation factor XIIIa, used as a marker in Figure 7C—displaying a ladder of multiples of the approximately 330 kDa protein monomer.

V. DECIPHERING THE POLYPEPTIDE COMPOSITIONS OF THE SDS–DTT-SOLUBLE CORES OF CROSS-LINKED ERYTHROCYTE STRUCTURES: IMMUNOLOGIC AND PROTEOMIC ANALYSIS

A. IMMUNOBLOTTING

There are no methods available for separating the nondisulfide-bonded, covalent cores of the large protein structures into the polypeptide units from which they were assembled under the catalytic influence of TG2 in the human erythrocytes; hence, its constituents can be inferred only by employing cross-reactive antibodies [31] or by a proteomic approach, using mass spectroscopy. Since the most recent results have not yet been published, these data have to be presented and analyzed in considerable detail.

Nitrocellulose blots of X2ASG and X2AE, after SDS electrophoresis on agarose (Figure 7), were probed with a variety of monospecific antibodies to individual human red cell proteins, including the anion transporter band 3 (B3), spectrins (Sp), catalase (Cat), ankyrin (2.1), band 4.1 (4.1), hemoglobin (Hb), and glycophorins (Gp). As shown in Figure 7D, the high-molecular-weight material from the stripped ghosts X2ASG was recognized by all of these antibodies, whereas positive cross-reactivity in the alkali extract X2AE was obtained only with antibodies to spectrins, catalase, and hemoglobin (Figure 7E). The absence of transmembrane proteins band 3 and glycophorins in X2B showed that the clusters recovered from the alkali extract were not anchored in the cell membrane.

A rabbit IgG raised against the polymeric material from Ca^{2+}-loaded human red cells [31] cross-reacted with several monomeric polypeptide constituents of the normal membrane skeleton (spectrins, ankyrin (2.1), and the band 4.1 [protein 4.1]) when these were used either as purified antigens or components found in the whole ghost preparation (WG). As revealed by the bands above that of protein 4.1, this antibody also reacted with two additional proteins on the electrophoretic profile of the ghosts at the normal positions of the anion transporter band 3 and adducin.

The Coomassie blue-stained protein profiles of ghosts from control and from Ca^{2+}-treated erythrocytes are shown in Figure 8A, lanes 1 and 2, respectively, and the alkali-stripped ghost profile of Ca^{2+}-treated cells in lane 3. A comparison of lanes 1 and 2 confirmed earlier findings [10, 13, 25, 29, 30] that intracellular TG2 activation by Ca^{2+} causes the essentially total disappearance of band 4.1, whereas the band 4.2 protein (seen below band 4.1 with a mass of about 75 kDa) and the approximately 42 kDa actin band staining intensities are unchanged from the control.

In the study of probing immunological cross-reactivities to the X1 and X2 high-molecular-weight polymers, a large panel of monospecific antibodies were used, and the results are presented in Figure 8. Figures 8B1–B13 are immunoblots for the three lanes of Figure 8A, with antibodies to transglutaminase (TG2; B1), ankyrin (b2.1, B2), dematin (B3), spectrin (alpha and beta, SP, B4), band 3 protein (b3, B5), band 4.1 protein (b4.1, B6), adducin (B7), p55 (palmitoylated membrane protein 1, B8), glycophorin A (GPA, B9), band 4.2 protein (b4.2, B10), glycophorin C (GPC, B11), stomatin (B12), and glucose transporter 1 (GLUT1, B13). Varying degrees of immunostaining of the polymers were obtained. While antibodies to spectrin (SP), band 4.1, and GPC recognized the X1 and X2 polymers essentially equally well in whole and alkali-stripped ghosts, those against band 2.1, dematin, adducin, and p55 seem to have reacted more strongly with the X1 polymers from whole ghosts. By contrast, immunostaining of polymers was considerably stronger in the alkali-stripped ghost preparations with antibodies to GPA and GLUT1. Variations of this kind may be due to differences in the concentrations of the building blocks of the polymers or due to differences

in accessibilities of antigen epitopes. In the cross-linked polymeric structures, epitopes may become more buried or more exposed for antibody recognition than in the monomeric proteins. Limitations inherent in the immunological approach were further underscored by our experience with two monoclonal mouse anti-transglutaminase antibodies, MAb H23 and MAb G92. Binding of our MAb H23 is at the second Ca^{2+}-ion-binding site in TG2 [33], whereas that of G92 is at the *N*-terminal *N*-acetyl-AEDLILER peptide of mature guinea pig TG2 protein or its equivalent *N*-acetyl-AEELVLER sequence in human TG2. This *N*-terminal octapeptide segment of TG2 is a conformationally sensitive epitope, inaccessible to G92 in the native protein in solution, but exposed on nitrocellulose or PVDF blots and ELISA plates. Immunostaining of purified TG2 with the two MAbs is essentially identical; thus, both antibodies can be employed for identification of the monomeric form of TG2 on immunoblots of ghosts from Ca^{2+}-treated human erythrocytes, but—unlike H23—G92 barely recognizes the TG2 that is incorporated into the membrane-associated polymers of Ca^{2+}-treated cells.

B. PROTEOMICS

Proteomics were employed for analyzing the γ:ε-bonded cores of polymers generated in red cells upon exposure to Ca^{2+} overload. Gel slices at the locations of X1 and X2 polymer bands were cut from eight parallel SDS-PAGE runs of whole ghosts (Table 1) and of alkali-stripped ghosts (Table 2) preparations, and were processed for mass spectrometric analysis. Mascot software was used to identify proteins from the ms/ms spectra of tryptic digests, and each spectrum was checked separately. Criteria for acceptance of a protein were the presence of at least two peptides with Mascot scores in the homology/identity range [34]. Molecular weight search (MOWSE) scores, representing probabilities for correct assignment of proteins, ranged from 3181 to 147 in Table 1 and from 4103 to 118 in Table 2; a significant ($p < 0.05$) match is defined as a score of 60 or higher. Among the proteins identified by immunological testing (Figures 7D–F) as constituents of the red cell polymers, the proteomics data, summarized in Tables 1 and 2, confirmed the presence of spectrin, ankyrin, band 3, band 4.1, adducin (adducin 1 alpha and ADD1 are nearly identical proteins), and hemoglobin peptides. However, glycophorin, as detected by immunostaining (Figure 7D), was not found by proteomics, whereas catalase, recognized by antibodies, was found by the proteomics screen only in the largest polymer of whole ghosts preparation (X2, Table 1). On the other hand, proteomics furnished evidence that transglutaminase itself was incorporated into the polymers. Importantly, proteomics revealed some previously overlooked membrane skeletal components, notably dematin (also known as band 4.9 or protein 4.9), glucose transporter 1 (GLUT1), and the palmitoylated membrane protein 1 (also known as p55 or MPPI), as substrates for cross-linking by the enzyme.

VI. INFERENCES FROM THE ANALYSIS OF POLYPEPTIDE COMPONENTS OF γ:ε-BONDED CORES OF MEMBRANE POLYMERS FOUND IN HUMAN ERYTHROCYTES WITH Ca^{2+} OVERLOAD

The band 4.2 protein and TG2 both belong to the transglutaminase gene family and share considerable sequence similarities [35, 36], except that the Cys/His/Asp/Trp residues of the catalytic tetrad—essential for transamidating enzyme activity in TG2 [23]—ismissing from band 4.2. Potentially, they compete for the same binding site at the *C*-terminal domain of the anion transporter band 3 protein [37]. Nevertheless, while TG2 was incorporated into the polymers of Ca^{2+}-enriched cells (Tables 1 and 2, and Figure 8B1), its close membrane skeletal protein relative, band 4.2, was not (Tables 1 and 2, and Figure 8B10).

Contrary to observations with immunoblotting (Figure 7D, Gp and Figures 8B9, B11, and B12), the proteomics data do not support the presence of glycophorins and stomatin in the

polymers. We have no explanation for this discrepancy, except to note that earlier immunoblotting experiments also failed to identify glycophorin as a transglutaminase substrate [31] and to suggest that hydrophobic or glycosylated single-pass membrane proteins might be difficult to detect by proteomics approaches [38]. If significant proportions of these proteins had been incorporated into the polymers, a greater reduction of staining of their monomeric forms would have been expected than observed in Figures 8B9–B11. In spite of a report that actin was an amine-incorporating substrate for TG2 in mouse erythrocytes [28], there is no evidence that this protein is involved in the cross-linking reaction taking place in human red cells (Tables 1 and 2). Properties and reactions of the rodent erythrocyte membrane are known to differ significantly from those of human cells [39]. Moreover, it should be noted that amine incorporation merely identifies only potential rather than actual cross-linking substrates of transglutaminases in cells. Prior data also showed that the staining of actin band (at around 40 kDa) on the SDS-PAGE of membrane protein profiles of human erythrocytes did not change with Ca^{2+} treatment of the cells [10, 13, 25, 29, 30].

It is not unreasonable to assume that the proteomics peptide counts are proportional to the monomeric masses that make up the covalently linked membrane polymers. This allows estimating the relative abundance of building blocks in the high-molecular-weight materials in the Ca^{2+}-loaded erythrocytes. The normalized peptide count, adjusted for the molecular mass of the smallest constituent—i.e., alpha hemoglobin—among the twenty different protein constituents of the X2 polymer (Table 2), indicates that a structure containing at least one copy of all monomers would have a total mass of about 9×10^6 Da and would be made up of 93 units. A similar calculation for the X1 polymer in Table 2 would yield an approximate mass of 11×10^6 Da of 110 polypeptide monomers. Such estimates exceed the masses of actually detected erythrocyte polymers (Figure 7C). Hence, as illustrated in Figure 9, we suggest that a variety of smaller cross-linked clusters are produced, each of which—in case of the alkali-stripped materials—would be covalently anchored to an intrinsic membrane protein, mainly band 3, but also GLUT1 and the Rhesus blood group CcEe antigen or to p55 that, through its fatty acyl moiety, is partially embedded in the lipid bilayer. Band 3, previously identified as a major target for cross-linking by TG2 [13, 40], accounts for about 33% of monomers that make up the X1 polymers and about 16% of monomers in the X2 polymers, confirming that band 3 is mainly responsible for anchoring the polymers covalently into the membrane.

The proteomics data indicate that the reaction of TG2 in the human erythrocyte is rather specific because, from the myriad of proteins in the cells, only the relatively few listed in Tables 1 and 2 seem to serve as substrates for the Ca^{2+}-triggered remodeling of the membrane skeleton. Approximately half of the polypeptide components that make up the covalent polymeric products are well-known membrane constituents, such as the band 3 anion transporter, α and β spectrins, ankyrin, band 4.1, GLUT1, dematin, adducin, and p55. On the other hand, one should also note an essentially total absence of glycolytic enzymes. In spite of the fact that aldolase and glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) are known to be functionally associated with band 3 [41–46] and GAPDH is an *in vitro* substrate for TG2 [47], neither was found to be incorporated into the cross-linked polymers.

Concerning the total number of polypeptide monomers built into the isopeptide-linked clusters of the cell, the best guideline seems to be the TG2-mediated disappearance of band 4.1, judged by the SDS-PAGE profiles of Ca^{2+}-treated human erythrocytes. Diminution of the staining intensity of band 4.1 on the electrophoretic protein profiles has long been considered to be a sensitive sign of TG2 activity in Ca^{2+}-loaded human cells, in comparison to controls [10,13] (see also Figure 6). Since there are 200,000 copies of band 4.1 per human

erythrocyte [43] and if all of these became partners in the cross-linking reaction, approximately 20 million copies of polypeptides of various types per cell would be built into the X1 and X2 polymeric superstructures.

While the combination of immunologic and proteomic screens revealed the composition of the γ: ε-bonded cores of peripheral and membrane-anchored polymers in Ca^{2+}-loaded erythrocytes, there is no analytical methodology for identifying nearest neighbors for the arrangement of the original polypeptide building blocks within the polymer matrix. It is unlikely that the structure would arise as a collection of homopolymers, comprised of polyTG2, polyband 3, polyband 4.1, and other polypeptides. In this context, it may be mentioned that in the *in vitro* reaction of TG2 with purified spectrin as a substrate, mostly cross-linked dimers and virtually no higher order spectrin polymers were found. Thus, we suggest that TG2 functions by catalyzing the tight attachment of monomers to monomers according to the noncovalent binding partnerships in which the individual units were arranged within the membrane skeletal framework of the cell (Figure 9).

The enzymatic cross-linking reaction would merely freeze existing assemblies by introducing zero-distance N^{ε} (γ-glutamyl)lysine protein-to-protein cross-links without inserting extra mass between the participating Gln and Lys residues that are in close contact. Other cytoplasmic proteins, such as Hb, would then be recruited more slowly to the structure. Altogether, the process would be similar to the final step of blood coagulation whereby a prior assembly of fibrin molecules into half-staggered arrays of overlapping filaments—forming the urea or monochloroacetic acid soluble, noncovalent provisional clot structure—is necessary for efficient cross-linking by factor XIIIa [48]. However, the erythrocyte membrane polymer clusters would have a more complicated, branched geometry.

While the peripheral membrane proteins dematin and adducin were not previously detected in the proteomics profile of alkali-stripped vesicles from human erythrocytes [38], the present study shows that dematin and adducin, along with GLUT1, participate in forming the TG2-mediated high-molecular-weight material (Tables 1 and 2). This observation implies that these proteins must be in close contact to be amenable for cross-linking by TG2, a view that fits in with the suggestion that dematin and adducin provide an alternate mechanism for anchoring the spectrin–actin junctions to the plasma membrane via GLUT1 in human erythrocytes [38].

VII. CONCLUSION

A. THE ERYTHROCYTE CELL DEATH PROGRAM IN HB KOLN DISEASE AND IN OTHER ANEMIAS

This review examines the biochemical basis of the premature death of erythrocytes occurring in anemias such as Hb Koln, one of the unstable hemoglobin diseases. The condition is characterized by an irreversible change from the normal discocyte to echinocyte shape and also by a stiffening of the cell membrane (loss of plasticity or loss of membrane deformability). These altered physical properties are considered to be the cause of splenic entrapment and removal of the abnormal cells from the circulation [16, 17]. As described in Section II, the membrane skeletal structure of Hb Koln cells is also modified by abundant, protein-to-protein N^{ε} (γ-glutaminyl)lysine crossbridges that are the footprints of transglutaminase action [18]. Hence, it is concluded that TG2—a latent enzyme present in red cells in an inactive state [10, 11, 13]—becomes activated in the Hb Koln erythrocytes and, based on the model studies presented in Section III, it appears to control a unique death program. Though change of cell shape and membrane rigidification [15] may be analogous to some events of apoptosis, inasmuch as human erythrocytes are devoid of nuclei and

mitochondria, the biochemical pathway leading to early cell death in Hb Koln disease and in similar erythrocyte disorders must be different from apoptosis. The TG2-led program does not seem to fit in with any of the categories currently listed among the various cell death modalities [49]; it may be regarded perhaps as a truncated, short segment of the apoptotic process ("meroptosis"?).

Experimental evidence supports the notion that GTP/GDP is the prime, if not the only, physiological allosteric regulator of TG2 [50, 51]. Since the ATP concentration is significantly lower than normal in Hb Koln cells [16], the inhibitory GTP/GDP concentration must also be considerably reduced, that is, TG2 would be activated more easily than that in normal cells. TG2 activation is brought about by elevation of the intracellular concentration of Ca^{2+} ions (see Section III and also [52]). In erythrocytes, the entry and exit of Ca^{2+} are controlled by ion pumps [53], and the influx is also facilitated by the shear stress on the cells in the circulation [54]. It is not known whether Ca^{2+} accumulates in Hb Koln cells as they become energy depleted over their very brief lifespans, lasting only 31 days [16], or whether the cation is delivered in distinct pulses over time. Nor is it known what concentration of Ca^{2+} would trigger the TG2-mediated membrane skeletal remodeling reaction in the Hb Koln cells with the reduced GTP content. The Ca^{2+} requirement for eliciting half-maximal velocity of TG2 activity in normal hRBC lysates, as measured with the extraneous *N*,*N*'-dimethylcasein:putrescine substrate pair, is about 0.3 mM [13]. This figure is in the range of the total Ca^{2+} concentration found in sickle cells: 0.1–0.3 mmoles per liter of packed sickle cells versus 0.016–0.039 mM in normal [55]. Indeed, N^{ε} (γ-glutaminyl)lysine-bonded protein polymers could also be isolated from the erythrocyte membranes of a sickle cell patient (Section II; [21]).

Model experiments with normal erythrocytes—exposed to Ca^{2+} stress—offer a remarkably good reproduction of the events that lead to the premature death of the abnormal Hb Koln and sickle cells. Influx of Ca^{2+} triggers the TG2-mediated covalent cross-linking of the membrane skeleton [10, 11, 13, 29, 30]—and, as illustrated in Section III B1, also some degree of proteolysis [25, 29, 30]—that, in turn, cause an irreversible change in cell shape and loss of membrane plasticity [15]. In order to speed up the TG2-mediated remodeling of membrane skeletal structures and to secure maximal response for incorporating as many proteins as possible into the polymeric products, the outside concentration of Ca^{2+} was set to fully saturate the enzyme (1.5–3 mM, i.e., 5–10 times of half-saturation requirement).

The polypeptide compositions of two categories of N^{ε} (γ-glutaminyl)lysine-bonded, SDS–DTT-soluble cores of membrane skeletal polymers (peripheral and integrally anchored) are discussed in Section V and VI. Though the work with Hb Koln cells revealed that the SDS–DTT-insoluble material (see Figure 2) contains the highest frequency of N^{ε} (γ-glutaminyl)lysine cross-links [18], this compartment has not yet been studied by the methodologies employed for obtaining the data presented in Figures 7 and 8 and in Tables 1 and 2. In pathological situations, the intensity and duration of the Ca^{2+} stress is expected to be less severe; hence, it is to be expected that the covalent polymeric clusters—produced by TG2 action—would probably be smaller, containing fewer of the polypeptide building blocks that participate in the enzymatic reaction as cross-linking substrates in the model experiment.

Finally, note should be made of the observations that competitive and noncompetitive inhibitors of transglutaminase (histamine, cystamine, and 2-[3(diallylamino)propionyl]benzothiophene) can be employed to block the protein chemical remodeling of the membrane skeleton [13, 30] as well as fixation of abnormal cell shape and loss of membrane plasticity [15], induced by the Ca^{2+} overload in erythrocytes (see Section I and III). This finding provided the necessary "proof of principle" that transglutaminase

activity can be interfered with inside erythrocytes and, also potentially, in other cell types; it carries far-reaching therapeutic implications. In relation to anemias, the effectiveness of a nontoxic inhibitor of TG2 in Hb Koln disease might conceivably be equivalent to what is currently accomplished by surgical splenectomy in lengthening erythrocyte survival by about 50% [16, 17].

Acknowledgments

This article is dedicated to the memory of Dr. Joyce Bruner-Lorand who contributed significantly to the early phase of our human erythrocyte research project [10, 11, 29]. We also thank Ms. Deanna Rybak for illustrating Figure 9. This work was partly supported by grants from the National Institutes of Health (HL-051445 and HL-095050 to AHC) and from the US Army Medical Research and Materiel Command (W81XWH-07-2-0034 to OL).

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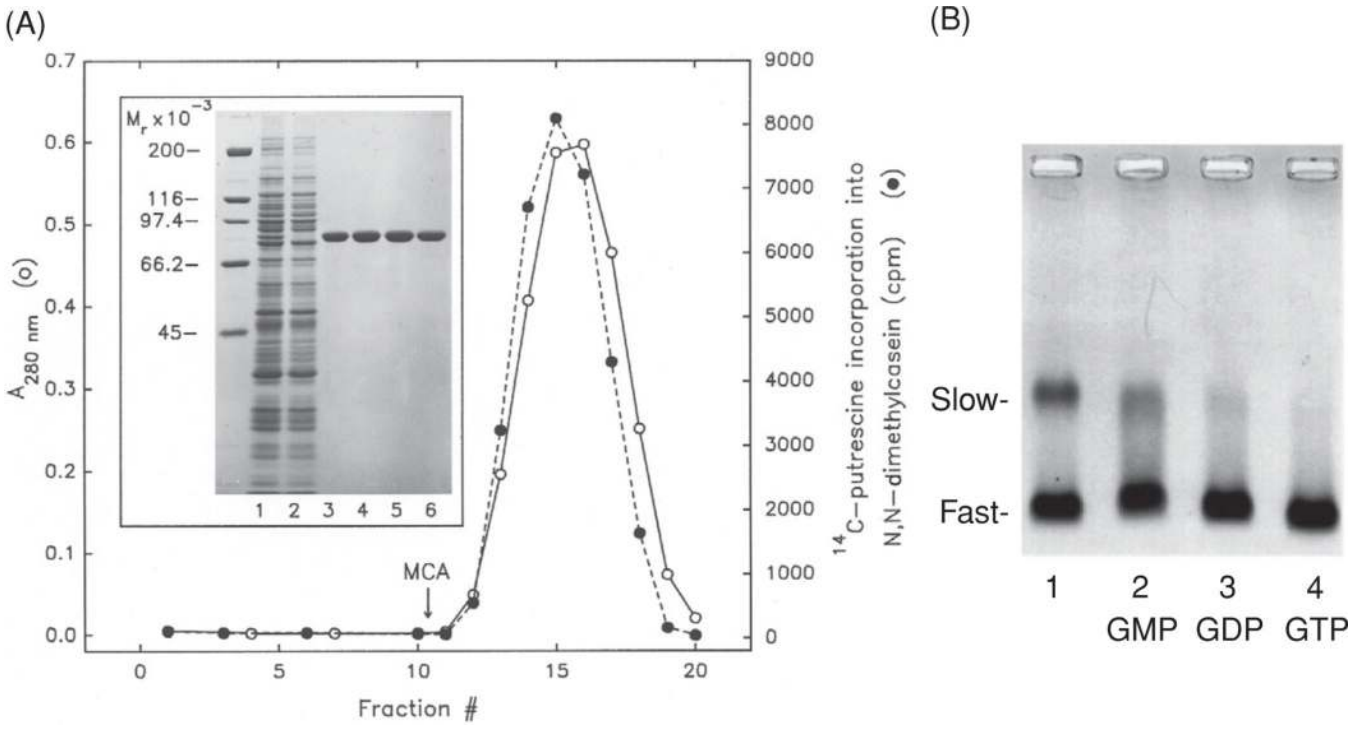


FIGURE 1.

(A) Affinity purification of TG2 by single passage of the hemoglobin-depleted lysate of hRBCs through a column of the 42-kDa gelatin-binding fragment of human fibronectin. The Hb-depleted cell lysate was applied to the affinity column. After extensive washing, the tightly held enzyme was eluted with 0.25% monochloroacetic acid (MCA). Fractions were neutralized and analyzed for protein (left ordinate, open circle) and enzyme activity (right ordinate, closed circle). Inset: eluted samples were examined by SDS-PAGE, stained with Coomassie blue R. Lanes: 1, hemoglobin-depleted lysate; 2, nonretained material passing through the column; 3–6, fractions 14–17 eluted with MCA. Molecular masses in kDa are indicated on the left. For experimental details, see [5]. In later experiments, with better preservation of TG2 activity, MCA was replaced by 80% ethyleneglycol [8]. (B) Purified hRBC TG2 appears to be preponderantly in the GDP-bound form in the hydrodynamically compact, fast-moving electrophoretic conformation. Mobility shifts of the purified protein (lane 1) from slow to fast forms were examined upon mixing with GMP (lane 2), GDP (lane 3), and GTP (lane 4) by nondenaturing electrophoresis (in 3% agarose). For details, see [9].

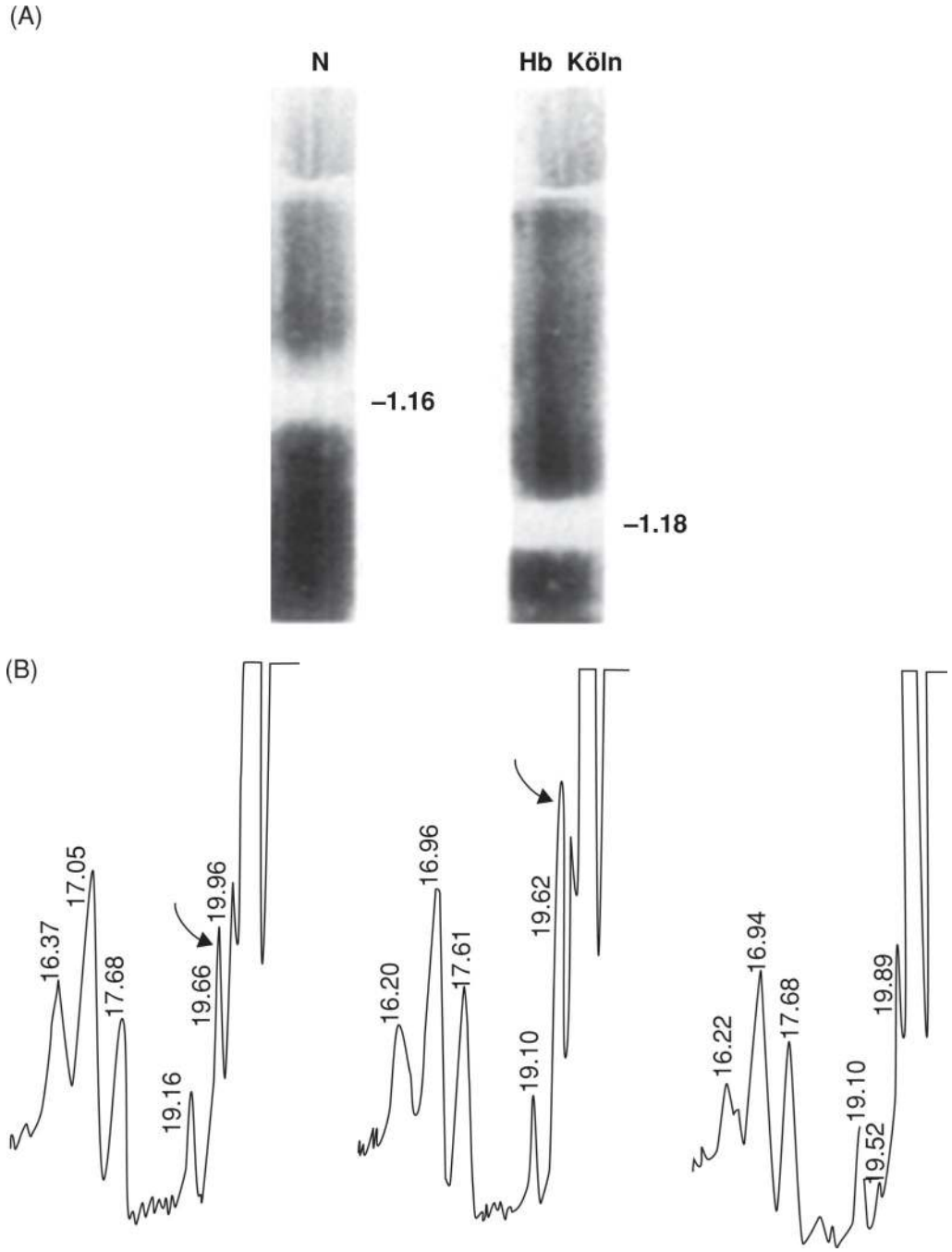


FIGURE 2.

(A) Increased density of the red cell membranes from a patient with Hb Koln disease (right) compared with that of a normal individual (left), measured by sucrose density centrifugation. Density values, marked in g/mL, were computed from the refractive indices of the solutions at the points of highest turbidities of cell membranes. For details, see [18]. (B) Skeletal proteins of the Hb Koln erythrocyte membrane are modified by N^{ε} (γ-glutaminyl)lysine side chain bridges, footprints of the activity of intracellular TG2. High-pressure liquid chromatography of the total proteolytic digest of SDS–DTT-insoluble membrane proteins reveals the presence of the isopeptide (left panel). The same is shown in the middle panel but with synthetic N^{ε} (γ-glutaminyl)lysine peptide added. The right-hand

panel corresponds to the first panel on the left, following treatment by γ-glutamylaminecyclotransferase, an enzyme specific for cleaving the isopeptide. Positions of N^{ε} (γ-glutaminyl)lysine are marked by curved arrows. For details, see [18].

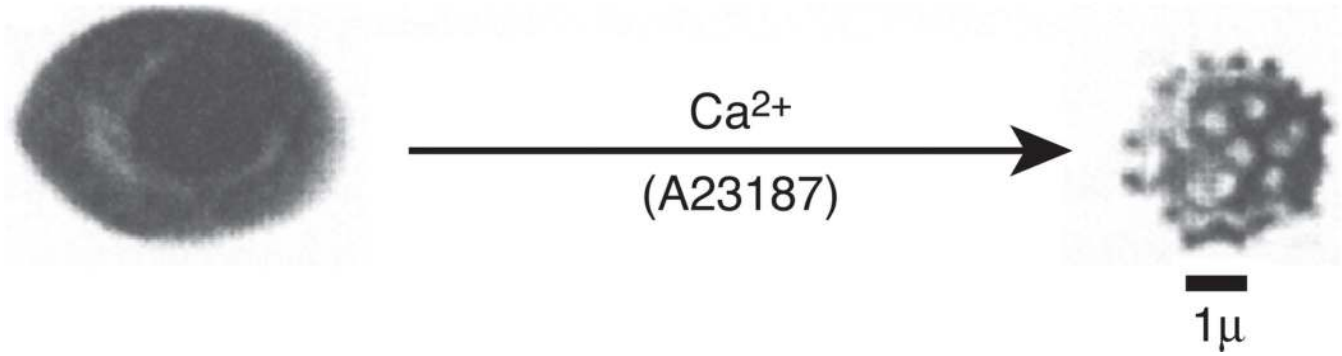


FIGURE 3.

Change of shape in human red cells from discocyte to spheroechinocyte, brought about by Ca^{2+} overload, is illustrated by scanning electron micrography. The process of shape change is reversible only if the intracellular Ca^{2+} is removed within a short period of time; however, it becomes irreversible by longer exposure to the cation. Fixation of the abnormal spheroechinocytic shape—seen in some erythrocyte diseases—is due to the cross-linking of membrane skeletal proteins by N^{ε} (γ-glutaminyl)lysine bonds under the catalytic influence of TG2. Inhibitors of the enzymatic reaction prevent loss of membrane plasticity, which otherwise would irreversibly freeze the shape of the red cell. For details, see [15].

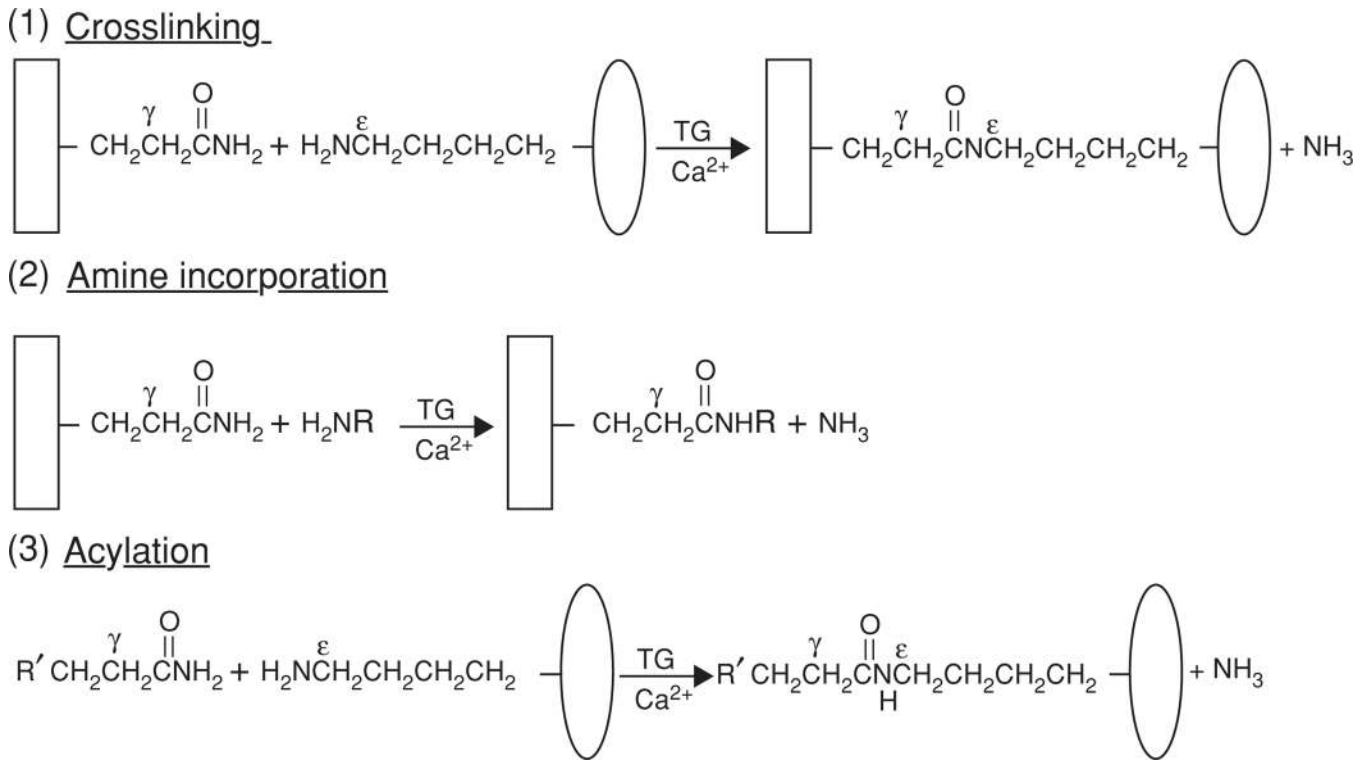


FIGURE 4.

Reactions catalyzed by hRBC TG2 and other transglutaminases. Cross-linking of two proteins by N^{ε} (γ-glutaminyl)lysine bonds (reaction 1) can be inhibited by the other two competing reactions shown. Incorporation of small primary amines (reaction 2) blocks the TG2-reactive γ-glutamyl cross-linking sites in the acceptor protein, whereas incorporation of small peptides with TG2-reactive glutaminyl residues blocks the ε-lysyl cross-linking sites of donor proteins (reaction 3). Labeled amines and glutamyl peptides are widely used for identifying and exploring potential protein substrates of transglutaminases; for a review, see [23].

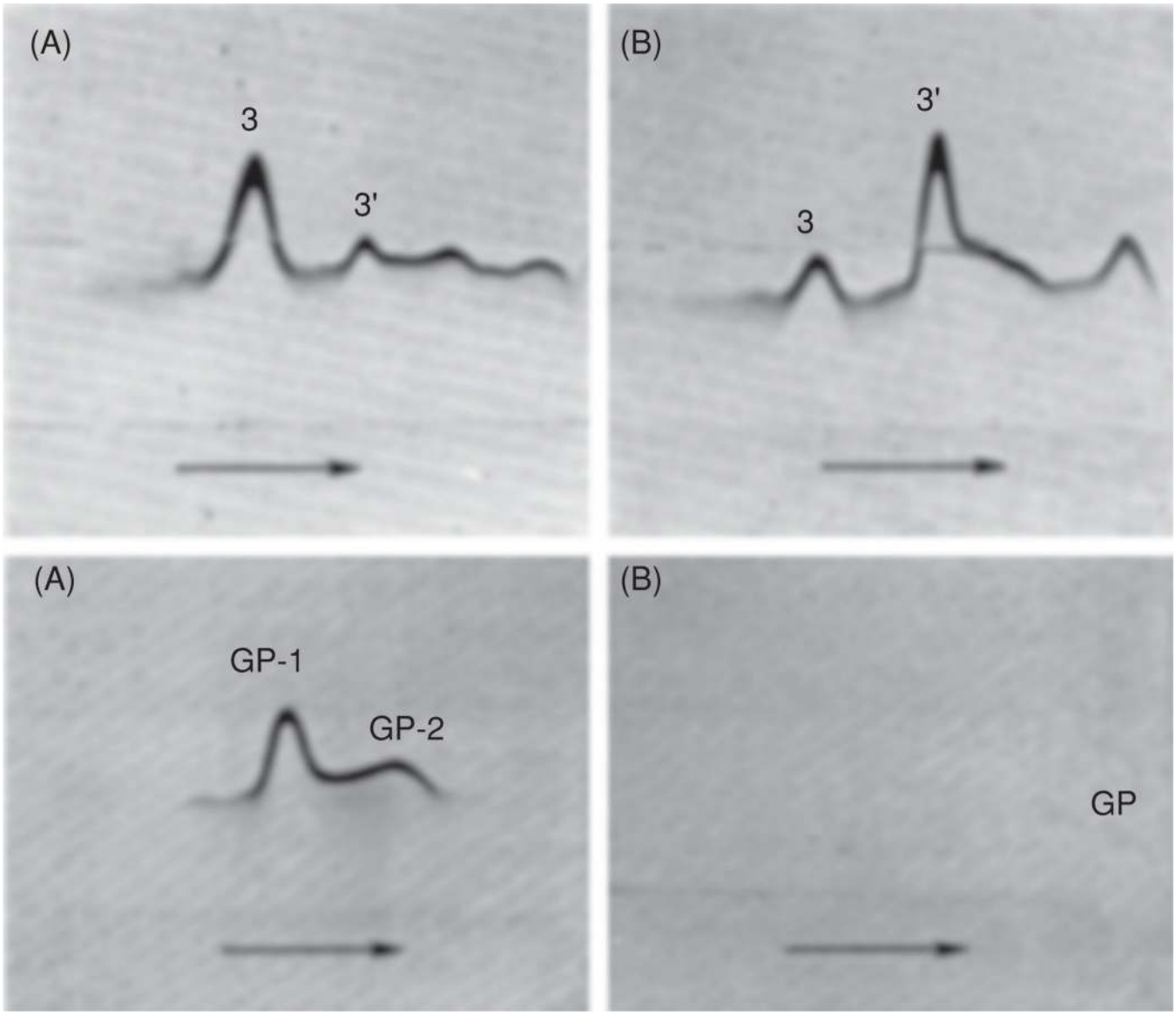


FIGURE 5.

Pepstatin inhibits the proteolysis of anion transporter band 3 (top panels A and B) and glycophorin (bottom panels A and B), triggered in freshly isolated human erythrocytes by the influx of Ca^{2+}. Red cells were incubated (12 h, 37°C) in Ca^{2+} (1.5 mM) and ionophore A23187 (10 μM) in the presence (A) and the absence (B) of pepstatin (2 mM). Membranes were isolated; proteins were extracted with SDS–DTT and electrophoresed in polyacrylamide gels horizontally (see arrows). After removal of SDS, cross-immunoelectrophoresis was performed in agarose (in the perpendicular direction) into which monospecific antibodies against the band 3 protein (top panels) or glycophorins (bottom panels) were incorporated. Immunoprecipitates corresponding to the intact anion transporter are marked as 3 and to its main breakdown product as 3′, whereas the intact glycophorins are denoted GP-1 and GP-2. The barely recognizable, small glycophorin fragments remaining in the absence of pepstatin are marked GP. For further details, see [25].

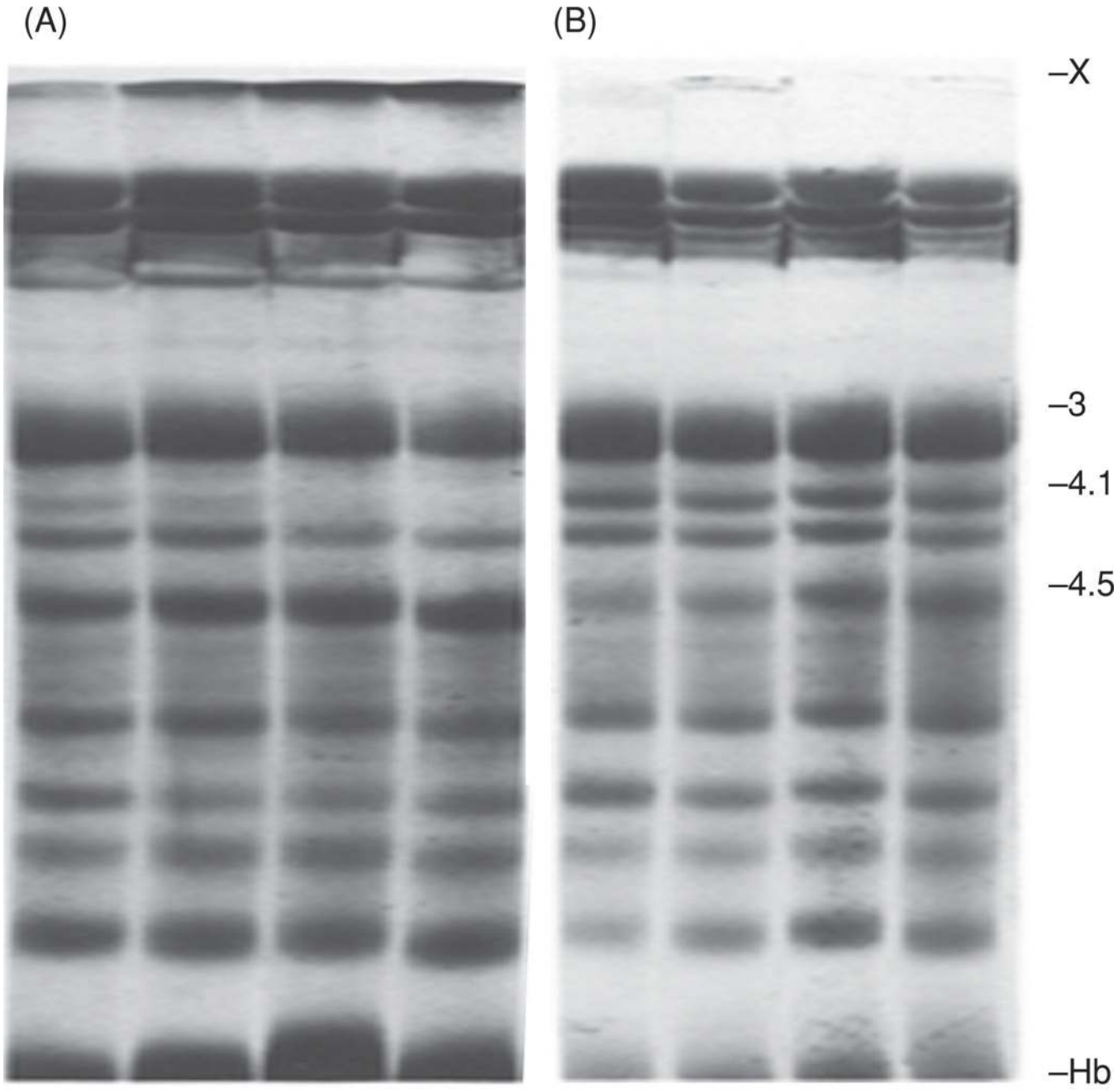


FIGURE 6.

A direct, noncompetitive inhibitor of TG2, 2-[3(diallylamino)propionyl] benzothiophene, or DAPBT, effectively blocks the cross-linking of membrane skeletal proteins brought about by this enzyme when activated in the human red cell by an overload of Ca^{2+}. The inhibitor also hinders proteolytic degradation of the band 3 anion transporter. Cells were incubated at 37°C with 2-mM Ca^{2+} and 20-μM ionophore A23187 for 1, 2, 4 and 8 h (corresponding to four gel profiles in each set from left to right) in the presence of 0.1 mM (set A) or 0.6 mM DAPBT (set B). The higher concentration of the inhibitor is seen to have prevented the formation of high-molecular-weight membrane protein polymers, marked X on top of the SDS-PAGE profiles, whereas lower concentration of DAPBT still allowed polymer production to proceed (albeit at a much slower rate than it would have without the presence

of the compound). In SDS-PAGE tests, a sensitive indicator of blocking the cross-linking reaction by TG2 is preservation of staining intensity of the 4.1 band, as in (B). This panel also shows, that—at a concentration of 0.6 mM—DAPBT hindered the proteolytic degradation of the anion transporter band 3 into its main fragment, appearing at the position of band 4.5 (which corresponds to that of 3’ in cross-immunoelectrophoresis in Figure 5). For details, see [30].

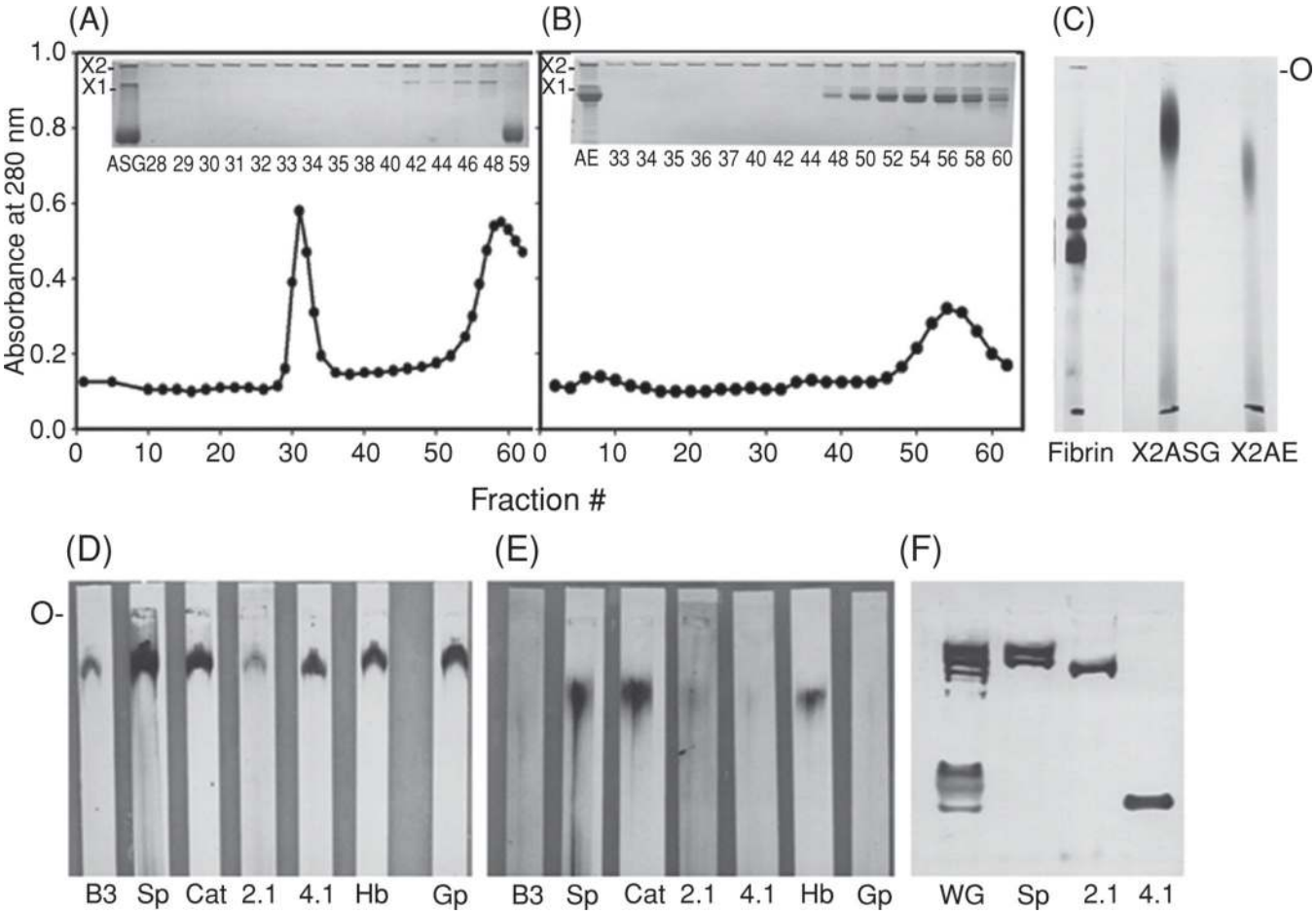


FIGURE 7.

Properties of high-molecular-weight polymers isolated from Ca^{2+}-treated human erythrocytes. Red cells were exposed to Ca^{2+} plus ionophore for 3 h; after hypotonic lysis, membranes were harvested and treated with 0.1 N NaOH. Proteins of the alkali-stripped ghosts (ASG) and alkali extract (AE) were fractionated by chromatography on Sepharose CL-4B; see (A) and (B). Insets to the panels show the SDS-PAGE profiles for the ASG and AE preparations applied to the column and for the fractions that contained X1 and X2 polymers, visualized on top of the separating and stacking gels. The X2 fractions from the ASG (A; fractions #29–35) and from the AE materials (B; fractions #33–40) were pooled, concentrated, and examined by SDS-agarose electrophoresis (C; O indicates origin). A nonreduced fibrin preparation, cross-linked by factor XIIIa, was employed as a molecular weight marker [32]. (D) and (E) are immunoblots of the purified X2 polymers of the alkali-stripped ghosts and from the alkali extract, following SDS electrophoresis on agarose (O marks origin), as in (C). Nitrocellulose blots were probed with various dilutions of antibodies, i.e., antisera to band 3 or B3 (1:300), spectrins or Sp (1:80,000), ankyrin or 2.1 (1:800), protein 4.1 or 4.1 (1:700), hemoglobin or Hb (1:13,000), glycophorin or Gp (1:7000), and an IgG to catalase or Cat (1:800). (F) A rabbit IgG raised against the cross-linked polymers from Ca^{2+}-treated human erythrocytes [31], in 1:5000 dilution, reacted with several monomeric membrane skeletal protein on the SDS-PAGE profile (5% acrylamide) of whole ghosts of normal red cells (WG), which co-migrated with purified spectrins (Sp), ankyrin (2.1), and protein 4.1 (4.1), each of which was also recognized by the antibody. Bands on the immunoblot of lane WG above the position of 4.1 probably correspond to adducin and the anion transporter band 3 protein.

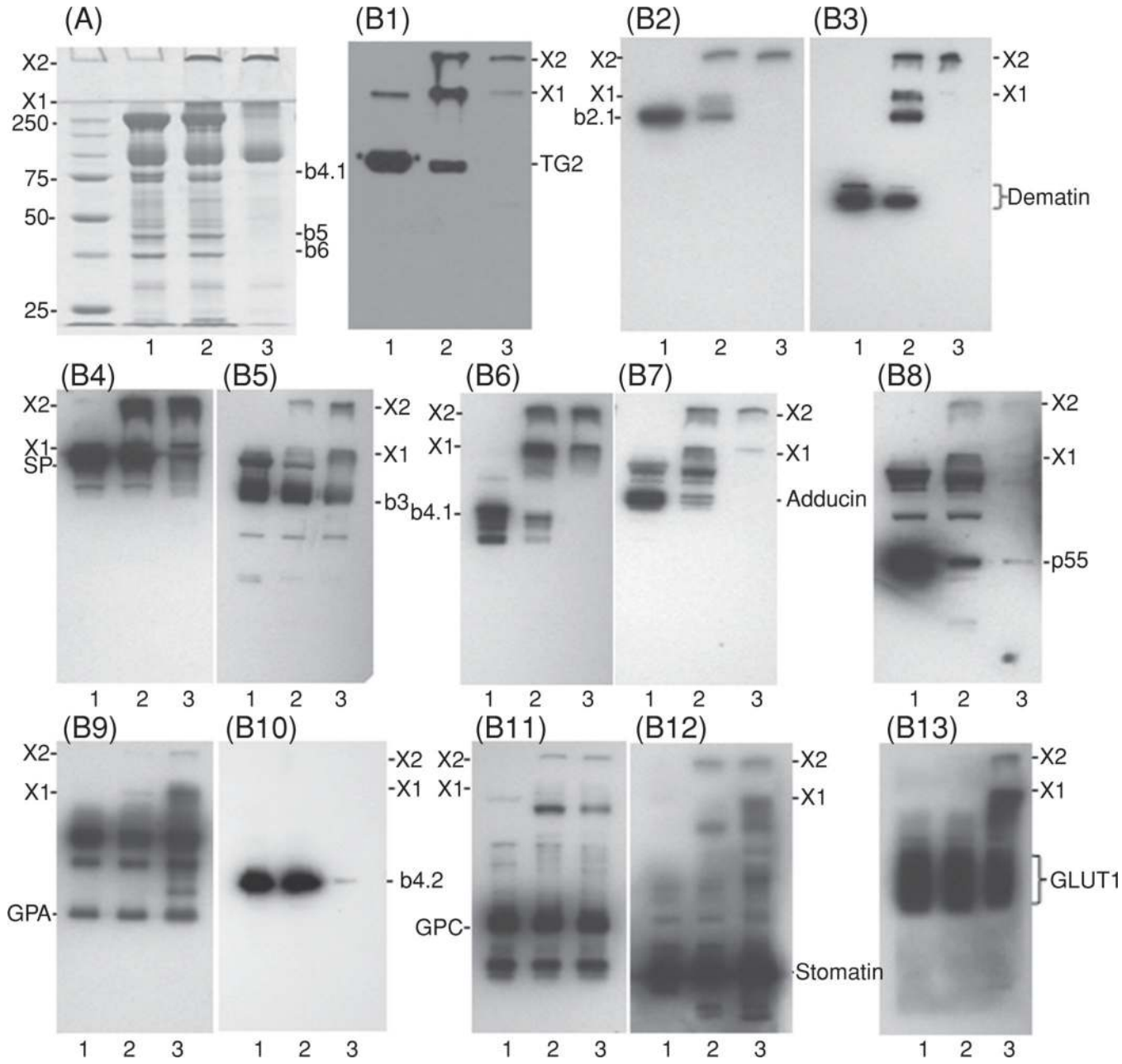


FIGURE 8.

Cross-linked polymers, formed in human erythrocytes upon elevation of the concentration of internal Ca^{2+}, are recognized by several antibodies from our new antibody repertoire. The polymers on the SDS-PAGE protein profiles of membrane proteins at the top of separating and stacking gels are marked as X1 and X2. Part (A) shows the Coomassie blue-stained gels of whole ghosts of control erythrocytes (lane 1), those with Ca^{2+} overload (lane 2), and of the alkali-stripped membranes of the latter (lane 3); molecular weight marker values are given in kDa; the position of actin is marked as band 5 or b5 and that of glyceraldehyde-3-phosphate dehydrogenase as band 6 or b6. PVDF transblots of the gels were probed with various dilutions of antibodies targeting transglutaminase 2 (TG2, B1; 1:20000), ankyrin (b2.1, B2; 1:20,000), dematin (B3; 1:5000), spectrins (SP, B4; 1:10,000), band 3 (b3, B5; 1:20,000), band 4.1 (b4.1, B6; 1:4000), adducin (B7; 1:3000), p55 (B8; 1:4000),

glycophorin A (GPA, B9; 1:1000), band 4.2 (b4.2, B10; 1:200,000), glycophorin C (GPC, B11; 1:1000), stomatin (B12; 1:100), and glucose transporter 1 (GLUT1, B13; 1:20,000). (See insert for color representation.)

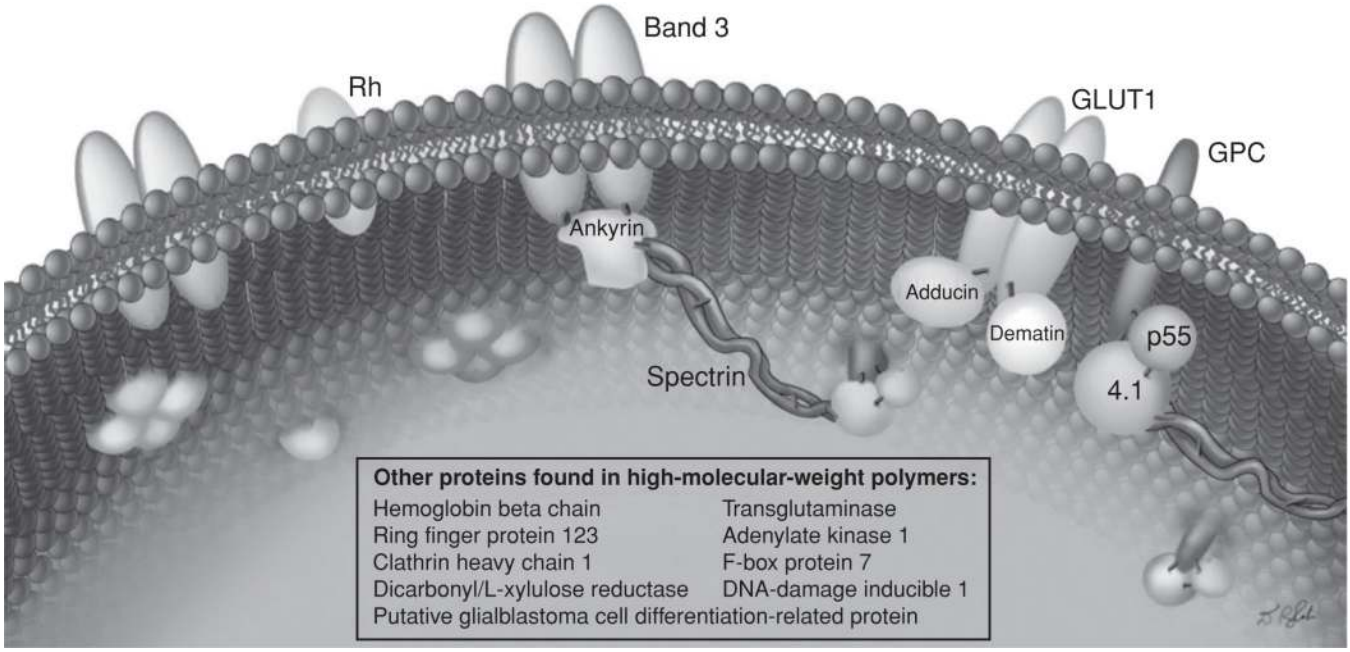
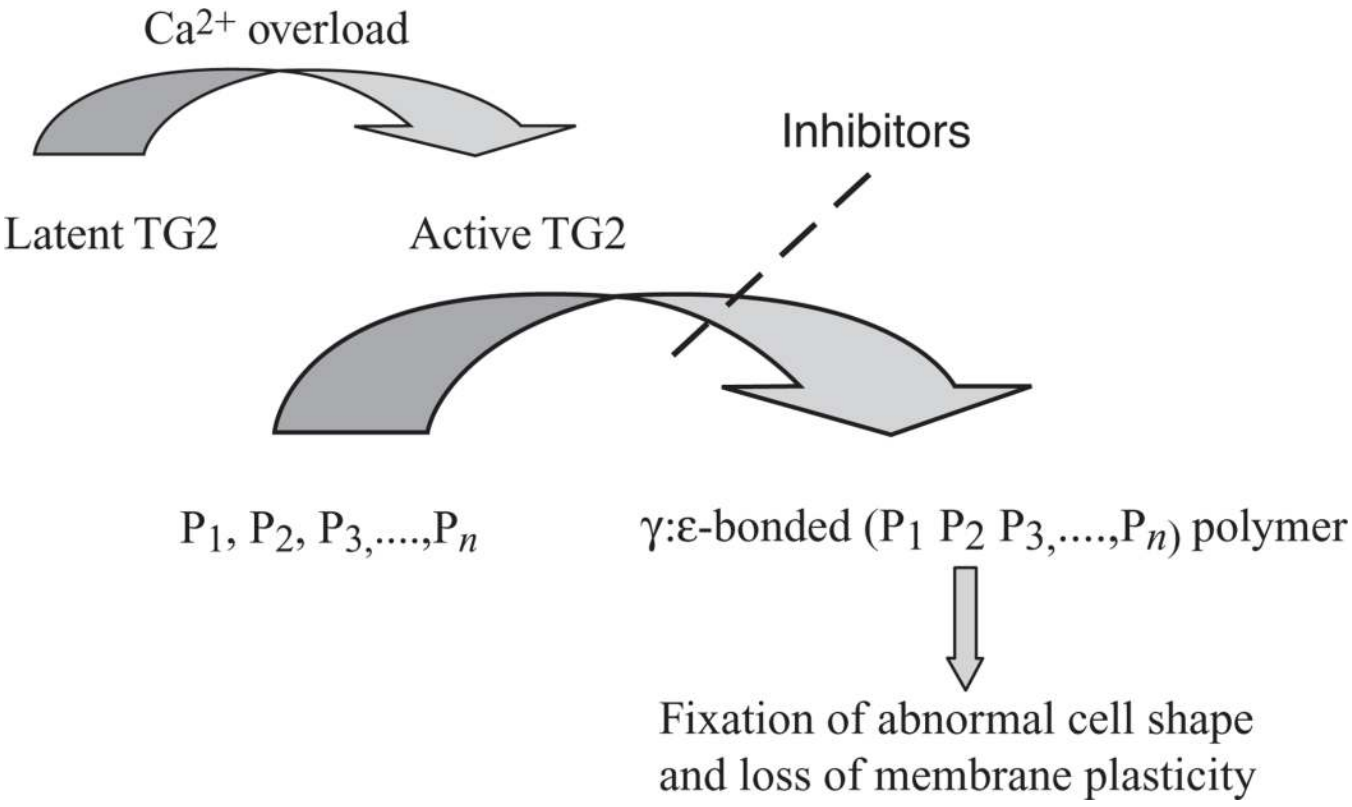


FIGURE 9.

Illustration of a section of the inner surface of alkali-stripped ghosts from Ca^{2+}-loaded erythrocytes indicating scattered clusters of protein polymers, covalently linked to transmembrane proteins or to p55, which—through its palmitoyl moiety—is partially embedded in the lipid bi-layer. The N^{ε} (γ-glutamyl)lysine side chain bridges between constituent polypeptide chains of the polymer are shown by red lines. Without remodeling of the membrane skeleton (as in the left portion of the picture), only the cytoplasmic domains of proteins such as band 3, GLUT1, the Rhesus blood group CcEe antigen, and p55 are seen. (See insert for color representation.)



SCHEME 1.
The Ca^{2+}-triggered, transglutaminase-mediated protein crosslinking cascade in cells.

Adv Enzymol Relat Areas Mol Biol. Author manuscript; available in PMC 2012 March 27.

TABLE 1

Proteins in the X1 and X2 polymers of whole ghosts, identified by mass spectrometry analysis of tryptic peptides.

gi Number	Whole Ghost, X1 Polymer	Score	Peptide Count	GI Number	Whole Ghost, X2 Polymer	Score	Peptide Count
338438	Alpha spectrin	3479	189	119573202	Alpha spectrin	4188	135
134798	Beta spectrin	2678	152	134798	Beta spectrin	3181	115
226788	Ankyrin	2461	139	226788	Ankyrin	2234	93
68563369	Band 3	1406	109	68563369	Band 3	1390	57
119628069	Band 4.1	595	27	119628069	Band 4.1	580	19
115502394	Glucose transporter	392	14	115502394	Glucose transporter	469	10
29826323	Adducin 1 (alpha)	372	11	37588869	Ring finger protein	424	7
28175764	ADD1 protein	338	11	39777597	Transglutaminase 2	388	10
1200184	Stimulator of TAR RNA binding	312	9	95115683	Hemoglobin alpha 1–2 hybrid	312	19
7705925	Dicarbonyl/*L*-xylulose reductase	259	3	438069	Thiol specific antioxidant protein	294	6
19070472	p600	218	27	23268449	Hemoglobin beta	288	10
37588869	Ring finger protein 123	217	12	19070472	p600	278	27
119588998	Importin 7	209	9	4826878	Oxidative stress responsive 1	265	8
4503581	Dematin, band 4.9	202	10	4503581	Dematin, band 4.9	256	13
4502011	Adenylate kinase 1	197	12	4505237	Palmitoylated membrane protein 1	238	7
39777597	Transglutaminase 2	178	7	29826323	Adducin 1 (alpha)	219	8
438069	Thiol specific antioxidant protein	171	8	7705925	Dicarbonyl/*L*-xylulose reductase	217	6
68533125	ACLY variant protein	138	5	68533125	ACLY variant protein	212	14
4378804	Hemoglobin beta	139	6	4502011	Adenylate kinase 1	159	8
4557395	Carbonic anhydrase	127	4	6164624	F-box protein Fbx7	155	3
6225268	Protein diaphonous homolog 1	125	6	4827050	Ubiquitin specific protease 14	150	6
				4557014	Catalase	147	6

The gi number is the accession number in the NCBI protein database for the protein sequence.

A score greater than 60 indicates a significant match (<0.05). The peptide count is the number of times a peptide is found for a particular protein; a finding of two peptides is sufficient to identify a protein with high confidence, provided that the peptides have a combined score of 60, at least.

Adv Enzymol Relat Areas Mol Biol. Author manuscript; available in PMC 2012 March 27.

TABLE 2

Proteins in the X1 and X2 polymers of alkali-stripped ghosts, identified by mass spectrometry analysis of tryptic peptides.

gi Number	Alkali-Stripped Ghost, X1 Polymer	Score	Peptide Count	GI Number	Alkali-Stripped Ghost, X2 Polymer	Score	Peptide Count
338438	Alpha spectrin	4073	138	338438	Alpha spectrin	5145	152
134798	Beta spectrin	2815	113	134798	Beta spectrin	4103	115
226788	Ankyrin	2317	128	226788	Ankyrin	2854	92
68563369	*Band 3	1963	207	68563369	*Band 3	1727	103
119628069	Band 4.1	709	37	119628069	Band 4.1	570	27
115502394	*Glucose transporter	594	28	115502394	*Glucose transporter	459	17
95115683	Hemoglobin alpha 1–2 hybrid	325	13	114619118	Dematin, band 4.9	327	12
29826323	Adducin 1 (alpha)	313	8	39777597	Transglutaminase 2	307	6
114619118	Dematin, band 4.9	260	12	3114508	Hemoglobin alpha	280	14
25573100	*Rhesus blood group CcEe antigen ces	242	7	25573100	*Rhesus blood group CcEe antigen ces	255	5
4378804	Hemoglobin beta	223	9	4929993	Hemoglobin beta	251	6
37588869	Ring finger protein 123	204	5	29826323	Adducin 1 (alpha)	214	7
39777597	Transglutaminase 2	204	8	4502011	Adenylate kinase 1	193	10
4502011	Adenylate kinase 1	189	13	7705925	Dicarbonyl/*L*-xylulose reductase	193	4
4505237	Palmitoylated membrane protein 1	172	5	4826878	Oxidative-stress responsive 1	188	4
119587177	*hCG1980844, isoform CRA_i	146	12	14249955	F-box protein 7	185	7
				4505237	Palmitoylated membrane protein 1	167	5
				4758012	Clathrin heavy chain 1	138	8
				119572126	DDI1, DNA-damage inducible 1	119	5
				3211975	Putative glialblastoma cell differentiation-related protein	118	5

The gi number is the accession number in the NCBI protein database for the protein sequence.

A score greater than 60 indicates a significant match (<0.05). The peptide count is the number of times a peptide is found for a particular protein; a finding of two peptides is sufficient to identify a protein with high confidence, provided that the peptides have a combined score of 60 or higher.

Transmembrane proteins are marked by an asterisk (*).