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Involvement of cytoskeletal proteins in the barrier function of the human erythrocyte membrane. II. Formation of membrane leaks in ghost membranes after limited proteolysis of skeletal proteins by trypsin

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Limited proteolysis of human erythrocyte ghost membranes by low levels of trypsin (10–240 ng/ml) added bilaterally at 0°C together with the proteinase inhibitor, phenylmethylsulfonyl fluoride (PMSF) before resealing at 37°C leads to a graded digestion of spectrin and ankyrin and the disappearance of band 4.1 protein, while band 3 is cleaved only to a very low extent. These alterations are accompanied by an increase of membrane permeability of the resealed ghosts to hydrophilic nonelectrolytes (erythritol to sucrose), taken to reflect impaired resealing. Moreover, the membrane begins to vesiculate. Shedding of vesicles during the efflux measurements can not be responsible for the increased release of test solutes, since the ghosts do not loose hemoglobin and discriminate the nonelectrolytes according to their size. Moreover, the vesiculation site itself does not seem to act as the leak site, since ghosts prepared from erythrocytes pretreated with a carbodiimide which induces membrane rigidification still exhibit a pronounced protein degradation and vesiculation while the permeability enhancement induced by trypsination is markedly suppressed. The trypsin-induced leak has the properties of an aqueous pore as indicated, besides size selectivity, by its inhibition by phloretin and the very low activation energy. In analogy with concepts developed in the preceding paper (Klonk, S. and Deuticke, B. (1992) Biochim. Biophys. Acta 1106, 126–136 (Part I in this series)) the impaired resealing after limited proteolysis is assumed to be related to a perturbation of interactions of membrane skeletal elements with themselves and/or with the bilayer domain constituting the permeability barrier.

Introduction

In the preceding paper [1] leaks formed in resealed ghosts after covalent modification of membrane SH groups at various stages of ghost preparation were assigned to altered interactions of modified membrane skeletal elements with themselves or with the bilayer.

In the present paper limited tryptic cleavage of erythrocyte membrane proteins is exploited to substantiate the influence of proteins at the cytoplasmic membrane surface on membrane resealing and on the barrier properties of the resealed membrane.

Abbreviations: BANA, *N*$^{\alpha}$-benzoyl-L-arginine-4-nitroanilide hydrochloride; DMSO, dimethylsulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; PMSF, phenylmethylsulfonyl fluoride.

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Materials and Methods

Materials

Fresh human blood from healthy donors was obtained from the local blood bank. Standard chemicals and compounds used as test permeants were from Merck (Darmstadt), Fluka (Neu Ulm), or Sigma (Munich).

^{14}C-labelled test permeants (erythritol, mannitol, sucrose, inulin) were from Amersham-Buchler (Braunschweig). EDC and trypsin (3.5 U/mg, substrate: BANA) were from Merck (Darmstadt), phloretin from Carl Roth (Karlsruhe), PMSF from Serva (Heidelberg).

Methods

Preparation of resealed ghosts was carried out as described in the preceding paper [1].

1. Treatment of ghosts with trypsin

Trypsin (final concentrations 0–240 ng/ml suspension i.e. 0–0.84 U/ml) was equilibrated with open ghosts during the period of loading with labeled test permeants at 0°C (see companion paper). After 15 min equilibration at 0°C the trypsin inhibitor PMSF (dissolved in a small volume DMSO, final concentration 0.8 mM) was added. After further 10 min isotonicity was restored as usual. Membrane proteolysis remains limited due to autoinactivation of trypsin [2] and inhibition by PMSF. Efflux measurements were carried out as described in the companion paper [1].

2. Polyacrylamide gel electrophoresis

Samples for electrophoresis were prepared by solubilisation of 0.9 volumes packed membranes with 0.1 vol. of 20% (w/v) SDS followed by addition of sucrose (100 mg/ml) and heating for 1 min at 100°C. Electrophoresis was performed according to Fairbanks et al. [3], using rod gels containing 5.0% acrylamide, 0.1% *N,N*-methylene-bisacrylamide and 1.0% SDS. Gels were stained with Coomassie blue. A marker kit (Fluka) was used for determination of molecular weights.

Results and Discussion

Influence of trypsin treatment on the membrane polypeptide pattern of resealed ghosts

Treatment of intact erythrocytes or resealed ghosts with trypsin externally is well established to cleave glycophorin A [4] while leaving all the other major membrane proteins intact. In contrast, substantial proteolysis after endofacial trypsination has been demonstrated by many investigators, although the time courses and cleavage patterns considerably diverge as a function of experimental details [5–10]. In our studies proteolysis from the internal side of the membrane was achieved by introducing trypsin and a trypsin inhibitor (PMSF) into the open ghosts at 0°C before reconstitution (for details see Methods). Limited proteolysis occurs under these conditions due to autoinactivation of trypsin [2] and the delayed action of the inhibitor.

As a consequence of this type of proteolysis, the time course of protein cleavage can be rather well resolved (Fig. 1). In line with earlier observations [5] both monomers of spectrin (band 1 and 2 (molecular mass 240/220 kDa)) are degraded. In contrast to other studies [8] the β subunit (band 2) was more sensitive towards trypsin than the α subunit. Owing to the use of a trypsin inhibitor (see Fig. 2) several new bands, differing in molecular mass by decrements of about 10 kDa, appear in the region of 120–200 kDa. This pattern was documented earlier for the tryptic digestion of isolated spectrin at low salt concentrations and 0°C, in contrast to the pattern obtained at high ionic strength, where three dominant fragments (80 kDa, 150 kDa, 170 kDa) are formed [11,12]. It can be concluded therefore, that spectrin cleavage by trypsin already started during the 0°C incubation of the ghosts at low ionic strength.

Cleavage of spectrin is one of the possible reasons for the onset of shedding of small membrane vesicles in

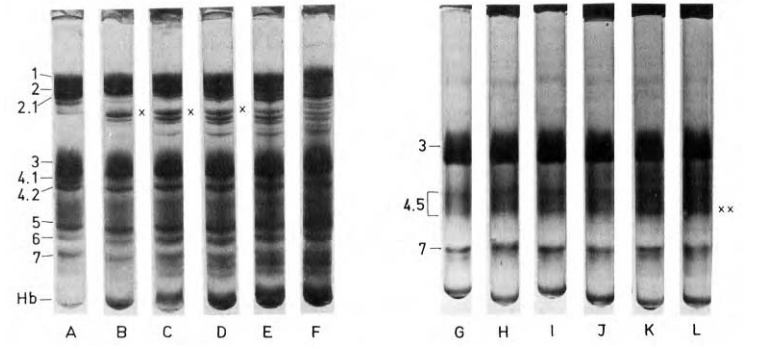

Fig. 1. Concentration dependence of the proteolytic cleavage of ghost membrane proteins by entrapped trypsin. Gels A–F before, G–L after removal of peripheral proteins (see in Results). Trypsin concentration in ng/ml: A, G: 0; B, H: 20; C, I: 40; D, J: 80; E, K: 160; F, L: 240. Experimental conditions described in Methods (removal of peripheral proteins (lanes G–L) by incubation of membranes in 0.1 mM EDTA, pH 12, 30 min, 0°C). Nomenclature of polypeptide bands in the SDS-PAGE gels (Coomassie staining) according to Ref. 3. Hb stands for hemoglobin, the asteriks marks the 165 kDa band (x), respectively, the 50–55 kDa region (xx).

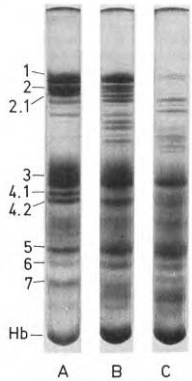

Fig. 2. Limitation of the trypsin induced-cleavage of ghost membrane proteins by the presence of the trypsin inhibitor PMSF. Gel A: 0 ng/ml trypsin, 0.8 mM PMSF (control). Gel B: 160 ng/ml trypsin, 0.8 mM PMSF. Gel C: 160 ng/ml trypsin, 0 mM PMSF. For details see Fig. 1 and Methods.

the presence of internal trypsin (Figs. 3A and B, see also Refs. 6, 13 and 14). In view of the geometry of these vesicles, the volume of the residual ghosts may be taken to remain nearly unaltered, while the surface area is probably slightly reduced.

The digestion products of spectrin appeared on the gels. Since the resealed ghosts were re-lysed again after trypsination and washed three times with 0.5 mM phosphate buffer prior to solubilisation and since even rather large proteins are known to pass the lytic holes in the erythrocyte membrane [15,16], at least the smaller spectrin fragments formed by tryptic cleavage would have been washed out if they were freely mobile in the ghost interior. Therefore these spectrin fragments may be assumed to remain associated with the membrane.

The ankyrin band (2.1), known to be very sensitive to tryptic digestion [7], was already greatly diminished in intensity after treatment with the lowest trypsin concentration used, while band 4.1 was even completely cleaved under these conditions, in agreement with earlier documentation [5,8]. Actin remained essentially intact.

Endofacial trypsin cleaves band 3 into a 50–55 kDa membrane-bound domain and a water-soluble 43 kDa cytoplasmatic domain under appropriate conditions [17]. Under our conditions this effect was not very

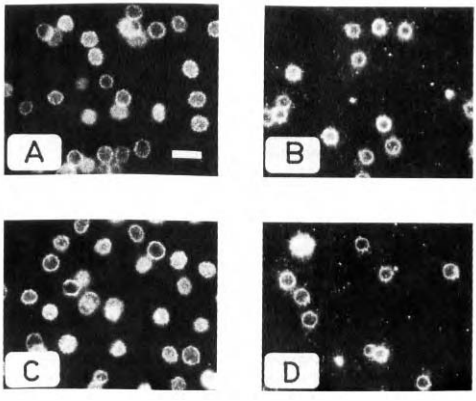

Fig. 3. Vesiculation of trypsin-treated ghosts. Dark field micrograph of ghosts suspended in the resealing medium (300 mosmol/l). Ghosts were prepared from normal (A,B) or EDC (5 mM) pretreated (C, D) erythrocytes. Trypsin (160 ng/ml) was introduced into the ghosts at 0°C prior to resealing time (B, D). All suspensions were photographed immediately after the resealing period at the same final dilution. Bar: 10 μm. For details see Methods.

TABLE I

Influence of trypsin treatment on the leak permeability of resealed ghosts

Leakiness quantified by rate coefficients ($k \times 10^3$, min^{-1}) of the efflux of various test solutes. Retention in % of the retention by control ghosts. Trypsin was introduced into the ghosts during the loading period (for details see Methods). k_0 = ground flux of unmodified ghosts. Efflux measured at 0°C. Number of experiments in brackets. n.d., not determined.

Test solute	Control	Trypsin			
		160 ng/ml		240 ng/ml	
	$k_0 \times 10^3$ (min^{-1})	$k \times 10^3$ (min^{-1})	Retention (%)	$k \times 10^3$ (min^{-1})	Retention (%)
Mannitol	0.79 ± 0.15 (5)	1.46 ± 0.1 (3)	58 ± 6 (3)	n.d.	n.d.
Sucrose	0.34 ± 0.1 (30)	0.91 ± 0.1 (7)	58 ± 9 (7)	2.3 ± 0.6 (4)	30 ± 5 (4)
Inulin	0.1 ± 0.02 (6)	0.36 ± 0.1 (3)	61 (2)	0.77 (2)	46 (2)

pronounced in spite of the extensive degradation of spectrin. Even after removal of peripheral protein (Fig. 1, lanes G–L) only a slight increase of staining intensity in the 50–55 kDa region could be detected. In this respect, the pattern of proteolysis observed under our experimental conditions differs from that reported by others [5]. The controversial reports on this subject (Refs. 5–10 and this paper) underline the possibilities provided by experimental details, when different patterns of limited membrane proteolysis are desired.

Formation of leaks in resealed ghosts subjected to endofacial trypsination

Effects of membrane modification by entrapped trypsin on the permeability to hydrophilic nonelectrolytes are shown in Fig. 4. While there is no increase of sucrose permeability up to 80 ng/ml trypsin (data not shown), higher trypsin concentrations enhance the rates of exit of sucrose and inulin. Prolongation of the trypsin exposure by extending the resealing period from 45 min to 90 min does not further increase permeability (data not shown), in line with the inactivation of the enzyme mentioned above.

The efflux kinetics for the trypsin treated ghosts, shown in Fig. 4, only refer to part of the ghost population (see retention data in Table I). Depending on the trypsin concentration a fraction of the ghost population already lost its trapped test solute during the washing process as indicated by the low retention values. In view of this heterogeneity, we applied only low trypsin concentrations (160 ng/ml) in most of the further experiments aiming at a characterisation of the defect.

The trypsin-induced leak pathway, most likely resulting from an impairment of resealing, slightly discriminates hydrophilic nonelectrolytes (mannitol, sucrose, inulin) according to size (Table I). Complete retention of hemoglobin (data not shown) during the efflux period clearly supports the assumption of the formation of a size-selective defect and excludes the possibility that the pinching off of vesicles is responsible for an indiscriminate loss of test solute during the efflux measurement. Most of this hemoglobin is freely

TABLE II

Suppression of leak permeability induced by trypsin in resealed ghosts by pretreatment of erythrocytes with a permeant carbodiimide

Rate coefficients ($k \times 10^3$, min^{-1}) of sucrose efflux of unmodified and modified ghosts. The conversion of the measured flux rate coefficients into permeabilities ($P \times 10^{10}$, cm/s) was carried out in the case of trypsin-treated membranes under the assumption of a loss of surface area due to vesiculation of about 50% of the surface excess. For a ghost with a volume of 51 μm^3 and an original unaltered surface area of 140 μm^2 (see the preceding paper [1]) this amounts to 38 μm^2. Erythrocytes were pretreated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (5 mM, 45 min, 37°C, pH 7.4, Hct 10%, see Ref. 1). Ghosts prepared from these cells were treated with trypsin as usual. Efflux at 0°C. Number of experiments in brackets.

Second treatment	First treatment					
	no EDC			EDC		
	$k \times 10^3$ (min^{-1})	$P \times 10^{10}$ (cm/s)	retention (%)	$k \times 10^3$ (min^{-1})	$P \times 10^{10}$ (cm/s)	retention (%)
None (Control)	0.34 ± 0.1 (30)	2.0	100	0.54 ± 0.1 (5)	3.24	100
Trypsin	0.91 ± 0.1 (7)	7.55	58 ± 9	0.52 ± 0.4 (3)	4.31	100
Trypsin minus Control		5.55			1.07	

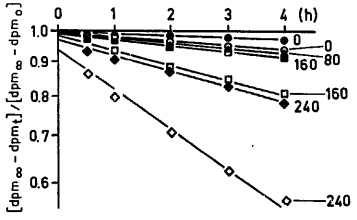

Fig. 4. Time course of the release of sucrose (open symbols) and inulin (closed symbols) from ghosts treated with various concentrations of trypsin (given in numbers at the lines in ng/ml suspension). Efflux measured at 0°C. For details see Methods.

mobile in the ghost interior and can be released by osmotic lysis of ghosts [1].

Artifacts resulting from a contribution of the content of small membrane vesicles to the fluxes can probably be neglected because of their low volume fraction. Nevertheless, vesiculation must be considered in calculating ghost permeabilities (see Table II).

The defect in resealing induced by tryptic cleavage may be regarded as an aqueous 'hole'. This is indicated not only by its size selectivity (see above) but also by the observation that phloretin inhibits the leak flux of mannitol and sucrose by about 35% (data not shown, see Ref. 18). Phloretin has been shown in recent studies to inhibit fluxes through aqueous leaks induced in erythrocytes [19] and ghosts [1] by various types of

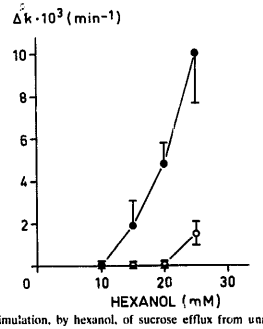

Fig. 5. Stimulation, by hexanol, of sucrose efflux from unmodified (○) and trypsin-treated (●) ghosts. Data given as the increase (Δk) of rate coefficients over the corresponding controls (without trypsin: $k_0 = (1.6 \pm 0.36) \cdot 10^{-3}$ min^{-1} ($n = 7$), with trypsin: $k_0 = (1.1 \pm 0.2) \cdot 10^{-3}$ min^{-1} ($n = 5$)). Fluxes measured at 37°C. Mean values ± S.D. from three experiments.

membrane modification. Moreover, the activation energy of the trypsin induced leak flux must be very low since the total sucrose efflux from trypsin-treated ghosts (i.e. the sum of ground and leak permeability) had no measurable temperature dependence between 0°C and 37°C (data not shown). Hexanol (15–25 mM, 37°C), which stimulates leak fluxes induced by various types of membrane modification [19] also increases the sucrose flux in trypsin-treated ghosts (Fig. 5). All these functional similarities between trypsin-induced leaks and leaks induced by oxidative modifications [19] suggest structural similarities between the two types of leaks.

Protection against trypsin-induced barrier damage by EDC

In the preceding paper [1] we described the suppression of diamide-induced leakiness after modification of the erythrocyte membrane with the carboxyl group modifying reagent EDC, which crosslinks and rigidifies the erythrocyte membrane [20]. In that case it was impossible to decide, whether the suppressive effect is due to a simple blockage of SH-groups by EDC or to a 'stabilisation' of the conformation of membrane skeletal elements. In order to further investigate this question, we studied the influence of an EDC pretreatment of erythrocytes on trypsin-induced leak formation. As evident from Table II, EDC decreases the enhancement of permeability and prevents the decrease of test solute retention induced by tryptic damage.

This normalisation of permeability can not be related to different extents of trypsin-induced vesiculation in control and EDC pretreated ghosts, since the extent of vesiculation proved to be the same under both conditions (Fig. 3). Moreover, an inhibitory effect of EDC on leak formation in spite of unaltered vesiculation contradicts the suggestive hypothesis that membrane vesicle shedding after trypsination might create the defects serving as a leak for small polar solutes.

Concluding remarks

The temporal correlation between a progressive cleavage, by trypsin, of membrane skeletal proteins and an increasing impairment of membrane resealing by trypsin is highly suggestive of a causal relationship between the two events, although the involvement of cleavage of other proteins, e.g. band 3, can not be excluded at present. This working hypothesis is also supported by the data on leak formation by SH group modification of skeletal proteins reported in the companion paper.

As a mechanism initiating trypsin-induced leak formation we propose a perturbation of the rearrangement of membrane skeletal elements and their interaction with the bilayer domain, assumed to occur in the

course of resealing of ghosts [21–23]. It remains to be seen, whether the impairment of the mechanical stability of the erythrocyte membrane observed after tryptic cleavage of membrane skeletal elements [5,24] bears any mechanistic relationship to leak formation under these conditions.

The site of proteolytic cleavage responsible for this leak formation (ankyrin?, Band 4.1?, spectrin?) remains to be localized. Secondary alterations in the lipid domain resulting from the protein perturbation are probably responsible for the ultimate formation of an aqueous leak, as indicated by the size selectivity of the leak fluxes, their low activation energy and their sensitivity to phloretin and alcohols (for a discussion of this subject see Ref. 19). Molecular models for these secondary alterations have been proposed earlier [25–27]. The molecular mechanism of protection by EDC remains to be explained.

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