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A RE-EXAMINATION OF THE EFFECTS OF CHYMOTRYPSIN AND TRYPSIN ON THE ERYTHROCYTE MEMBRANE SURFACE TOPOLOGY

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Summary: Silver/Coomassie blue staining of human erythrocyte membrane electrophoretograms permits simultaneous visualization and color differentiation of asialoproteins, sialoglycoproteins and lipids in the same gel. Using this technique evidence is provided that chymotrypsin cleaves glycophorin A as well as band 3. The chymotryptic fragmentation pattern of glycophorin A in situ intact cells was different from that generated by trypsin treatment. Chymotryptic cleavage of band 3 generated two Coomassie blue stained fragments at 62,000 and 38,000 Mr, whereas simultaneous cleavage of glycophorin A dimer and glycophorin A B heterodimer yielded yellow silver stained fragments at 68,000 and 47,000 Mr. Trypsin cleaved glycophorin A dimer (88,000 Mr) and monomer (38,000 Mr) to form membrane associated fragments of Mr = 40,000 and 18,000 respectively.

Selective protease digestion of the external surface of the intact erythrocyte has been used extensively in topological analysis of the erythrocyte membrane. The membrane's surface architecture can thus be described in terms of protease cleavage sites located within the external domains of susceptible membrane polypeptides. Specific membrane cleavage sites for various proteases such as trypsin, pronase, and papain have been studied (1-5). Chymotrypsin has been shown to cleave band 3 into Mr = 58,000 and Mr = 35 - 45,000 membrane associated fragments (3, 6, 7, 8). Conclusive evidence that chymotrypsin simultaneously cleaves glycophorins is lacking, however. Results of previous studies (3, 9) though suggestive of chymotrypsin cleavage of glycophorin A,

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The abbreviations used are:
PAS, Periodic acid Schiff stain for sialoglycoproteins; CB, Coomassie brilliant blue R-250, PMSF, phenylmethylsulfonyl fluoride, YSS, Yellow Silver stained; TPCK, tosyl-L-phenylalanylcholoromethyl ketone; TLCK, tosyl-L-lysyl-chloromelthyl ketone; SDS, sodium dodecyl sulfate.

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were somewhat inconclusive because the major periodic acid Schiff (PAS) stained chymotrypsin cleavage product was electrophoretically indistinguishable from that generated by trypsin treatment. Because most commercial chymotrypsin preparations, unless treated with specific inhibitors, do contain residual trypsin activity, it is impossible to ascertain if cleavage of glycophorin A in intact erythrocytes is in fact due to chymotrypsin activity per se. In addition, these studies were limited by the relatively low sensitivity of the Coomassie blue (CB) and PAS stains commonly used to identify these proteolytic fragments.

We have recently described a sensitive Silver/Coomassie blue (Ag/CB) double staining technique that differentially detects by a simple color code human erythrocyte membrane proteins, sialoglycoproteins and lipids in the same gel (10). This has made feasible re-evaluation of the effect of chymotrypsin on intact erythrocytes since the fates of band 3, sialoglycoproteins and their membrane associated cleavage products can be differentially detected and followed in the same gel.

MATERIALS AND METHODS

Materials - Soybean trypsin inhibitor, neuraminidase type VIII and chymotrypsin preparations were purchased from Sigma Chemical Co. (St. Louis, MO) including alpha-chymotrypsin type VI, Diisopropylfluorophosphate (DFP) treated, three times crystallized; and alpha-chymotrypsin type II, three times crystallized. N -tosyl-L-phenylalalanylchloromethyl ketone (TPCK) treated trypsin was obtained from Worthington Diagnostic Systems, Inc. (Freehold, NJ) and silver stain kit was purchased from Bio-Rad Laboratories (Richmond, CA). Heparinized blood samples were obtained from normal healthy volunteers with informed consent.

Isolation of Erythrocytes - Whole heparinized blood was passed through cotton wool to remove platelets and leukocytes (11). The erythrocytes were sedimented by centrifugation at 1000 x g for 5 min and then washed 3 times in (10 vol/wash) 5 mM Tris-HCl/140 mM NaCl, pH 7.4 at 25°C.

Proteolysis - Protease digestion of intact erythrocytes was carried out as previously described (5). Trypsin or each chymotrypsin preparation was added to the erythrocyte suspension (20% hematocrit) from stock solution (10 mg/ml) to give a final enzyme concentration of 200 ug/ml. The cell suspensions were incubated at 25°C for 2 h. Digested cells were washed 5 times (10 vol/wash) in ice-cold 5mM Tris-HCl/150 mM NaCl/1.0 mM EDTA/2 mM PMSF, pH 7.4. Soybean trypsin inhibitor was added to the trypsin digest. The final inhibitor to trypsin ratio was 1.3: 1(w/w) and the suspension was incubated for 15 min at room temperature before the ice-cold Tris-saline wash. A control erythrocyte suspension without protease treatment was similarly processed.

Membrane Preparation - Creamy white ghosts were prepared in ice-cold 5 mM Tris-HCl/7 mM NaCl/1.0 mM EDTA/1.0 mM PMSF, pH 7.4 as previously described (12).

Isolation of Integral Membrane Proteins - Ghosts from control and protease treated erythrocytes were stripped of peripheral membrane proteins by cold 0.1 M NaOH treatment (13). The ghost NaOH pellets were dissolved directly in Laemmli (14) electrophoresis sample buffer and stored at -20°C until used.
Isolation of Crude Sialoglycoprotein Fraction - The procedure of Hamaguchi and Cleve (15) was used to obtain a crude isolate of the erythrocyte membrane sialoglycoprotein fraction from control and protease treated samples.
SDS/Polyacrylamide Gel Electrophoresis - Electrophoresis was carried out on isotropic 11% (w/v) acrylamide slab gels using the discontinuous buffer system of Laemmli (14). Molecular weight protein standards used were myosin, B-galactosidase, phosphorylase B, bovine serum albumin and ovalbumin. Gels were stained with Coomassie blue R-250 or PAS reagent by the method of Fairbanks et al. (16).
Double Staining - Gels were stained first with the Ag stain then followed by CB as previously described (10). Photographs were taken of the wet gels using both color and black/white films.
Neuraminidase Treatment of Intact Erythrocytes - Washed intact erythrocytes, control and protease-treated, were incubated with neuraminidase as described by Aminoff et al. (17). Cells were suspended at 50% hematocrit in 5mM sodium acetate/140 mM NaCl/1.0 mM $CaCl_2$/0.1 mM PMSF, pH 5.1. Neuraminidase was added to a final concentration of 1.1 units/ml packed cells. Incubations were at 37°C for 3 h after which white ghosts and derivatives were prepared from the washed cells.

RESULTS AND DISCUSSION

Protease Treatment of Intact Erythrocytes - Site specific cleavages occurred in exposed domains of susceptible membrane polypeptides upon exposure of intact cells to chymotrypsin or trypsin. No hemolysis occurred during these incubations. It is therefore unlikely that the proteases gained access to the cell's interior. This premise was confirmed by the observation that digestion under these conditions was highly restricted in contrast to extensive proteolysis noted with exposure of unsealed ghosts to these proteases (18).

After the digestions, isolated ghosts and derivatives were analyzed by SDS-gel electrophoresis (14). Gels were stained first with Ag stain then followed by Coomassie blue R-250 as recently described (10). In this Ag/CB staining format, all conventional CB sensitive membrane polypeptides (19) stained blue whereas the sialoglycoproteins stained yellow with Ag. Cleavage products derived from CB-stained polypeptides (band 3 in this study) also stained with CB. Cleavage products derived from sialoglycoproteins still stained yellow. Parallel gels stained with PAS reagent (16) confirmed the premise that the yellow Ag stained cleavage products were fragments of sialoglycoproteins.

Control Ghosts and Derivatives - Figure 1 A-C shows typical Ag/CB staining patterns of control ghosts (G), (Lane A); insoluble ghost NaOH pellet (N), (Lane B); and crude sialoglycoprotein extract (S), (Lane C). All conventional blue CB stained erythrocyte membrane polypeptides and yellow Ag stained (YSS) sialoglycoproteins were directly visualized and distinguished in the same gel (Lane A). YSS sialoglycoprotein bands were observed at Mr x

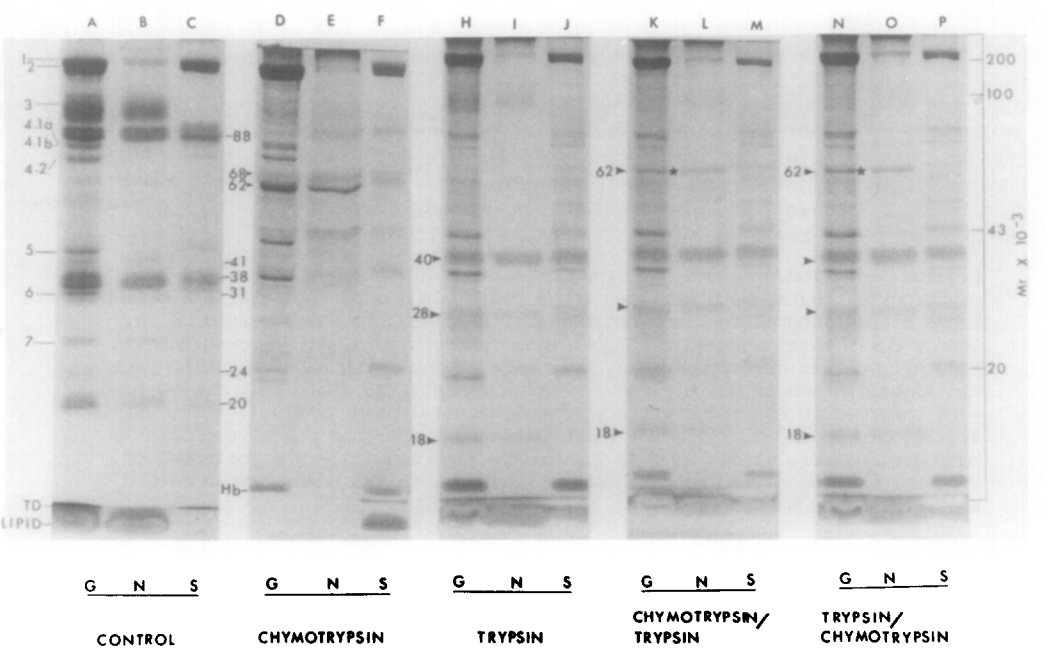

Figure 1 - Silver/Coomassie blue double stained electrophoretograms of human erythrocyte ghosts, NaOH stripped ghost pellets and crude preparations of membrane sialoglycoproteins from untreated control and protease digested cells. Control cells were incubated without proteases (A-C). Test samples were erythrocytes treated with chymotrypsin (D-F), trypsin (H-J) or cells subjected to protocols of sequential digestion with chymotrypsin followed by trypsin (K-M) or trypsin treatment followed by chymotrypsin digestion (N-P). Details and conditions of protease treatment were as described in Materials and Methods. Ghosts (G), NaOH stripped ghost pellets (N) and crude isolates of the membrane sialoglycoprotein fractions were prepared as described. Electrophoresis was performed on a 1.5mm thick slab gel containing isotropic 11% (w/v) acrylamide with a stacking gel of 3% (w/v) acrylamide in the discontinuous buffer system (14). Fifteen ul packed ghost equivalents were solubilized in Laemmli sample buffer and applied to each gel slot. After electrophoresis (12h at 25 mA) each gel slab was stained first with Ag stain then followed by Coomassie blue staining as recently described (10). Membrane polypeptides stained blue with Coomassie blue and were labeled according to the nomenclature of (19). Membrane sialoglycoproteins and lipids stained yellow. The sialoglycoproteins migrated with estimated Mr x (10^{-3}) = 88, 65, 41, 38, 31, 24 and 20 (control A,C). The 62,000 Mr chymotrypsin cleavage product of band 3 was stained blue with Coomassie blue and marked with (*). Yellow Ag stained chymotryptic cleavage products were observed at 47,000 and 68,000 Mr (D-F). Trypsin cleavage products which stained yellow with Ag were noted at 40,000, 28,000 and 18,000 Mr. For other details, see Results and Discussion.

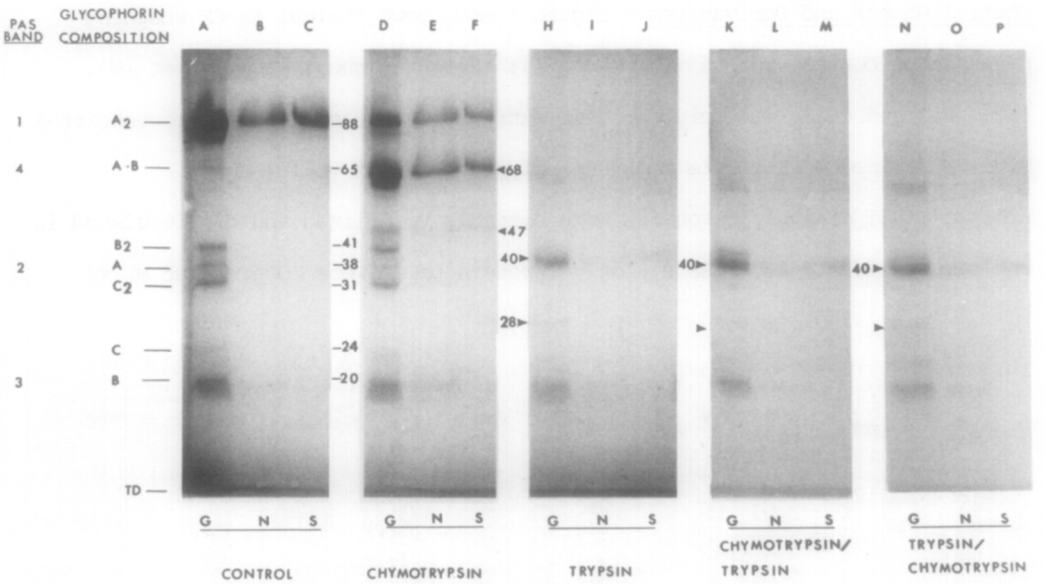

Figure 2 - Periodic acid-Schiff reagent stained electrophoretograms of erythrocyte ghosts, NaOH stripped ghost pellets and crude preparations of membrane sialoglycoproteins from control untreated and protease treated cells. Intact cells were digested and subsequent manipulations were as described in Figure 1. After electrophoresis, gels were stained with PAS reagent to permit visualization of the sialoglycoprotein bands. PAS reaction positive bands were observed in control erythrocyte membranes (A) at Mr x (10^{-3}) = 88, 65, 41, 38, 31, 24 and 20. The PAS band numbering system and the glycophorin compositions were illustrated as previously reported (5). The positions of the various proteolytic products which stained with PAS reagent were marked by their estimated Mr values.

10^{-3} = 88, 65, 41, 38, 31, 24, and 20. (Lane A). Parallel gels stained for sialoglycoproteins showed PAS reaction positive bands at Mr x 10^{-3} = 88, 65, 41, 35, 31, 24 and 20 (Fig. 2A-C). About four times as much sample (60ul packed ghost equivalents) was loaded on the PAS stained gels as was applied to the Ag/CB gels in order to obtain comparable staining intensities. The yellow Ag stained bands are directly superimposable on the PAS positive bands. Consistent with earlier results (13), cold 0.1 M NaOH stripped ghost pellets comprised lipid vesicles containing integral proteins. The pellet (Fig. 1B) comprised CB stained band 3 and 7 in addition to yellow Ag stained glycophorins. The PAS stained gel (Fig. 2B) showed only the corresponding yellow Ag stained bands. The asialoglycoprotein band 3 was neither stained by PAS nor with Ag.

The crude sialoglycoprotein extract was comprised of glycophorin A in monomeric and dimeric forms, but was completely devoid of band 3 protein (Fig. 1C). Some contaminating CB stained bands, notably spectrin, also were observed.

Simultaneous Cleavage of Glycophorin A and Band 3 by Chymotrypsin - Figure 1D-F shows the Ag/CB stained digestion patterns of ghosts and derivatives after intact cells were digested with DFP pretreated chymotrypsin. Digestion produced two differentially stained and prominent fragments which migrated at 62 - 68,000 Mr (Fig. 1D-F). Careful relative molecular weight measurements indicated that the CB stained component migrated at 62,000 Mr whereas the yellow Ag stained band was centered at 68,000 Mr. These fragments were observed in ghosts and the NaOH stripped pellet (Fig. 1D, E). The crude sialoglycoprotein extract (Fig. 1F) contained the 68,000 Mr fragment but not the 62,000 Mr CB stained fragment. A second YSS chymotryptic fragment was observed at 47,000 Mr (best seen in the NaOH pellet (Fig. 1E). Concomitant with the appearance of these new fragments, reduction of staining in the band 3 and PAS-1 (YSS) were noted (Fig. 1D,E).

Parallel gels stained with PAS reagent showed positive staining of fragments at Mr = 68,000 and 47,000 but not at Mr = 62,000 (Fig. 2D-F). Residual undigested parent PAS-1 was clearly shown at Mr = 88,000. However, glycophorins B and C were refractory to cleavage by chymotrypsin (Fig. 2D). The PAS positive bands were directly superimposable on the yellow Ag stained bands. These results suggest that the yellow 68,000 Mr and 47,000 bands were derived from glycophorin A dimer (88,000 Mr) and glycophorin A - B heterodimer (65,000 Mr). The 62,000 Mr CB stained band was derived from band 3, however. Several commercial chymotrypsin preparations including DFP-chymotrypsin, TLCK-chymotrypsin and twice crystallized Sigma Type II gave identical membrane surface polypeptide fragmentation patterns (not shown).

In accord with published results (7, 15) the 35 - 45,000 Mr carboxyl-terminal chymotrypsin fragment of band 3 was often not seen in these gels (Fig. 1D,E,F). This fragment stains poorly with CB perhaps because of

its high carbohydrate content or its augmented sensitivity to further proteolysis (3, 7, 20, 21).

Digestion of Intact Erythrocytes with Trypsin - To rule out the possibility that the observed cleavage of glycophorin A by chymotrypsin was due to residual contaminating trypsin, intact cells were digested with TPCK-trypsin as described in "Materials and Methods" section. Figure 1 H-J shows the fragmentation patterns obtained for ghosts and derivatives. The Ag/CB stained gels showed complete disappearance of the yellow 88,000 Mr sialoglycoprotein band with concomitant appearance of yellow 40,000 Mr cleavage product. This cleavage product is clearly seen in the ghosts and derivatives (Fig. 1H-J). The fidelity of the yellow Ag stain indicates a direct precursor - cleavage product relationship between glycophorin A dimer (88,000 Mr) and its cleavage product of 40,000 Mr. As previously reported (22) and confirmed in this study glycophorin C both monomer and dimer were cleaved by Trypsin (Fig. 2H-J). Neither was band 3 cleaved nor did the ghost and NaOH stripped ghost pellet show any unusual CB stained bands. These results demonstrate the strict specificity of trypsin towards glycophorin A and its lack of activity towards band 3.

Sequential Chymotrypsin/Trypsin Digestion of Intact Erythrocyte - The absence of overlap between glycophorin A dimer (88,000 Mr) fragmentation pattern produced by trypsin on the one hand, and chymotrypsin on the other was interpreted as evidence that these proteases cleave the same glycophorin substrate at different loci on the cell's external surface. Because the 40,000 Mr trypsin cleavage product of PAS-1 (Fig. 1H-J and Fig. 2H-J) is smaller than the 47,000 and 68,000 Mr chymotrypsin cleavage products, treatment of intact cells with chymotrypsin followed by trypsin digestion should convert the 47,000 and 62,000 Mr fragments to residual lower Mr fragments. The rationale of this experiment was that the 47,000 and 68,000 Mr fragments were each composed of segments of glycophorin A which contain trypsin sensitive sites. Accordingly, washed intact cells were first treated with chymotrypsin then followed by trypsin sequentially. Figure 1K-M shows

that this prediction was verified. Conversely, intact cells treated with trypsin then followed by chymotrypsin treatment would not be expected to show the intermediate 47,000 and 68,000 Mr fragments. By prediction, it should generate a pattern similar to that obtained with trypsin alone, which we also found (Fig. 1N-P). Verification of these predictions on the basis of the Ag/CB stained gel profiles was clearly supported by parallel gels stained with PAS, see Fig. 2K-P.

Effect of Neuraminidase on Intact Cells Pretreated with Trypsin or Chymotrypsin - To provide further evidence that the 47,000 and 68,000 Mr fragments obtained with chymotrypsin were cleavage products containing sialyl moieties, the effect of neuramindase on intact cells pre-digested with chymotrypsin was examined. Experimental controls comprised untreated intact cells and cells treated with neuraminidase alone. We observed shifts in Mr of the 47,000 and 68,000 fragments towards lower Mr values upon removal of sialyl groups (results not shown). Control erythrocytes treated with neuramindase alone showed shifts in all sialoglycoprotein bands as reported (10). In parallel experiments with cells pre-digested with trypsin, neuraminidase treatment caused similar shifts in the apparent Mr of the 40,000 and 18,000 Mr fragments to lower values indicating that these fragments also contained residual sialic acid.

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