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CHROMOSOMA

A Pepsin-Revealed Material Possibly Related to Chromosomal Banding

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Abstract. The enzymes pepsin, α-chymotrypsin, trypsin, RNase and DNase were applied to preparations of human metaphase chromosomes before staining to study whether dissociable materials related to the formation of G-, Q- and C-bands would be seen. Treatment with active pepsin but not the other enzymes revealed material with ribonucleo-protein properties which dissociated from the chromosomes and formed a halo. — Lateral extensions from the chromatids stretched to the rim of the halo and appeared at positions corresponding to G-bands. A G-band may be defined as a ring of stable chromatid-matrix binding at positions where the chromatids coil to form lateral extensions.

Introduction

The formation of G-, Q-, and C-bands in relation to metaphase chromosome structure has been extensively investigated but remains controversial (see for example Comings et al., 1973; McKay, 1973; Schwarzacher, 1976; Burkholder, 1976). I report here observations which suggest the presence of a chromosomal material which may be involved in the formation of the chromosomal banding pattern.

Materials and Methods

Cell Culture. Chromosomes were prepared from human peripheral blood lymphocytes by standard methods (Schwarzacher and Wolf, 1974), using chromosome medium 1 A (Gibco) containing phyto-haemagglutinin (Gibco) for a 3-d culture, incubation with 1 μg/ml colcemide (Gibco) 2 h before harvest, 0.075 M KCl for hypotonic treatment, and fixation with methanol-glacial acetic acid (3:1) and air-drying.

Treatment and Reagents. Soon after air-drying, the slides (except those planned to age) were treated with solutions of various enzymes for different durations as described in detail below. All enzymes used in this study were dissolved in 0.9% NaCl solution and incubated at room temperature

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except where otherwise stated. Preparations were incubated in pepsin (Merck), specific activity of 1.000 units/g at a concentration of 10 and 20 mg/ml, pH 1.6 for 10, 30 and 60 min, respectively. The 10 mg/ml concentration was also used at pH 6.5 for 10 and 30 min; 10 mg/ml of the same enzyme were inactivated by boiling at 100° C for 10 min and cooled to room temperature. The resulting turbid solution was adjusted to pH 1.6 with 1 N HCl and slides incubated in this solution for 30 and 60 min. Normal saline at pH 1.6 was used to test the pH effect. α-Chymotrypsin (Serva Heidelberg), specific activity of 45 units/mg at a concentration of 0.22 mg/ml and pH 4.55 and 8.0, was placed on chromosome preparations for 1 and 30 min, respectively. DNase (Boehringer Mannheim), specific activity of 1.000 units/mg, was applied at a concentration of 50 µg/ml for 2 and 10 min at 37° C. Trypsin (Flow Laboratories, Bonn) had a concentration of 0.25% and was used for 30 and 60 s. RNase (Serva Heidelberg), specific activity of 20 units/mg, was used at a concentration of 100 and 500 µg/ml for 60 min at 37° C. Ethylendiamintetra-acetic-acid-Na_2-salt (0.02% EDTA, Serva Heidelberg) was dissolved in distilled water and used for 60 min at 37° C.

Staining. After the desired pretreatments (see Table 1), slide preparations were stained with the following substances and washed with distilled water after staining except where otherwise stated:

a) 1% 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dansyl chloride, Serva) in acetone plus an equal volume of 0.2 M $NaHCO_3$ at a resulting pH of 9.3 overnight at 37° C and washing with acetone/water (1:1).

b) 0.3 mg/ml of quinacrine mustard (Sigma St. Louis USA) in McIlvaine's buffer, pH 6.0 for 20 min and washing in the same buffer.

c) 10% Giemsa (Merck) in phosphate buffer pH 6.88 for 10 min.

d) 4,5,4,5-Dibenzo-3,3-diethyl-9 methyl-thiacarbocyanin-bromide (Stains-all, SA, Serva) at a concentration of 0.005% in a mixture of formamide and water 1:1 (v/v) for 1 and 10 min.

e) 0.1% fast green (Serva) in distilled water for 30 min at pH 8.2.

f) 0.125% acridine orange (Sigma) in Sørensen buffer pH 6.0 for 5 min and washing in the same buffer for 15 min.

In order to have a handy standard of the colors produced by the stain "stains-all", commercially purified samples of DNA, RNA and albumin (Serva) were electrophoretically fixed in 1.5% agarose gel using 0.05 M barbital buffer (Merck). The gel was dried and stained with "stains-all" for 10 min.

Analysis. Chromosome preparations were made from 25 different cultures. Thirteen of the cultures were analyzed in detail to give a total of 1,300 metaphases randomly selected as the first 100 metaphases along the preparation slide. The remaining 12 cultures were spot checked by analyzing 10–15 metaphases for each culture. All observations were done with light microscopy and photography.

Results

The effects of enzymes on fixed human metaphase chromosomes from 25 (7♀, 18♂) cultures were examined by a variety of staining techniques. The results are summarized in Table 1. Most of the preparations treated with active pepsin revealed material from the chromosomes which dissociated in situ to form a halo around them (Fig. 1). Nearly all (99%) of the analyzed metaphases showed the dissociated material while 1% did not show a separation. The latter did not always involve the same chromosomes.

The rim of the halo stained reddish with "stains-all", blue with Giemsa, did not stain with alkaline fast green (presence of halo checked by restaining with Giemsa), was green-fluorescent with dansyl chloride and quinacrine mustard and red fluorescent with acridine orange (Fig. 2). Strand connections which stained like the dissociated material were frequently seen between the chromatids

Table 1. Effect of enzymes and reagents on fixed human metaphase chromosomes

Treatment	Matrix separation	Staining	
		Matrix	Chromatid
Pepsin + quinacrine mustard	+	Green-fluorescent	Green-fluorescent
Pepsin + dansyl chloride	+	Green-fluorescent	Green-fluorescent
Pepsin + acridine orange	+	Orange-fluorescent	Green-fluorescent
Pepsin + stains-all (SA)	+	Red	Blue
Pepsin + Giemsa	+	Blue	Red
Pepsin + alkaline fast green	+	Not stained	Faint green
Pepsin + trypsin + Giemsa or SA	+	Matrix reduced	G/C-banded
Pepsin + EDTA + Giemsa	+	Blue	Red
EDTA + Pepsin + Giemsa	+	Blue	Red
Pepsin + RNase + Giemsa	+	Pepsin-halo disappears	
Pepsin + RNase + trypsin + Giemsa	+	Halo disappears only	C-bands form
Pepsin + DNase + Giemsa	+	Halo does not disappear	
RNase + Pepsin + Giemsa	−	No halo formed	
RNase + Giemsa	−	Uniformly stained	
Inactivated pepsin + Giemsa	−	Uniformly stained	
Pepsin at pH 6.5 + Giemsa	−	Uniformly stained	
Trypsin + Giemsa	−	G-bands	
Trypsin + SA	−	G-bands deep blue, interbands light blue	
Stains-all alone	−	Uniformly stained/G-band	
Quinacrine mustard alone	−	Q-bands	
α-Chymotrypsin + Giemsa or SA	−	G-bands	
DNase + Giemsa or SA	−	G-, C-bands or digested	
Normal saline at pH 1.6 + Giemsa	−	Uniformly stained	

and the rim of the halo, more prominent at the centromeres of sub- and metacentric chromosomes (Fig. 1, arrow). The chromatids inside the halo were generally loosened, stained blue with "stains-all", faint green with alkaline fast green, red with Giemsa, green-fluorescent with dansyl chloride and quinacrine mustard. About 90% of the metaphases stained with quinacrine mustard, fluoresced generally uniformly except for the distal part of chromosome Y, satellites of some D- and G-group chromosomes and the centromere of chromosome 3 in certain cultures. The chromatids fluoresced green with acridine orange also but showed a tinge of red in interband regions. Lateral extensions (of chromatid nature according to staining) from the chromatids traversed the halo and connected to the rim of it. The more easily identifiable ones corresponded to the G-band positions.

Exposure of chromosomes to the inactivated enzyme as well as to normal saline at pH 1.6 and to pepsin at pH 6.5 did not reveal the halo. In 8 d old preparations, 40% of the metaphases also failed to show the halo, and the dissociated material in the others was not as well separated as in the fresh slides. Chromosomes incubated in EDTA and subsequently treated with pepsin (10 mg/ml) still showed a dissociated material. Figure 3 shows results of chromo-

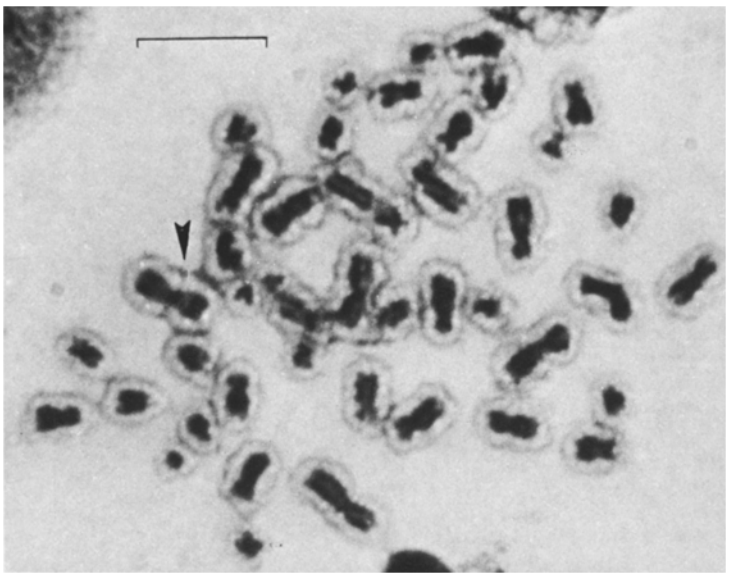

Fig. 1[1]. Metaphase chromosomes treated with pepsin (10 μg/ml) for 60 min and stained with "stains-all". The arrow shows connections (staining like the matrix) between the centromere and the rim of the halo

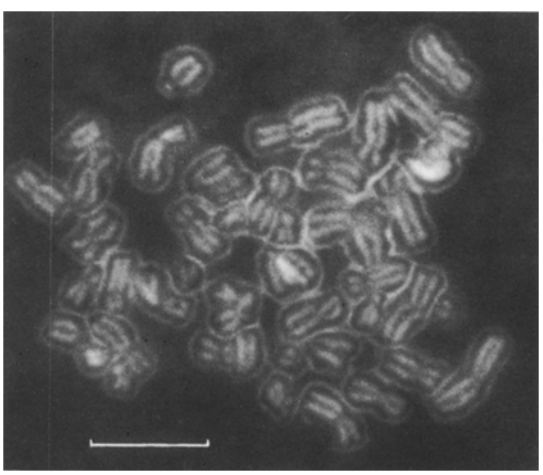

Fig. 2. Quinacrine mustard stained chromosomes after treatment with pepsin for 10 min

[1] Each bar on the Figures represents 10 μm

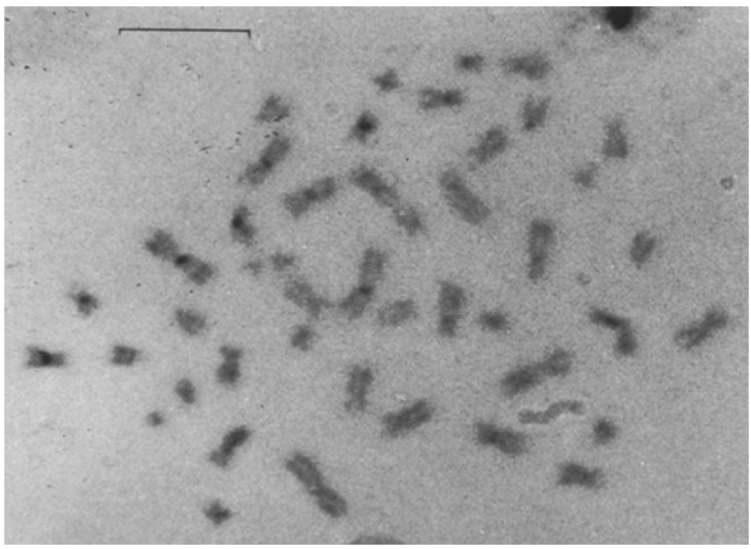

Fig. 3. The same metaphase treated as described in the legend to Figure 1, destained with fixative (see methods), incubated in trypsin for 1 min and restained with Giemsa. Note C-Bands

somes pretreated with pepsin for 60 min (metaphase in Fig. 1), destained with fixative and subsequently exposed to trypsin for 1 min and restained with Giemsa whereby the halo disappeared and the chromosomes became C-banded. About 30% of the metaphase still showed G-bands. Shorter treatments with pepsin (10 min) followed by trypsin (30 sec) also gave G-bands. The rim of the halo was faint but the bands could still be observed to be continuous with the lateral extensions from the chromatids to the rim of the halo (Fig. 4). Pepsin dissociated the material from the chromosomes dependent on the duration of treatment. Thus the size of the halo increased over 10, 30, and 60 min at high as well as at low pepsin concentrations until the halo disrupted in some metaphases.

A film of material around many interphase nuclei dissociated from the nuclear mass after pepsin treatment, stained in a similar way to the rim of the halo around chromosomes, and disappeared or was considerably reduced after trypsin treatment.

Incubations with DNase for 2 min gave G-bands in 25% of the 100 observed metaphases, 50% were C-banded, and the rest uniformly stained. In longer incubations for 10 min, only the contour of the chromosomes and the midline between the chromatids stained red with "stains-all". Treatment with α-chymotrypsin at both high (8.0) and low (4.55) pH and staining with Giemsa or "stains-all" gave G-bands but no dissociation of the chromosome material. When chromosomes with the dissociated material after pepsin treatment were incubated with RNase at low (100 μg/ml) and high (500 μg/ml) concentrations, the dissociated material (Fig. 5a) disappeared or became very faint on restaining

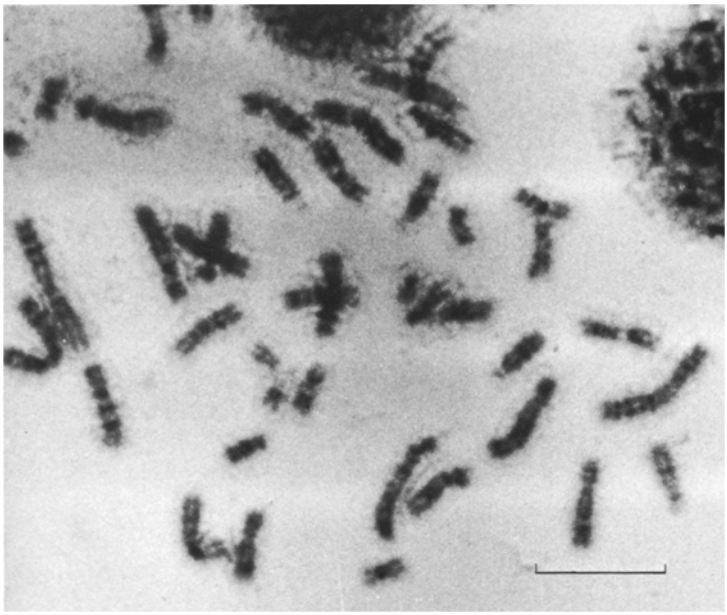

Fig. 4. Metaphase treated with pepsin for 1.0 min, washed with normal saline and incubated in trypsin for 30 sec. G-bands extend to the rim of the halo

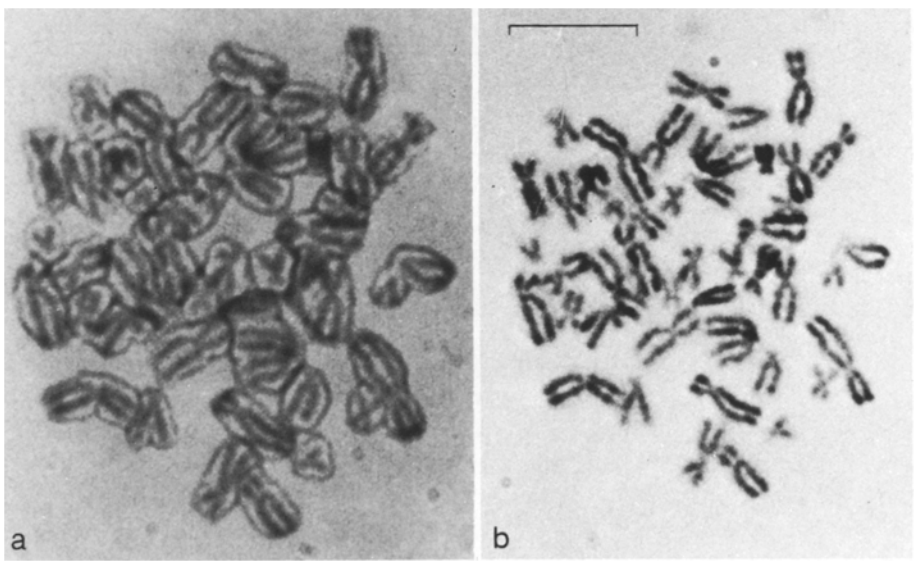

Fig. 5a and b. a Dissociated material with pepsin treatment. **b** The dissociated material disappears after incubation with RNase (100 μg/ml) for 60 min at 37° C and staining with Giemsa

with Giemsa (Fig. 5b). When the same metaphase was then exposed to trypsin and restained, the chromosomes were C-banded. The pepsin dissociated material was, however, not labile to DNase (50 μg/ml) after incubation for 60 min at 37° C.

Staining for 1 min with "stains-all" alone without pretreatment resulted in approximately 50% of the metaphases staining red along the periphery of relatively swollen chromosomes, more densely staining at centromeric positions. The remaining metaphases also stained red in the periphery but in addition the chromatid strands stained brilliantly blue.

Discussion

This study indicates the presence of a chromosomal matrix that is stainable and dissociates easily from the chromatids following pepsin treatment. Although the mechanism involved in the matrix separation was not investigated, it is possible that a fixed structure or a highly viscous material is involved in its formation. Support is given by the presence of strands of matrix-similar nature (according to staining) which still connect to the centromeric regions of sub-meta and metacentric chromosomes, and by the results of Ghosh et al. (1978) reporting the presence of a filamentous matrix system in nuclei and chromosomes.

The matrix probably contains RNA and protein. The stain "stains-all" is reported to stain proteins pink or red, RNA bluish-purple and DNA blue according to the manufacturer's information and Dahlberg et al. (1969). Although the separated matrix stained predominantly reddish, it was difficult to unambiguously identify its color as that seen with either RNA or protein in the standard samples. The matrix fluoresced with dansyl chloride, a dye which reacts with amino acids, peptides and proteins to give sulfonamide conjugates which fluoresce under UV irradiation as reported by Utakoji (1974). The susceptibility of the matrix to trypsin indicates its protein nature. Its failure to stain with alkaline fast green would indicate that histones are absent although it may be heterogeneous. The matrix was resistant to DNase but labile to RNase, suggesting the RNA is a component of the matrix. Thus the chromosomal matrix may be a ribonucleoprotein. This supports the results of Pierpont and Yunis (1977) who found a wide-spread distribution of chromosomal RNA in human chromosomes and those of Ghosh et al. (1978) who have reported a matrix with acidic proteins. Pepsin at high pH did not separate the matrix. Since pepsin is a protein, is was thought that it could have been non-specifically accumulated around the chromosomes and nuclei. This assumption was disproved when the inactivated enzyme was applied and did not cause the halo. Since EDTA removes contaminants from the surface of the chromosomes, the dissociated material must have come from the chromosomes themselves because the halo still appeared or persisted after EDTA application. In any case, the rim of the halo stained differently from the background artifact material on the slide whenever present.

A relationship between the pepsin-revealed matrix and chromosomal banding seems to be based on the differential binding between the matrix and the chromatids along their length. Observations that support this arguments are: 1. the thicker lateral extensions through the halo occur at positions corresponding to G- and Q-bands, whereby in some chromosomes the contact points on the rim of the halo are indented. 2. positions on the chromosomes where the matrix is not removed by pepsin stain more densely than the others. After RNase application the halo disappears but these areas remain densely stained. 3. on addition of trypsin for brief periods to pepsin-treated chromosomes, the halo disappears or becomes faint and G-bands form at positions where the halo is indented.

It is thus suggested that dark G-banding takes place preferentially where the chromatid-matrix binding is very stable, in this case where the chromatid lateral extensions are present. G-bands may thus be rings of stable matrix-binding around the chromatids, similar to the ridges reported by Gormley and Ross (1976). On introducing trypsin or other G-band producing agents, the chromatid-matrix association in the interband regions gets easily distorted in such a way that the chromosomes stain much less. If the observed lateral extensions from the chromatids are produced when the chromatids coil (cf: epichromatin of Stubblefield, 1973), the staining of a G-band should also be related to the relatively more amount of chromatin present at these points.

C-bands would be best considered as positions where the chromatid-matrix is so stable that the chromatid hardly releases the matrix as indicated by the matrix strand connections persistent at the centromeres (cf: Burkholder, 1976). The chromosomal matrix also appears to have a role in the formation of Q-bands. The uniform fluorescence obtained on applying quinacrine mustard to pepsin-treated chromosomes could be interpreted to mean that when the matrix is removed by pepsin, the stain gains unhindered access to chromatin where it interacts with DNA along the chromatids. The lateral extensions might not have been identifiable because of the bright fluorescence. The dissociated matrix fluoresced with quinacrine mustard probably through the RNA content. There is a close similarity between the chromosomal matrix and the film of material that surrounds the interphase nuclear mass with response to trypsin, RNase and all the staining applied. It is conceivable that this film of material is derived from the nuclear matrix.

The fact that DNase produced G-bands in some metaphases also hints to the protective nature of the matrix at the G-band positions and their staining ability. Evidence that supports C-bands as areas a degree more stable than G-bands (McKay, 1973) is the fact that when chromosomes are subjected to the enzymes pepsin, RNase and trypsin sequentially, the chromosomes are only C-banded. Since the enzyme α-chymotrypsin is chemically similar to pepsin it was expected that similar results would be obtained, but this was not the case.

In conclusion, it was shown that with the use of pepsin, a matrix possibly of ribonucleoprotein nature can be dissociated from the chromosomes without drastic morphological changes of the latter. The matrix seems to be involved in the formation of G-, Q-, and C-bands of chromosomes.

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Note Added in Proof

After this paper had been accepted for publication, the stainability of the pepsin-dissociated material with silver nitrate was tested using the Ag-NOR method [S.E. Bloom and C. Goodpasture: An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. Hum. Genet. **34**, 199–206 (1976)]. Only the dissociated material around individual chromosomes stained with silver just as the nucleolar organiser regions (NORs) on control chromosomes. Thus it may be deduced from my observations that what stains in NOR are non-structural (loose) ribonucleoproteins.