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Profiling of the CD4 Receptor Complex Proteins

G. I. Krotov[1]*, M. P. Krutikova[1], V. G. Zgoda[2], and A. V. Filatov[1]

[1]*National Research Center Institute of Immunology, Federal Biomedical Agency of Russia, Kashirskoe Shosse 24/2, 115478 Moscow, Russia; fax: (499) 617-7765; E-mail: grkrotov@rambler.ru*
[2]*Orekhovich Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, Pogodinskaya ul. 10, 119121 Moscow, Russia; fax: (495) 246-1641; E-mail: vic@ibmn.msk.ru*

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Abstract—Intermolecular complexes produced by the CD4 molecule were studied. To preserve the integrity of weak protein–protein interactions of the CD4 antigen, cells were lysed in a mild nonionic detergent Brij97. Protein constituents of the complex were identified by our previously proposed fluorescence immunoprecipitation assay with subsequent mass spectrometry. In total, 26 proteins associated with CD4 were identified on CEM cells. The CD4 complex included the following major components: tyrosine phosphatase CD45, transferrin receptor CD71, tyrosine kinase Lck, and a lymphocyte phosphatase-associated phosphoprotein LPAP. The CD4 complex also contained some components of cytoskeleton and heat shock proteins. The association between CD4, CD71, and CD45 molecules was confirmed by immunoblotting. The CD4 complexes were not detected on the U937 myeloid cells lacking Lck and LPAP. We attempted to quantitatively characterize the CD4 complex composition.

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Key words: protein complexes, nonionic detergents, immunoprecipitation, mass spectrometry, CD4

The majority of proteins function through interactions with other molecules: proteins, lipids, carbohydrates, and nucleic acids. Characterization and identification of protein complexes are now in the forefront of biochemical studies. New protein–protein interactions are most often detected using molecular biological systems of two-hybrid screening [1, 2], luminescence-based interactome mapping [3], and also fluorescence resonance energy transfer (FRET) [4]. But these highly sensitive approaches have some disadvantages. Thus, two-hybrid systems give a high percentage of false positive results and do not allow membrane protein interactions to be studied. Moreover, these systems may be used to study only binary protein–protein interactions, whereas real protein complexes can be much more complicated and consist of more than two interacting molecules [5]. Protein complexes are now actively studied using affinity approaches, when a complex is physically isolated from the cell lysate and its components are identified by mass spectrometry. Such approaches include, in particular, the so-called tandem affinity purification, the epitope tagging method using recombinant proteins that were fused with different tags (Myc, HA, Flag, KT3), and also a "pull-down" method [6]. However, these strategies require preparation of fusion proteins and their subsequent transfection into the cell, which can give false positive results. Therefore, co-immunoprecipitation remains the most reliable method to prove the existence of protein associations [7]. In this case, interacting proteins are isolated from cell lysate using immobilized antibodies, which recognize an epitope on the known component of the complex, and then associated proteins are usually revealed by immunoblotting [8]. Currently mass spectrometry is most frequently used to identify components of protein complexes purified by affinity methods [9, 10].

In spite of numerous studies on protein–protein associations, usually only the qualitative composition of complexes is under study and their quantitative composition is studied very seldom [11]. We proposed earlier a method of fluorescence immunoprecipitation assay (FIPA) [12]. An advantage of this method is a simultane-

Abbreviations: FIPA) fluorescence immunoprecipitation assay; FITC) fluorescein isothiocyanate; FRET) fluorescence resonance energy transfer; LS ESI-MS/MS) tandem mass spectrometry with electrospray ionization with peptide pre-separation by liquid chromatography; MS) mass spectrometry; MS/MS) tandem mass spectrometry; PBS) phosphate-buffered saline; R6G) rhodamine 6G succinimide ester.

* To whom correspondence should be addressed.

ous visualization of all co-precipitated components of a complex. The method has a wide dynamic range that promotes measuring and comparing quantities of the co-precipitated proteins. Finally, the method is compatible with mass spectrometry (MS), which provides for identification of the detected proteins. In this article, the potential of FIPA is shown, exemplified by investigation of CD4 complexes.

The keen interest in studies on protein complexes of the surface glycoprotein CD4 is due to its function in the immune system. The 55-kD transmembrane protein CD4 consists of an N-terminal extracellular part, which includes four immunoglobulin-like domains, a transmembrane part, and a short cytoplasmic part [13]. CD4 protein is expressed only on immune system cells—T-helpers and monocytes. This protein plays an important role in development and activation of T-cells; as a co-receptor, it increases the T-lymphocyte antigen receptor affinity for peptides of class II histocompatibility antigens. CD4 is also involved in signal transmission through the associated tyrosine kinase Lck [14, 15]. Moreover, CD4 in a complex with chemokine receptors CXCR4 and CCR5 is an HIV-1 receptor [16, 17].

In the present work, we attempted to directly isolate the intermolecular complex produced by CD4 and identify components of this complex. Using mass spectrometry, we detected for the first time the presence of LPAP molecule in the complex.

MATERIALS AND METHODS

Cell cultures, antibodies, and flow cytometry. Human cell lines CEM and U937 were cultured in DMEM medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, and gentamicin (80 mg/liter) at 37°C in a humid atmosphere with 5% CO_2. The antibody OKT4 (IgG1, anti-CD4) was kindly presented by M. B. Zaitseva (CBER, USA). Antibodies EM4 (IgG2a, anti-CD4), LT71 (IgG2b, anti-CD71), and LT45 (IgG2a, anti-CD45) were earlier produced in our laboratory.

For immunofluorescence staining, a pellet containing (1-5)·10^5 cells was mixed with 25 µl of unlabeled primary antibodies and incubated at 4°C for 30 min. The cells were washed twice in phosphate-buffered saline (PBS) by centrifugation at 320*g* for 5 min. Then the cells were incubated with FITC-labeled secondary antibodies representing $F(ab')_2$ fragments of sheep antibodies to mouse Ig (Sigma, USA) and subsequently washed. The stained cells were analyzed using a FACScan flow cytometer (BD Biosciences Immunocytometry Systems, USA). The fluorescence brightness was measured in arbitrary units on determination of its average intensity. The cells treated only with the secondary antibodies were used as a negative control.

Fluorescence immunoprecipitation assay (FIPA). The protein complex produced by CD4 molecules was precipitated from lysate of cells prelabeled with the aminoreactive fluorescent dye Rhodamine 6G (R6G) (Sintol, Russia). The CEM cells were washed three times in PBS (pH 7.4) and 4·10^7 cells were resuspended in 1 ml of PBS (pH 8.0). The cells were labeled at room temperature by two additions to 1 ml of the cell suspension of 50 µl of R6G succinimide ester solution in dimethyl sulfoxide (Serva, Germany) with the concentration of 3 mg/ml at zero time and a second addition 10 min later. Ten minutes after the second addition of the fluorochrome, the reaction was stopped by washing the cells in 10 mM glycine in PBS (pH 7.4). Then the labeled cells were washed three times in cold PBS. Cell lysate was prepared on an ice bath by suspending the cell precipitate (4·10^7 cells) in 1 ml of cold lysing buffer consisting of 20 mM Tris-HCl (pH 8.0) supplemented with 1% detergent, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Fluka, Switzerland), 0.7 µg/ml pepstatin A, and 1 µg/ml leupeptin (Sigma). Triton X-100 (Ferak, Germany) or Brij97 (Sigma) was used as the detergent. After 30 min of incubation on ice, the nuclei and cellular debris were removed by centrifugation at 2800*g* for 10 min at 4°C. The resulting supernatant was centrifuged again at 20,000*g* for 10 min at 4°C. Then the lysate was sequentially clarified by incubation for 1 h on an ice bath with BSA and normal mouse IgG covalently immobilized on BrCN-Sepharose (Pharmacia, Sweden) (200 µl of 50% v/v suspension of each reagent per ml of the lysate) and on Affi-Gel Protein A Gel protein A-agarose (Bio-Rad, USA) (100 µl of 50% v/v suspension per ml of the lysate). For immunoprecipitation, first rabbit antibodies to mouse Ig and then a specific monoclonal antibody were applied on protein A-agarose. The sorbed antibodies were immobilized by covalent cross-linking in the presence of dimethyl pimelimidate [8]. For immunoprecipitation, 1 ml of the clarified lysate was added to 50 µl of protein A-agarose with the antibody complex preformed on it and incubation overnight at 4°C. The immunoprecipitates were washed thrice, each time in 800 µl of lysing buffer. The bound fluorescence-labeled protein complexes were eluted from the carrier by heating of the immunoprecipitates for 5 min at 95°C in buffer for Laemmli electrophoresis. The eluted proteins were separated by electrophoresis in 10% SDS-polyacrylamide gel under reducing conditions in the Laemmli buffer system. Pre-stained High Range or Low Range proteins (Bio-Rad) were used as molecular weight markers. After electrophoresis, the proteins were visualized in the gel using a Molecular Imager FX Pro fluorescence scanner (Bio-Rad) without additional gel fixation, staining, or drying. On preparing specimens for subsequent mass spectrometry, the antigen complex was isolated on a column with antibodies immobilized on protein A-agarose. The volume of anti-CD4 beads was 300 µl. The elution was performed using an acidic buffer:

50 mM glycine-HCl (pH 2.5) supplemented with 150 mM NaCl and 0.1% Triton X-100. The eluate was collected in 100 µl fractions. The protein yield from the column was recorded by fluorescence of the fractions measured with a Gene fluorimeter (DNA-Technology, Russia).

Identification of proteins by LC ESI-MS/MS mass spectrometry. Protein bands (~5 mm^3) were excised from the gel and washed thrice in deionized water. The gel pieces were incubated in 50% (v/v) acetonitrile in 200 mM ammonium bicarbonate (pH 8.9) for 20 min and then in 100% acetonitrile for 20 min. The gel pieces were dried for 1 h at room temperature. Depending on the size, the gel slices were supplemented with 5-8 µl of sequencing grade modified trypsin (Promega, USA) solution (25 ng/µl) in 50 mM ammonium bicarbonate. After rehydration of the gels for 40 min at 4°C, the proteins were hydrolyzed overnight at 37°C. Tryptic peptides were extracted with 12-17 µl of a solution consisting of 5% (v/v) acetonitrile (Merck, Germany) and 0.5% formic acid in deionized water for chromatography (Merck) for 30 min. The peptide mixture in the volume of 1.5-2.0 µl was separated for 60 min by reversed-phase HPLC on an Agilent 1100 instrument (Agilent Technologies Inc., USA) using a linear gradient of 5-80% (v/v) acetonitrile supplemented with 0.1% formic acid. Then the peptides were ionized by electrospray and detected by ion trap (Agilent 1100 SL Series MSD Trap; Agilent Technologies Inc.) in the range from 200 to 1800 m/z (LC-ESI-MS/MS). The proteins were identified by tandem mass-spectra (MS/MS) of tryptic peptides using the MASCOT program (www.matrixscience.com), and all MS/MS spectra were identified by correlation with a sequence of human protein peptides presented in the NCBI database (www.ncbi.nlm.nih.gov). For the search the following parameters were pre-assigned: *Homo sapiens* taxon, trypsin for hydrolysis of peptide bonds, mass tolerance for the monoisotopic peptide window was set to ±2 daltons, the MS/MS tolerance window was set to ±0.6 dalton, one missed cleavage was allowed, possible different acryl-induced cysteine modifications, and oxidation of methionines. The criteria of positive identification were set as following: minimum score is 55 and at least three positive identifications from three different gels.

Immunoblotting. Proteins resulting from the immunoprecipitation were separated by electrophoresis in 10 or 7% SDS-polyacrylamide gel under nonreducing conditions in the Laemmli buffer system. The separated proteins were transferred by blotting for 1 h onto a nitrocellulose membrane using a Mini Trans-Blot set (Bio-Rad) at 100-120 V in buffer containing 25 mM Tris, 192 mM glycine (pH 8.3) and 20% ethanol (v/v). The membranes were blocked overnight at 4°C with 5% (w/v) dry defatted milk in PBS with 0.1% Tween 20 and incubated for 1 h at room temperature with biotinylated primary antibodies LT45 (anti-CD45) or EM4 (anti-CD4). The membranes were washed and incubated in the presence of streptavidin conjugated with horseradish peroxidase (Amersham, USA). Visualization was performed by chemiluminescence using commercial ECL reagent (BioRad).

RESULTS

Co-precipitation of CD4-associated proteins occurs on lysis in the mild nonionic detergent Brij97. Two CD4-positive cell lines were used: the T-cell lymphoblastoid line CEM and myeloid line U937. To identify the group of polypeptides capable of interacting with CD4, surface proteins expressed on these cells were determined by immunofluorescence using a panel consisting of 63 monoclonal antibodies against different membrane antigens. The surface protein composition of these cell lines was rather varied. Both cell lines carried on the surface proteins as follows: CD4, CD43, CD44, CD45, CD47, CD71, CD98, CD99, CD184, and HLA I. Some polypeptides (CD7, CD38, CD53, CD59, CD81, CD82, CD147, and CD162) were expressed only on the CEM

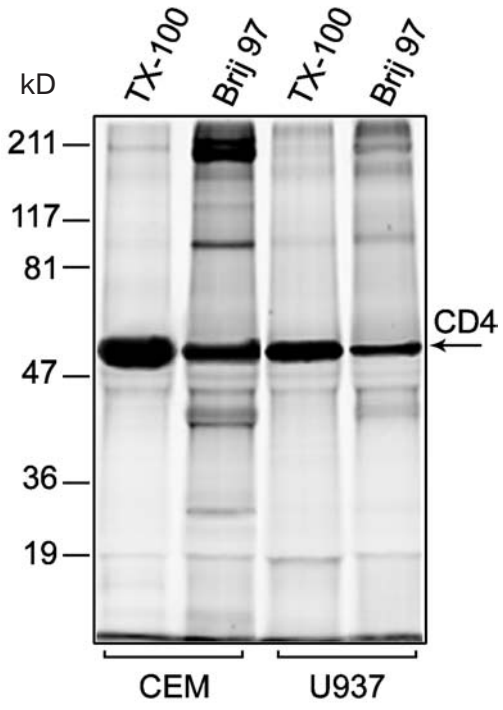

Fig. 1. Immunoprecipitation of CD4 after lysis of CEM and U937 cell cultures in detergents Triton X-100 and Brij97. The CEM and U937 cells labeled with Rhodamine 6G were lysed in these detergents. CD4 protein was isolated from the lysate using the OKT4 antibody (anti-CD4). The resulting immunoprecipitates were separated by electrophoresis in 10% polyacrylamide gel under reducing conditions in the Laemmli buffer system. A sample equivalent to $40 \cdot 10^6$ cells was applied onto each lane. The band images were obtained by fluorescence scanning of the gels.

Table 1. Identification by mass spectrometry of proteins co-isolated with CD4 and CD45 upon lysis of the CEM cells in Brij97

Gel band (Fig. 3)	Protein	Number in the Swiss-Prot database	Mw, kD	MASCOT Score*	Number of peptides					
					OKT4			LT45		Σ**
1	tyrosine phosphatase CD45	P08575	180-220	165-369	8	7	9	22	17	32
	myosin-9 (heavy chain, non-muscle IIa)	P35579	230	1084-1674	–	23	–	33	–	41
2	transferrin receptor CD71	P02786	190	56-414	2	9	4	–	2	11
3	CD4	P01730	55	55-311	5	4	4	–	–	8
	Lck	P06239	56	282-552	10	10	6	–	–	14
	vimentin	P08670	54	395-1295	–	–	22	–	10	25
	tubulin α6	Q9BQE3	55	78	3	–	–	–	–	3
	tubulin β	P07437	55	118-325	9	–	3	–	–	9
4	actin	P60709	42	707	n.i.	16	n.i.	n.i.	n.i.	16
5	LPAP	Q14761	32	69-231	2	–	–	4	4	4

Note: n.i., not identified by mass spectrometry; "–", no peptides of the protein detected.

* The minimal and maximal reliability coefficient (S) is indicated; at $S > 55$ it gives agreement error ($p < 0.05$) for a series of experiments.

** The total number of unlike peptides recorded in three experiments.

cells, whereas other proteins (CD11a, CD15, CD29, CD31, CD36, CD48, CD54, CD58, CD63, CD185, and HLA II) were specific only for the U937 cells. Because antigens CD4, CD71, and CD45 were the most interesting for subsequent study, their surface expressions were quantitatively compared by flow cytometry. The fluorescence intensity of the CEM cells on staining with a corresponding antibody was 185 for CD4, 420 for CD45, and 117 for CD71; for the U937 cells it was 152, 222, and 105, respectively. The fluorescence intensity was expressed in arbitrary units, and the autofluorescence level was no more than 10 arbitrary units.

Upon lysis of both cell types in Triton X-100 detergent and subsequent precipitation on the OKT4 antibody (anti-CD4) a single major band was observed in the electrophoregram (Fig. 1), which corresponded to molecular weight of 55 kD. Immunoblotting indicated this band to correspond to CD4 protein. Upon lysis of the CEM cells in the milder detergent Brij97, which is believed not to destroy intermolecular protein complexes, the picture was more complicated. In addition to the CD4 protein itself, in the electrophoregram there were three other intense co-precipitation bands with molecular weights corresponding to 180-200, 90, and 32 kD, and also some minor bands. However, immunoprecipitation of CD4 from the U937 cell lysate in Brij97 gave no pronounced co-precipitation bands (Fig. 1). A band in the region of 45 kD was observed in all gels and also in the negative control. Later it was identified as the cytoskeletal protein actin (Table 1) nonspecifically bound with the beads. The set of protein bands resulting from the CD4 immunoprecipitation was different from the bands obtained by isolation of antigens CD47, CD38, and HLA I (Fig. 2) and CD44 (data not shown), which were present on the membrane of the CEM cells together with CD4.

Identification of CD4-associated proteins by mass spectrometry. To isolate the complex in sufficient amount for MS, ~$3 \cdot 10^8$ fluorescently labeled CEM cells were lysed in Brij97 detergent. The CD4 complexes were isolated by immunoaffinity on the OKT4 antibody. Proteins specifically bound with the beads were eluted in acidic buffer. The protein yield from the column was recorded by fluorescence. The major peak fractions were pooled, concentrated, and separated by electrophoresis. After scanning the gels, protein bands indicated in Fig. 3 were cut out and subjected to in-gel trypsinolysis; the resulting peptides were extracted and analyzed by LC-ESI-MS/MS. We succeeded in reliable identification of several membrane proteins. The major 55-kD band was found to contain, in addition to CD4, proteins with similar electrophoretic mobilities, such as tyrosine kinase Lck, tubulin, and vimentin. In the 180-200-kD band, tyrosine phosphatase (CD45) was identified, and a heavy chain of non-muscle myosin was also found in one experiment. A transferrin receptor (CD71) and a tyrosine phosphatase C-associated protein also termed lymphocyte phosphatase-associated phosphoprotein (LPAP) were determined in the 90- and 32-kD bands, respectively.

To confirm that CD71 and CD45 are components of CD4 complexes, in further experiments we used recipro-

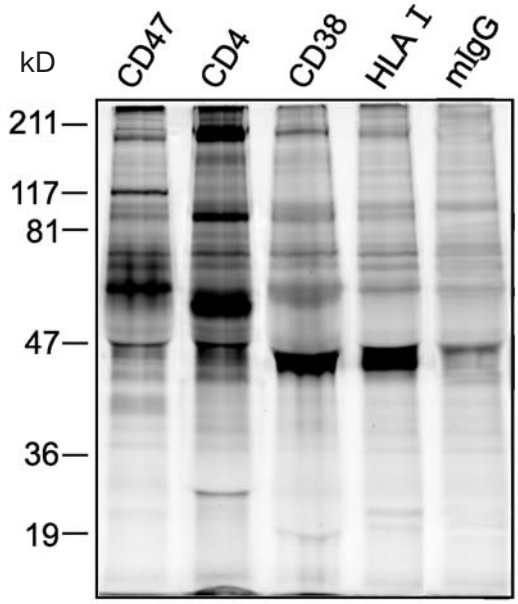

Fig. 2. Protein profiles obtained on immunoprecipitation of CD4, CD47, CD38, and HLA I from lysate of the CEM cells in 1% Brij97. The CEM cells labeled with Rhodamine 6G were lysed in 1% Brij97. The lysate was immunoprecipitated using the indicated antibodies. Normal murine mIgG was used as a negative control. Precipitated proteins were separated by electrophoresis in 9% polyacrylamide gel under reducing conditions.

cal immunoprecipitation with anti-CD71 and anti-CD45 antibodies (Fig. 3). Proteins with molecular weights of 180-200, 90, 55, and 32 kD were obtained on the LT45 antibody. By mass spectrometry we identified only three of these proteins: CD45 (180-200 kD) and co-precipitated with it CD71 (90 kD) and LPAP (32 kD). The 55-kD band was not identified because of low protein concentration. But the CD4 antigen was detected in this band by immunoblotting. Immunoaffinity purification revealed on the LT71 antibody in the gel a major protein band of 90 kD and a minor band of 180 kD. By mass spectrometry, both bands were identified as a transferrin receptor CD71. Thus, the band with the higher molecular weight was a dimer of the CD71 protein. Identical results were obtained in three independent experiments. The identified proteins, which co-precipitated with CD4 and CD45 molecules, are presented in Table 1.

To more completely determine the group of proteins possibly associated with CD4, we analyzed a whole lane obtained by electrophoresis of the CD4 precipitate. The lane was cut into pieces of about 1.5 mm in width and treated with trypsin. In addition to proteins listed in Table 1, subsequent MS/MS revealed the following proteins: spectrin, heat shock proteins Hsp90, Hsp71, and Hsp40, translation and elongation eukaryotic factor, tropomyosin, desmin, fibroblast growth factor receptor, carboxymethyl transferase isoAsp, pyruvate kinase, multifunctional protein CAD, valosin-containing protein, human elongation factor 2, binding stimulator TAR RNA, catalase, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein γ- and τ-polypeptides, β-inhibitor of dissociation Rho GDP, ZC3H12B protein, interferon-induced transmembrane protein, and the light regulatory chain of non-muscle myosin.

Existence of CD4 complexes confirmed by immunoblotting. Association between CD4, CD71, and CD45 molecules was confirmed by immunoblotting. Using corresponding antibodies, proteins CD4, CD71, and CD45 were isolated from lysates of the CEM and U937 cells in Brij97. Each of the resulting precipitates was developed with antibodies to both CD45 and CD4. Figure 4 shows that CD4 protein from the CEM cell lysate was co-precipitated with CD45, and in turn CD45 was co-isolated with CD4. And intensity of the CD45 co-precipitation band was higher than that of the CD4 co-precipitation band, and this was consistent with the FIPA results. In the immunoprecipitate obtained with the antibody to CD71 only the CD45 antigen was revealed and not CD4. Thus, the CD4–CD45 complex was isolated by reciprocal immunoprecipitation and detected by both FIPA (Fig. 3, lanes *CD4* and *CD45*) and immunoblotting (Fig. 4, lanes *OKT4* and *LT45*). In contrast, the interaction between the CD4 and CD71 antigens was observed only in FIPA on the precipitation with the antibody OKT4 (Fig. 3, lane *CD4*) and not the reverse (Fig. 3, lane *CD71*; Fig. 4, lane *LT71*).

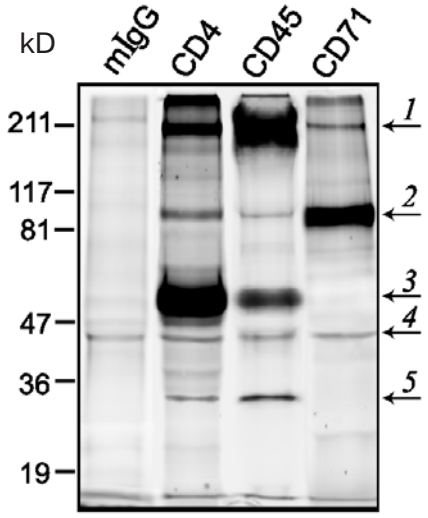

Fig. 3. Visualization of proteins isolated by immunoaffinity and identified by mass spectrometry. The CEM cells labeled with Rhodamine 6G were lysed in 1% Brij97. Immunoprecipitation was performed using antibodies OKT4 (anti-CD4), LT45 (anti-CD45), and LT71 (anti-CD71). Normal murine mIgG was used as a negative control. The resulting immunoprecipitates were separated by electrophoresis in 9% polyacrylamide gel under reducing conditions. The bands indicated with arrows (*1-5*) were cut out to be analyzed by mass spectrometry.

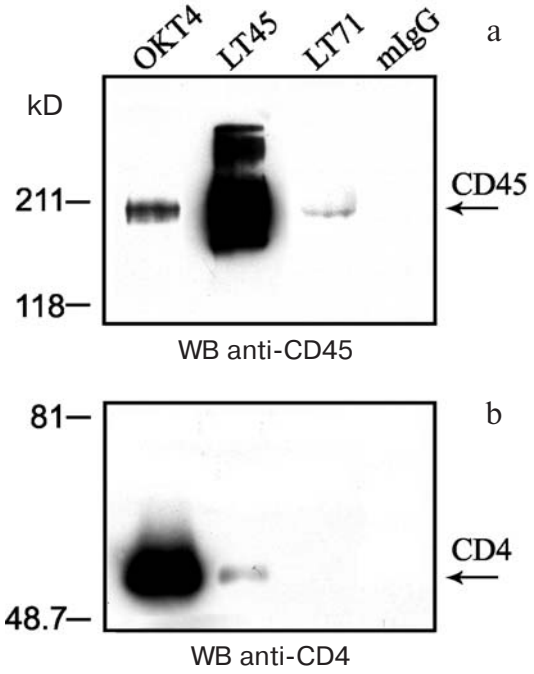

Fig. 4. Reciprocal co-precipitation of CD4 and CD45. The CEM cells were lysed in 1% Brij97, and the complexes were isolated by immunoprecipitation with antibodies OKT4, LT45, LT71, or mIgG. The proteins were identified by immunoblotting (WB) with antibodies LT45 (anti-CD45) (a) and EM4 (anti-CD4) (b).

Some discrepancies between the mass spectrometry and immunoblotting data may be explained by different sensitivities of these methods and also by different efficiencies of antibodies in the immunoprecipitation and immunoblotting tests. According to results obtained by FIPA, only the major proteins were precipitated from lysates of the U937 cells and no co-precipitation bands were found. Note that for immunoblotting the cell lysates were used without preliminary fluorescence labeling. Therefore, the CD4 association with other molecules found in the CEM cells was not an artifact of the labeling with Rhodamine.

Stoichiometry of CD4 complex. Comparison of the co-precipitation band intensities allowed us to quantitatively evaluate the ratios of proteins isolated in the CD4 complex. Integral fluorescence intensities were measured in the bands of CD4, CD71, and CD45 proteins. Because the dye was bound by the NH_2-group on the N-terminus of a polypeptide and also in lysine residues, it was supposed that the intensity of protein staining and fluorescence should be proportional to the total number of amino groups in the polypeptide. To evaluate molar ratios of the proteins involved in formation of the CD4 complex, the intensities of band fluorescence were normalized corresponding to the number of NH_2-groups in each protein. Molecular weights of CD4 and Lck are similar, and their bands are completely overlapping. In our calculations we assumed that CD4 and Lck molecules are in a complex at the ratio of 1 : 1 [15]; therefore, to normalize the CD4/Lck band intensity the total number of amino groups in these proteins was used.

First of all, we tried to evaluate the ratio of free molecules and molecules inside the complex. The protein fluorescence intensities were compared in two lanes resulting from immunoprecipitation on the antibody either to this protein or to some other component of the complex. We found that the complexes included about 30% of the total pool of CD4 molecules and 1-2% of CD45 and CD71 molecules.

Molar ratios of proteins forming the CD4 complex were calculated for six independent experiments (Fig. 5). A calculation for one experiment is exemplified in Table 2. The CD4/CD45 and CD4/CD71 ratios varied within rather wide limits. On average, 7 and 4.5 CD4 molecules corresponded to one molecule of CD45 and CD71, respectively. As noted above, on precipitation of the CD4 complex both CD4 molecules involved in the complex and free CD4 molecules were isolated. Having in mind that the complex includes no more than 30% of the total number of CD4 molecules, the real CD4/CD45 and CD4/CD71 ratios are to be approximately threefold lower that the above-presented ones. The CD45/CD71 ratio was calculated more correctly. The co-precipitation bands of CD45 and CD71 consisted only of the CD4-associated molecules, and free CD45 and CD71 molecules did not contribute to them because the complex was isolated on the antibody against CD4. Note that the molar ratio CD45/CD71 insignificantly varied near the value 2 : 1. The calculated CD45/CD71 ratio seems to characterize the real stoichiometry of these molecules inside the CD4 complex.

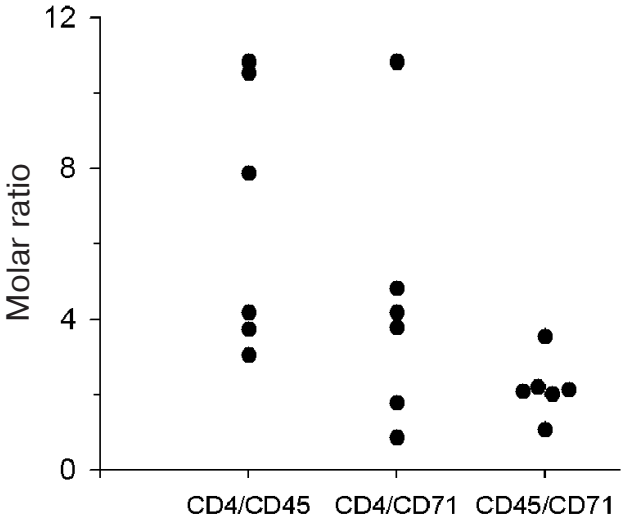

Fig. 5. Molar ratios of the CD4 complex components isolated by immunoprecipitation of CD4 protein. Results of six independent experiments are presented.

Table 2. Calculation of numbers of CD4 and CD45 molecules per CD71 molecule exemplified by one experiment

Protein	N	A, arbitrary units	$I = A/N$	$R = I_{CDX}/I_{CD71}$
CD4-Lck	(43 + 23)*	253,280	3838	7.86
CD45	86	87,446	1016	2.08
CD71	94	45,901	489	1.00

Note: N, number of lysine residues; A, band intensity; I, normalized intensity; R, ratio of molecule numbers.
* The 55-kD band was normalized using the total numbers of lysine residues in CD4 and Lck molecules.

DISCUSSION

Although there are more than twenty different membrane proteins on the CEM cell surface, CD4 molecule can produce stable complexes only with some of them. We have shown that CD45, CD71, LPAP, and Lck were the main partners on complexing with CD4. However, some other proteins, such as CD38, CD44, CD47, and HLA I, which are present on the CEM cell membrane together with CD4 do not form such complexes. The interaction between CD4 and CD45 was demonstrated by both direct (on the anti-CD4 antibody) and reverse (on the anti-CD45) immunoprecipitation. Nevertheless, in the first case the complex was isolated in the greatest amount. This seems to be caused by different ratio between free CD4 and CD45 molecules and those inside the associations. Because the expression level of CD45 on the CEM cells is two-to-three times higher than that of CD4, the majority of CD45 molecules remain free. Although CD71 pronouncedly co-precipitated with CD4, it is still unclear why the reverse precipitation on the anti-CD71 antibody failed to give a visible co-precipitation of the CD4 antigen. Perhaps the antibody used by us for isolation of CD71 prevented the formation of CD4–CD71 complex. Similar examples are known from the literature. Thus, the A1 antibody reacts only with the monomeric form of CD130 and does not bind with it after dimerization and complexing with CD126 [18].

CD4 is shown to produce complexes with other proteins [19, 20], but the data are contradictory. By confocal microscopy, the CD4 co-localization was shown on living lymphocytes with a number of surface proteins, including CD3, CD5, CD25, CD28, CD44, CD53, and some others [19]. A selective complexing of CD4 with definite isoforms of CD45 protein was shown. Thus, CD4 seemed to closely associate with CD45RC, weakly associate with CD45RB, and not interact with CD45RA and CD45R0 isoforms. But the authors themselves remarked that their technique allowed them to detect even weak affinity interactions, which could be lost on solubilization of the cell membrane. Therefore, the list of proteins associated with CD4 may be considered as the maximum evaluation of kinds of the interacting molecules. On the contrary, the study using FRET revealed that CD4 interacted only with the CD45R0 isoform but not with CD45RB or CD45RC [20]. But it must be taken into account that the cell lines transfected with CD4 and CD45 proteins were used in this study. These results must be looked at with a certain caution because conditions of increased expression of heterologous protein can be associated with non-physiological conditions for association of proteins. And finally, CD4 complexes were isolated by immunoaffinity and characterized by mass spectrometry [21]. In this case proteins on the cell surface were covalently cross-linked in the presence of the bifunctional reagent dithiobis(succinimidyl propionate). Proteins Lck, CD45, and CD71 were identified as major partners interacting with CD4.

To retain the integrity of weak protein–protein interactions, cell lysis can be performed with mild nonionic detergents (CHAPS, digitonin, Brij58, and Brij97), which are used for isolation of proteins interacting with the tetraspanin network [22-24] and other weak membrane lateral associations [25, 26]. Our findings are in good agreement with results of the work by Bernhard et al. [21]. Note that our results with the mild detergent Brij97 were the same as with the strong detergent Triton X-100 combined with chemical cross-linking of surface proteins. But unlike our data, in the above-cited work CD98 and annexin II proteins were also found to be constituents of the complex. The authors concluded that these molecules were nonspecifically cross-linked into the complex due to their high expression on the cell [21]. Moreover, the chemical cross-linking caused a partial loss of the CD4 antigen reactivity to specific antibodies. Our approach avoided these disadvantages and allowed us to detect not only lateral protein associations available for chemical cross-linking but also proteins associated through an intracellular moiety. In particular, as differentiated from the cited work [21], we detected in the CD4 complex LPAP molecules, which have a very short extracellular region [27]. We also found some cytoskeleton components and heat shock proteins among the co-precipitated molecules. They may have occurred in the precipitate nonspecifically because of their high expression. But a specific association of the CD4 complex with these proteins must not be ruled out. Thus, CD45 was earlier

shown to specifically bind with the cytoskeleton protein fodrin (non-erythrocyte spectrin) [28] responsible for the association of CD45 with actin.

It was earlier shown by immunoblotting that chemokine receptors CXCR4 (CD184) and CCR5 (CD185) were immunoprecipitated from the lysate of lymphocytes together with CD4 [25, 26]. On the CEM cells, only the CXCR4 chemokine receptor is expressed. On isolating CD4 from the CEM cell lysate, we have not found CXCR4 in the CD4 complex. Possibly, the fraction of CD4 molecules associated with CXCR4 is low and they are insufficiently represented in the immunoprecipitates for being recorded by the method used. Moreover, chemokine receptors have seven transmembrane regions, which determine their high hydrophobicity. This can prevent effective trypsinolysis and the subsequent identification of these proteins by mass spectrometry.

Intermolecular bonds in the complex and the order of entry of individual molecules into it are especially interesting. The CD4 and Lck molecules are known to directly associate to one another due to formation of a coordination bond between two N-terminal cysteines of Lck and two C-terminal cysteines of CD4 [29]. The bond involves zinc ions. Lck is a cytosolic protein, but it is located on the inner surface of the plasma membrane, where it is anchored by its palmitoylated N-terminus. Experiments with chimeric proteins revealed that CD45 and LPAP interact directly via their transmembrane domains [30]. Moreover, the cytoplasmic region of LPAP enriched with acidic residues can also bind with the catalytic site of Lck [31].

Two variants of formation of CD4 associations are supposed. Either the CD4 complex includes all the detected components, or there is a set of associations each having a specific composition. The absence of complexes between CD4, CD45, and CD71 on the U937 cells suggests that the presence of Lck and/or LPAP molecules is crucial for production of associations because these molecules are not expressed on the U937 cells [32]. But here is a contradiction. There are many data indicating that CD45 and Lck are located in different compartments of the surface membrane. CD45 protein is located only in the fluid phospholipid moiety of the membrane, whereas Lck is preferentially located in more ordered regions of the membrane, the so-called lipid rafts [33, 34]. And CD4 itself is nearly equally distributed between these membrane regions [33, 35]. Thus, the detected complexes are to be formed only at the cost of the CD4 and Lck located in the fluid moiety of the membrane.

We attempted to determine the ratio of molecules constituting the CD4 complex. Two CD45 molecules were shown to correspond in the complex to one CD71 molecule. Because CD71 is a homodimer formed due to a disulfide bond, for two CD71 chains there are really two chains of CD45, which is also prone to dimerization [36, 37]. To more accurately answer the question about the CD4 complex stoichiometry, we are planning to separate the complex from the free CD4. The *in vitro* reconstruction of the CD4 complex is also promising for determination of the order in which individual proteins enter the complex.

Thus, a new proteomic approach allowed us to show for the first time, without artificial chemical cross-linking, that CD45, CD71, Lck, and LPAP molecules and also some heat shock proteins and cytoskeleton components constitute the CD4 complexes.

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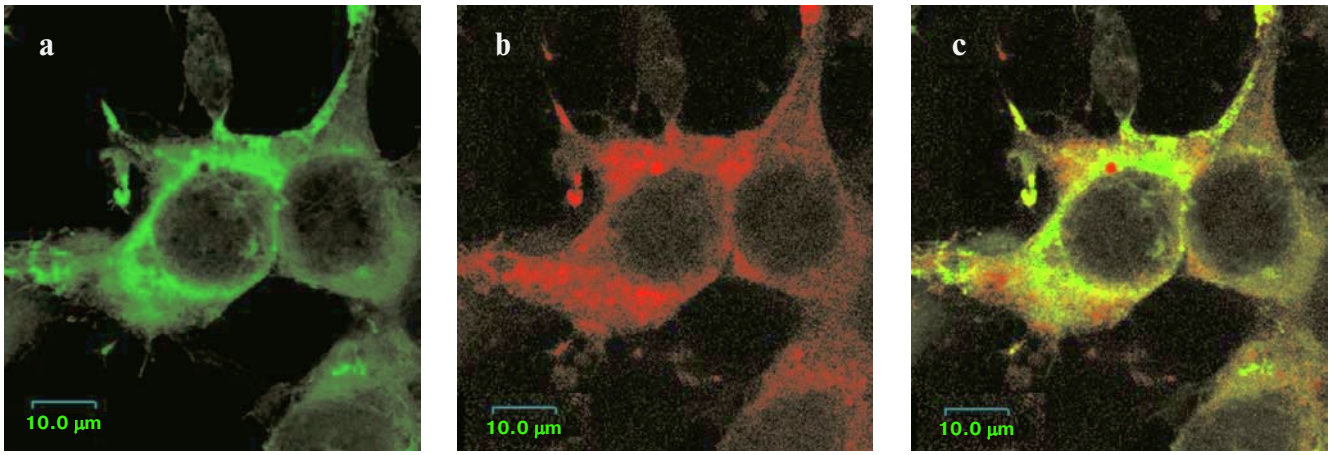

Fig. 2. (Yan Feng et al.) Colocalization of Xpress-tagged AOP-1 and GFP-TNNI3K. HEK-293FT cells were cotransfected with pEGFP/TNNI3K and pcDNA4-Xpress/AOP-1. Twenty-four hours after transfection, cells were fixed. a) GFP-TNNI3K was detected by its green fluorescence (green); b) Xpress-AOP-1 was visualized with the anti-Xpress antibody and a TRITC-conjugated secondary antibody (red); c) overlay of (a) and (b) (yellow). Magnifications are 10×60×2.5.

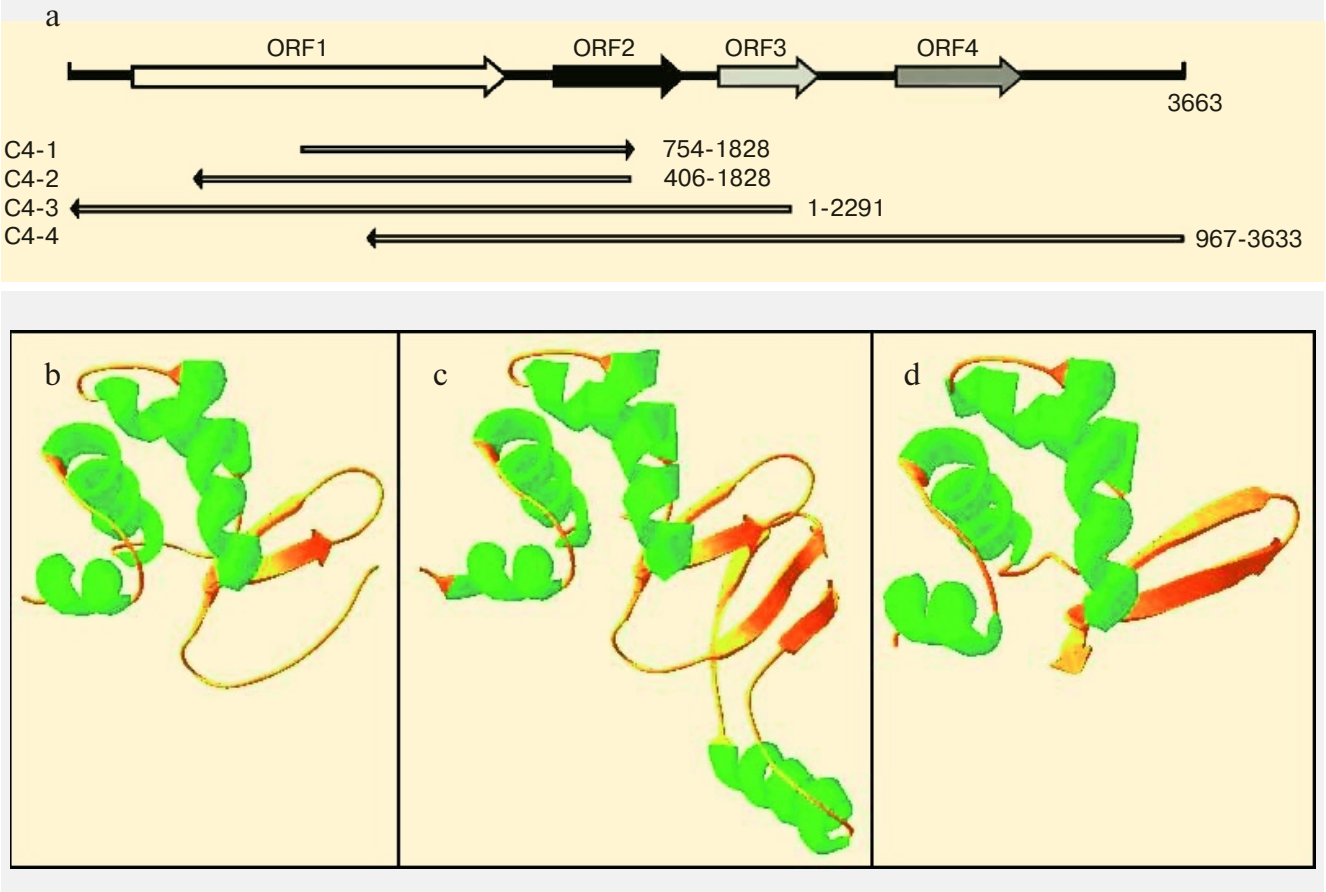

Fig. 1. (Huang Yijun et al.) Schematic assembly of overlapping fragments and ORF organization and comparison of the predicted tertiary ribbon structure of *Mg*-Fur monomer was drawn by SWISS-MODEL (http://swissmodel.expasy.org/). a) Four positive fragment sequences identified from the sub-clone library were assembled into a 3633-bp fragment with DNAMAN6.2 software containing four tentative ORFs. ORF1 encodes a hypothetical 2-nitropropane dioxygenase, ORF2 encodes a hypothetical Fur protein, and both ORF3 and ORF4 encode unknown proteins. b) Predicted structure of *Mg*-Fur. c) Crystal structure of *Pa*-Fur. d) N-Terminus tertiary structure model of *Ec*-Fur (*E. coli*). Four α-helixes and two reverse β-sheets occur in the N-terminal of *Mg*-Fur, but C-terminus structure was not present and with low similarity to *Pa*-Fur and *Ec*-Fur. The *Pa*-Fur was provided by the SWISS-MODE repository (ID: Q52083-C00001), *Ec*-Fur by NCBI (ID: 2FU4).

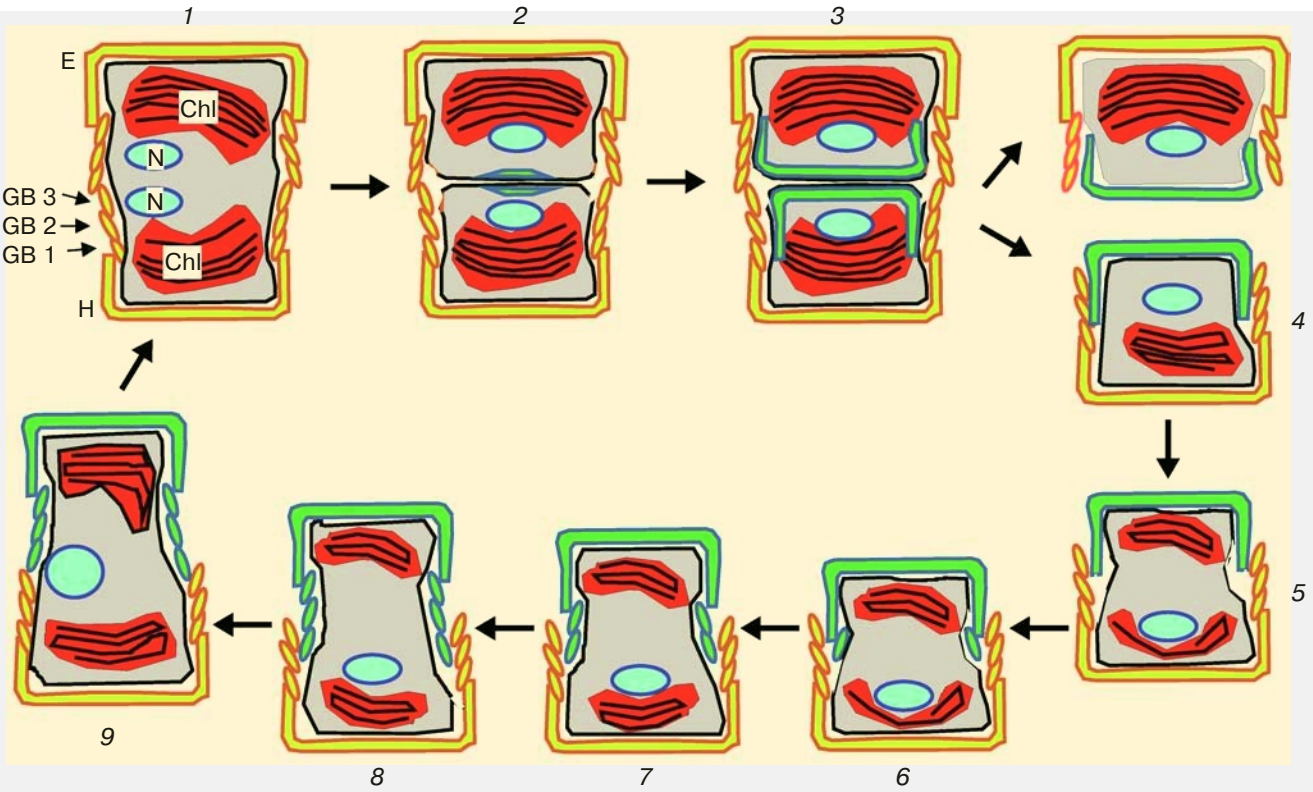

Fig. 2. (Safonova et al.) Scheme of development of the siliceous frustule of the diatom *S. acus* subsp. *radians* during the cell cycle. Transverse section. Stages: *1*) a cell with two mature siliceous valves after mitosis; *2*) beginning of the daughter valve laying upon formation of the cleavage furrow; *3*) daughter valves upon maturation and exocytosis; *4*) divergence of daughter cells; *5*) division of chloroplast; *6-8*) synthesis of girdle bands; *9*) migration of the nucleus before cell mitosis. E, epitheca; H, hypotheca; GB 1-3, girdle bands of the hypotheca; Chl, chloroplasts; N, nuclei.

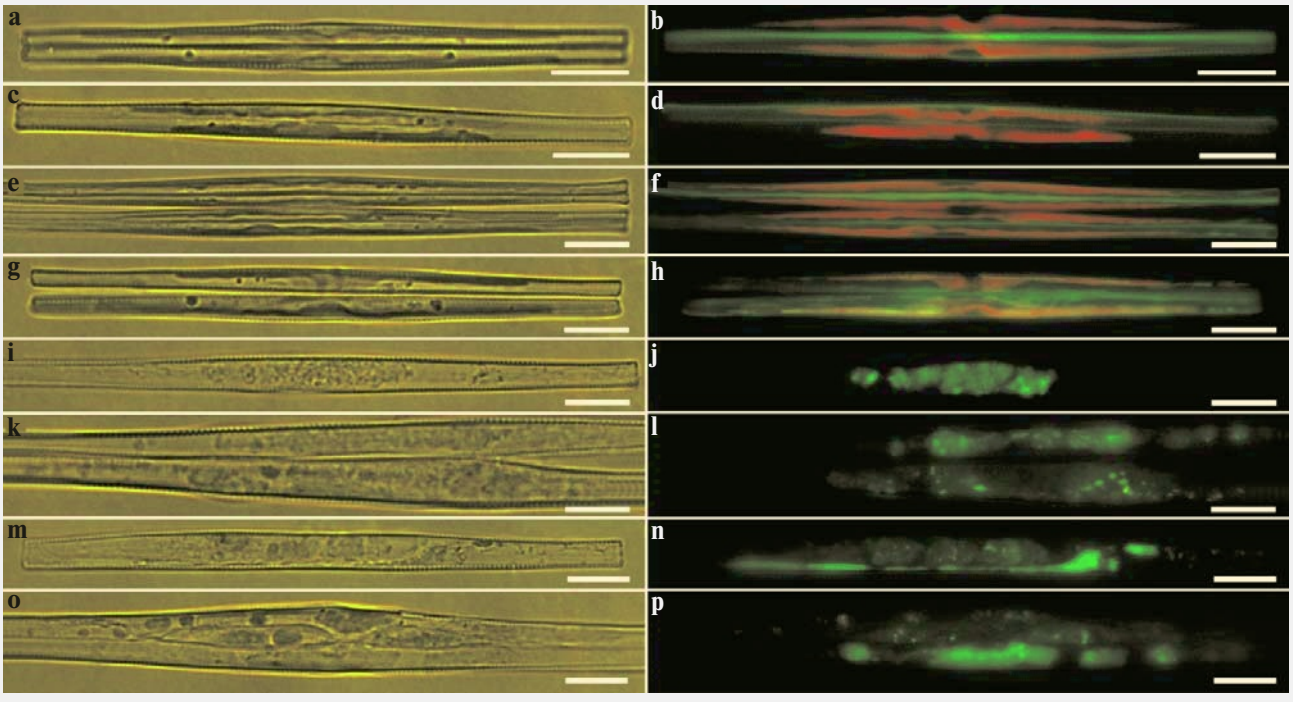

Fig. 6. (Safonova et al.) Effect of germanic acid on morphogenesis of daughter valves of the diatom *S. acus* subsp. *radians* cells (K1). Light (to the left) and fluorescent (to the right) microphotographs (staining with rhodamine 6G): red fluorescence, chloroplasts; green fluorescence, siliceous formations. a-d) Control; e-h) Ge/Si 0.01; i-l) Ge/Si 0.05; m-p) Ge/Si 0.1. Scale: 10 μm.