J. Membrane Biol. 172, 77–88 (1999)

The Journal of
Membrane
Biology

Hydrophobic and Hydrophilic Radio-Iodination, Crosslinking, and Differential Extraction of Cell Surface Proteins in *Paramecium tetraurelia* Cells

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Received: 5 April 1999/Revised: 19 July 1999

Abstract. We combined widely different biochemical methods to analyze proteins of the cell surface of *P. tetraurelia* since so far one can isolate only a subfraction of cell membrane vesicles enriched in the GPI-anchored surface antigens ("immoblization" or "i-AGs"). We also found that i-AGs may undergo partial degradation by endogenous proteases. Genuine intrinsic membrane proteins were recognized particularly with lipophilic 5-[^{125}I]-iodonaphthalene-1-azide (INA) labeling which reportedly "sees" integral proteins and cytoplasmic cell membrane-associated proteins. With INA (+DTT), bands of ≤55 kDa were similar as with hydrophilic iodogen (+DTT), but instead of large size bands including i-AGs, a group of 122, 104 and 94 kDa appeared. Several bands of the non i-AG type are compatible with integral (possibly oligomeric) or associated proteins of the cell membrane of established molecular identity, as we discuss. In summary, we can discriminate between i-AGs and some functionally important minor cell membrane components. Our methodical approach might be relevant also for an analysis of some related protozoan parasites.

Key words: Cell membrane — Ciliates — Crosslinking — Iodination — *Paramecium* — Proteins

Introduction

Our goal was to obtain some biochemical baseline information on different components of the cell surface membrane of *Paramecium* cells since so far, with a very few exceptions, only proteins with a glycophosphatidylinositol-(GPI)-anchor or cell membrane fraction enriched in these components can be isolated. Taking into account the widely different molecular constitutents and their functional implications, we combined a variety of different methods.

Generally the cell membrane consists not only of the lipid bilayer with integral proteins and internally and externally associated proteins, but some proteins can also be externally attached by a GPI-anchor [18]. In the particular case of *Paramecium*, the entire cell surface is covered by GPI-anchored surface variant antigen or "immobilization antigen" (i-AG), as reviewed by Jones [23], Prat et al., [37], Preer [39], and Schmidt [41]. The i-AGs contribute ~3.5% to total cell protein and, thus, by far outnumber any other cell membrane proteins [27]. Though they may by far dominate in cell membrane fractions obtainable by the only protocol available [44] to a disturbing extent, the function of these i-AGs in *Paramecium* remains enigmatic [6, 13], and such proteins represent a serious challenge to some closely related parasitic protozoa [28]. In the *Paramecium* cell, surface AGs follow a complicated biosynthetic and degradative pathway [19] which also requires more detailed analysis.

The i-AGs belong to a multigene family with mutual intergenic [3] and interallelic [8] exclusion, only one type of i-AG being expressed at a time [4, 45]. The i-AGs consist of one polypeptide chain with a remarkably high cysteine content of ~10% [23, 37] whose SH-groups are all involved in disulfide bond formation [23]. Their apparent molecular mass is reported as ranging between ~230 to 330 kDa [7, 23, 37]. The reasons for fragmentation of i-AGs during isolation have not yet een elucidated in much detail. The C-terminal GPI-anchor of i-AGs is inserted into the outer leaflet of the cell membrnane lipid bilayer [2, 9, 10, 11, 12, 13, 14].

Our goals were to clarify conditions of fragmentation of i-AGs, to set a baseline for further protein analysis, and thus, to obtain access to less numerous but functionally important proteins of the surface of *Paramecium*

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cells, and to see whether correlation with formerly established molecular components (*see* Discussion) may have a chance. We combine widely different methods, including protein extraction, radio-iodination or crosslinking, as outlined in Figs. 1 and 2. In particular we compare [^{125}I]-labeling with either lipophilic 5-iodonaphthyl-1 azide (INA), as introduced by Bercovici and Gitler [5] and Schwaller et al. [42], or with hydrophilic iodogen according to standard methods. Analyses of related parasites may also profit from the methods once established, particularly since isolation of cell membranes on the basis of GPI-anchored proteins is selective and may involve membranes from many intracellular compartments, according to *in situ* labeling studies [19].

Materials and Methods

Cell Cultures

Paramecium tetraurelia 7S wild-type cells [46] obtained from the CNRS in Gif-sur-Yvette were cultivated at 25°C in a sterile medium [24]. Until reaching the early stationary phase the cells were harvested as previously described by Plattner et al. [33].

Chemicals

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate = CHAPS, Boehringer, Mannheim, Germany. E64 (L-trans-epoxysuccinyl-leucylamido-[4-guanidinobutan]), Calbiochem, LaJolla, CA. 5-Aminonaphthyl-1-azide, Fluka, Buchs, CH, Aprotinin, TAME (p-tosyl-L-arginin-methylester-HCl), high molecular weight (MW) kit, polyethyleneglycol (P-3640), Sigma, Deisenhofen, Germany. Low MW kit, dextran T500, Pharmacia, Freiburg, Germany. Iodogen, 1,5-difluoro-2,4-dinitrobenzene (DFDNB), ethylenglycol-bis[succinylimidylsuccinate] (EGS), N-hydroxysuccinimidyl-4-azidobenzoate (HSAB), Pierce, St. Augustin, Germany. Na[^{125}I] carrier free, ICN, Eschwege, Germany. Pefabloc SC, Pepstatin A, Serva, Heidelberg, Germany. Leupeptin, Biomol, Hamburg, Germany. Bis-Tris, Roth, Karlsruhe, Germany. All other compounds listed in Materials and Methods were of reagent grade and obtained from local suppliers.

Isolation of Surface Antigens

An outline is given in Fig. 1. We largely followed the method by Preer [38] as refined by Jones [23] to prepare i-AGs. Briefly, 2 liters of cell culture (~2 × 10^3 cell/ml) were washed in Dryl's solution (1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 mM Na_3 citrate and 1 mM $CaCl_2$, pH = 6.8). Then cells were kept suspended for 1 hr in extraction medium (12% ethanol, 7.7 mM NaCl) at 4°C and briefly centrifuged after incubation (2 min, 180 × *g*, 4°C). The supernatant was further processed by centrifugation (5 min, 20,000 × *g*, 4°C). From the resulting supernatant, proteins were precipitated by adding $(NH_4)_2SO_4$, up to 75% saturation, under constant stirring on ice for 3 hr. The precipitated material was centrifuged (15 min, 10,000 × *g*, 4°C), resuspended in 6 ml distilled water, and dialyzed overnight against distilled water. Any precipitated material appearing during dialysis was removed by centrifugation. Protein concentrations were determined by the Bradford method.

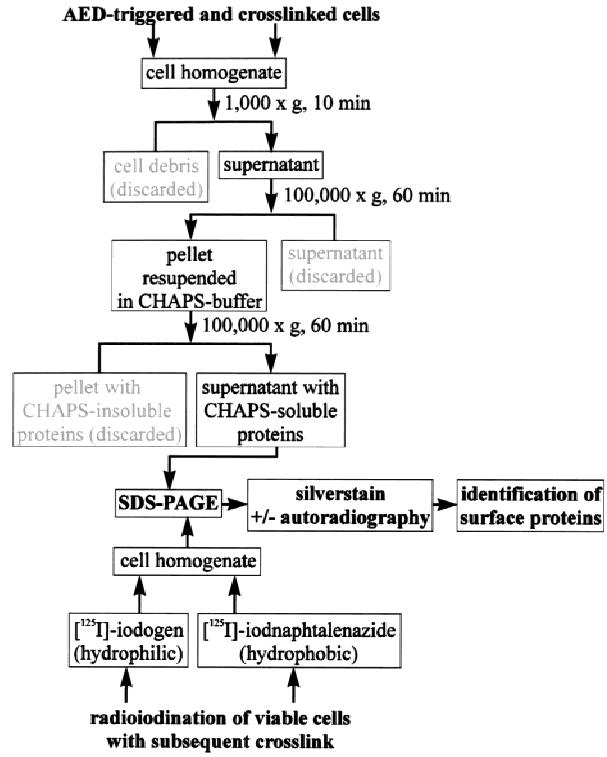


Fig. 1. Survey of different radio-labeling procedures and isolation of cell membrane proteins, with differential centrifugation and CHAPS extraction.

Gel Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Laemmli-type system. After brief boiling in sample buffer (0.4 M Tris-HCl, 0.035 M SDS, 80 μM EDTA, 1 mg bromphenolblue, pH 8.0) with or without preceding reduction of disulfidebonds by 0.5% dithiothreitol (DTT) samples were alcylated for 30 min in the dark with 2% iodacetamide. Most samples were analyzed on linear gradient (5 to 15%) SDS-PAGE gels, with a 4% stacking gel, while 7.5 to 15% gels were used for cell membrane vesicles. Gels were stained with silver. Markers varied between 14.5 and 94 kDa or 97 and 584 kDa, in low or high MW kits.

Cell Surface Labeling by Hydrophilic Radio-Iodination In Vivo Followed by Crosslinking

5×10^4 cells in 5 ml Pipes buffer (5 mM Pipes, 1 mM KCl, 1 mM $CaCl_2$, pH 7.0) were transferred into propylene vials coated with iodogen according to the manufacturers advice. Iodination, as outlined in Fig. 2, was started by adding 500 μCi (1.8×10^7 Bq) of carrier-free Na[^{125}I], specific activity = 100 mCi/ml (3.6×10^9 Bq/ml). After 15 min at 22°C, cells were removed, washed 6-times with Pipes buffer, homogenized, and subjected to SDS-PAGE and ARG using Reflection™ ARG film (NEN, Köln, Germany). Crosslinking of the iodinated and washed cells was started by adding EGS or DFDNB, each in a final

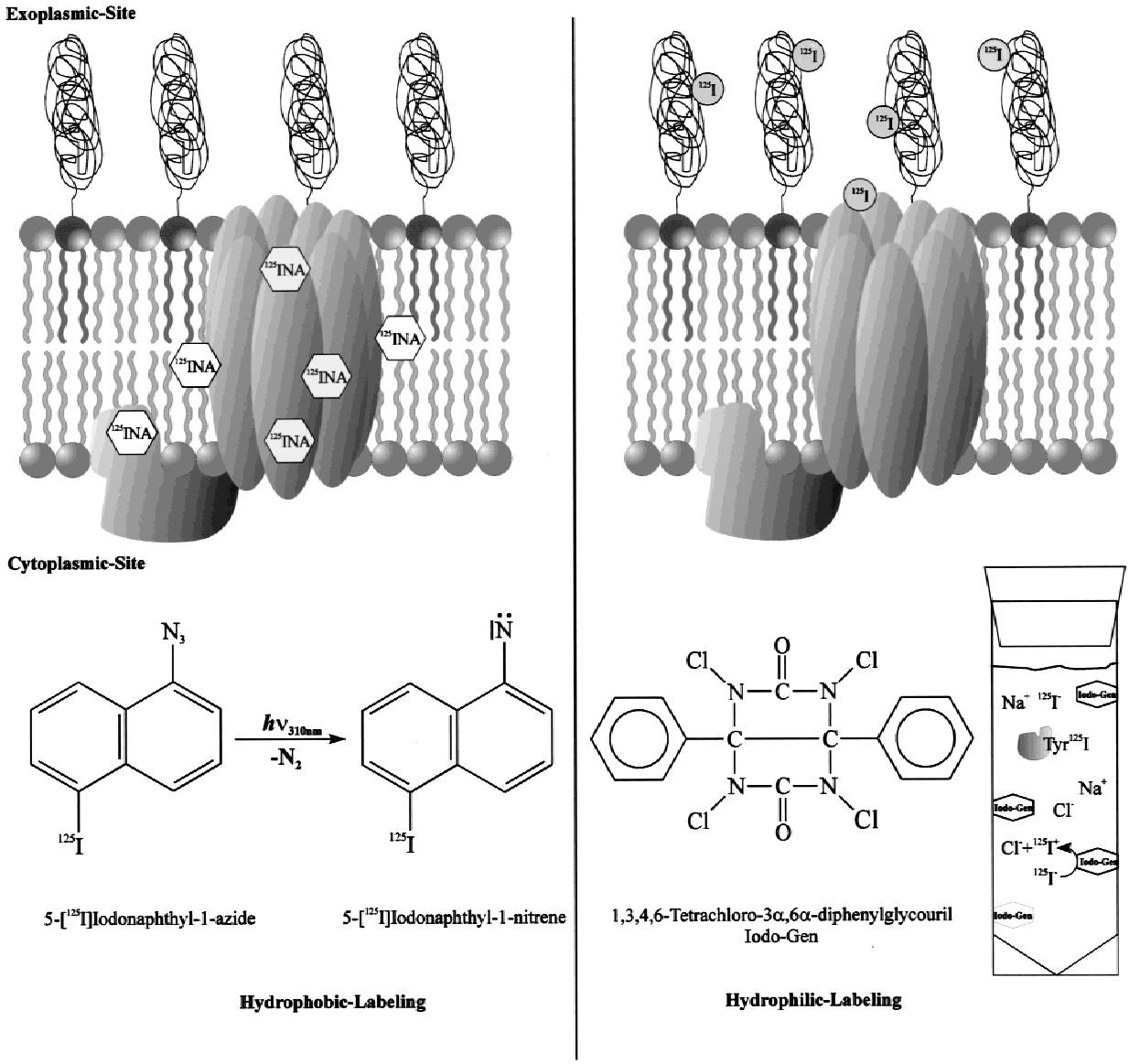


Fig. 2. Principles and targets of the two different radio-iodination procedures used.

concentration of 0.2 mM. Crosslinkers were initially dissolved in DMSO (final concentration <1%). After 30 min incubation at 4°C the reaction was quenched by adding cystein to a final concentration of 50 mM for further incubation, 30 min at 4°C. After crosslinking cells were treated as described below for noncrosslinked aliquots.

Cell Surface Labeling by Lipophilic Radio-Iodination In Vivo with Simultaneous Crosslinking

[^{125}I]-INA was synthesized from 5-aminonaphthyl-1-azide and Na[^{125}I] according to "method 3" indicated by Bercovici and Gitler [5]. Purified [^{125}I]-INA was dissolved in ethanol, stored in the dark at −20°C, and used within 4 days after synthesis. ~10^4 cells in Dryl's solution (*see* above) were transferred into 1.5 ml reaction vials and incubated for 1 min at 25°C under dimmed light ($\lambda \geq 600$ nm) with 500 μCi (1.8×10^7 Bq) [^{125}I]-INA per probe (specific activity 150 μCi/ml [0.55×10^7 Bq], final ethanol concentration <1%). For labeling, as outlined in Fig. 2, reaction vials were irradiated with a 150 W mercury lamp for 20 sec at a distance of 10 cm. Photoactivated crosslinking with HSAB was carried out by simultaneously incubating living cells with [^{125}I]-INA and 0.2 mM HSAB, which has been first dissolved in DMSO (final concentration of DMSO <1%). After labeling all further steps were made on ice. Cells were incubated for 15 min with Dryl's solution containing 0.1% BSA and washed three times with the same solution and two times, without BSA added. After labeling the cells were transferred in HM-Buffer containing the protease-inhibitor cocktail described below. They were then homogenized with 70 strokes in a glass-homogenizer with teflon-pestle (Braun, Melsungen, Germany). Cell debris were removed by brief centrifugation (1,000 × *g*, 10 min) and the supernatants were subjected to SDS-PAGE and ARG (*see above*). The protease inhibitor cocktail used consisted of 3 μg/ml Aprotinin, 10 μM E64, 100 μM Leupeptin, 0.6 mM Pefabloc SC, 1 μM Pepstatin A, 0.3 mM TAME.

Preparation of Chaps-Soluble Membrane Proteins

See survey in Fig. 1. Cells (~4 liters) were harvested and washed in Pipes buffer pH 7.3 (5 mM Pipes, 1 mM KCl, 1 mM $CaCl_2$) at 22°C. With some aliquots exocytosis was triggered by mixing cells with an equal volume of Pipes buffer containing 0.01% aminoethyldextran (AED). For details see Plattner et al. [33]. Cells were transferred into a mixture of 30 ml deciliation buffer, pH 7.5 ("STEN," containing 500 mM sucrose, 20 mM Tris, 4 mM EDTA, 6 mM NaCl) and 20 ml Pipes buffer. Cells were then slowly cooled to 4°C with 0.4°C/min to avoid exocytosis due to harsh temperature shift in control cells. Then 2 ml of a 1 M $CaCl_2$ solution was added to start deciliation (final Ca^{2+} concentration ~40 mM). When 95% of the cells were deciliated under light-microscope control, they were transferred into fresh STEN (4°C) without EGTA but with 1 mM $CaCl_2$. Crosslinking was then carried out by adding the crosslinkers dissolved in DMSO (final concentration <1%). After 30 min at 4°C the reaction was quenched by adding 50 mM cystein for 30 min. Cells were then transferred, with two wash steps, into homogenization-buffer (HM-buffer) with protease inhibitors at 4°C. The HM-buffer contained 250 mM sucrose, 20 mM Tris-maleate and 20 mM NaOH, pH 7.0. Homogenization of the cells was carried out by 100 strokes in a glass-homogenizer with a Teflon-pestle. From resulting crude extracts cell debris was removed by brief centrifugation (10 min, 1,000 × *g*). Supernatants were subjected to a 100,000 × *g* centrifugation for 60 min at 4°C. Resulting pellets were resuspended in 2 ml of 50 mM bis-Tris, 5 mM CHAPS and 600 mM ε-aminocapronic acid, pH 7.0. To obtain dissolved membrane proteins, suspensions were spun once again with 100,000 × *g* for 60 min at 4°C and the resulting supernatants were subjected to SDS-PAGE.

Isolation of Cell Membrane Vesicles by Two-Phase Aqueous Polymer Separation

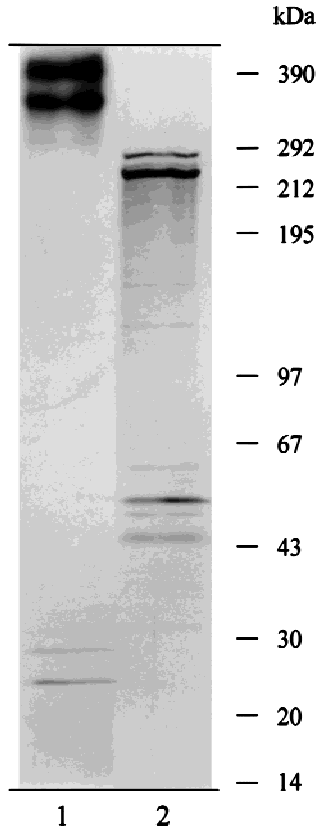
The method of Smith and Hennessey [44] was applied. Briefly, deciliated cells (*see above*) were homogenized in the presence of protease inhibitors (*see above*) to obtain a crude microsome suspension of intracellular and cell surface membranes. Preparations were done at 4°C. Microsomes were first suspended in a mixture of two immiscible polymers, polyethyleneglycol P-3640 and dextran T500. During phase separation of the two polymers, right side-out cell membrane vesicles, due to their surface properties, partition exclusively into the upper phase which was removed by centrifugation, 10 min, 1,000 × *g*. Finally, cell membrane vesicles were collected by centrifugation, 60 min, 100,000 × *g*.

Results

Experiments presented were repeated at least twice, eventually up to four times.

Extracted Surface Antigens

Surface antigens, largely representing high MW i-AGs, have been isolated from living cells by ethanolic extraction according to established methods [10, 23, 38]. On SDS-PAGE (Fig. 3, lane 1, −DTT) the main set of proteins migrates as two bands, 382 and 335 kDa, with two additional minor bands of 28 and 24 kDa. The two large main protein bands show a significant MW shift under reducing conditions (Fig. 3, lane 2, +DTT). In fact, antibodies prepared against these bands recognize just these bands on Western blots and in immuno-EM analysis they intensely label the glycocalyx [19]. This is not inhibited by the protease inhibitor cocktail used (*see* Materials and Methods and below) and, hence, may indicate inherent proteolytic activity, possibly activated by DTT (*see* below). On the gel DTT then allows fragmentation to become manifest due to reduction of the abundant disulfide bonds and the release of fragments thus produced. How to explain the double high MW bands in Figs. 3 and 4 (−DTT)? Although individual *Paramecium* cells can express only one serotype at a time, two different ones may be expressed in a population when cells shift from one serotype to another.

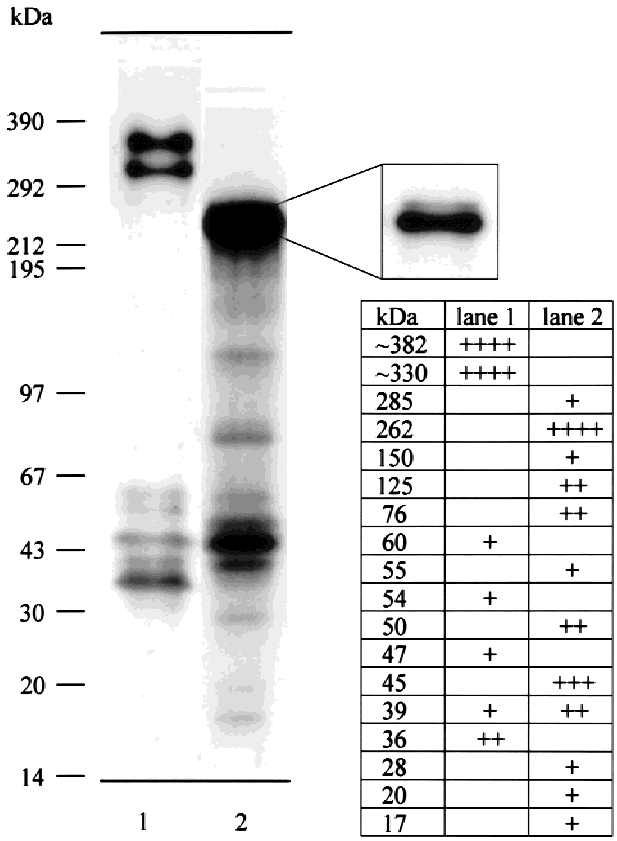


kDa	lane 1	lane 2
~ 382	++++	
~ 330	++++	
285		+++
262		++++
150		+
125		+
63		+
55		++
52		+
45		++
32		+
28	++	
24	++	

Fig. 3. Silver stained SDS-PAGE gel from ethanol extracted GPI-anchored surface proteins, nonreducing (lane 1) or reducing conditions (lane 2), 3 min DTT. Iodacetamide (2%) treatment for 30 min at 22°C. Note shift of high MW bands and occurrence of additional medium-sized MWs in lane 2. Horizontal line on top marks start of separation gel.

Hydrophilic Labeling of the Cell Surface

We then labeled living cells with [^{125}I]-iodogen. As Fig. 4 shows, elecrophoretic pattern (±DTT) of labeled proteins is almost identical as with isolated surface antigens (shown in Fig. 3), with the exception that, under nonre-



kDa	lane 1	lane 2
~382	++++	
~330	++++	
285		+
262		++++
150		+
125		++
76		++
60	+	
55		+
54	+	
50		++
47	+	
45		+++
39	+	++
36	++	
28		+
20		+
17		+

Fig. 4. Autoradiography of SDS-PAGE from cell homogenates (+ protease inhibitors) showing electrophoretic pattern of surface proteins labeled in vivo with [^{125}I]-iodogen. Nonreducing (lane 1) or reducing conditions (lane 2), 3 min DTT. Iodacetamide (2%) treatment for 30 min at 22°C. In lane 2 the 285 kDa close to the 262 kDa band is visible only after brief exposure (inset). Note that labeled bands comprise light MW forms (lane 1 and 2), as in Fig. 3, and some additional medium-sized bands (lane 2). Horizontal line on top marks start of separation gel.

ducing conditions (Fig. 4, lane 1), an additional set of proteins of 60, 54, 47, 39, and 36 kDa appears. Under reducing conditions (Fig. 4, lane 2) a strong 262 kDa band preodominates over a weak 285 kDa band which becomes visible only after short exposure (Fig. 4, inset). It has to be noted that so far, similarities can be discussed only on the basis of MW. The major extracted surface proteins seen after silver staining (Fig. 3) are also radio-iodinated. In Fig. 4 (lane 2), under reducing conditions, lower MW bands appear slightly smaller than under nonreducing conditions, perhaps due to S-S unfolding. Though such small differences are difficult to ascertain, the 60 kDa band may correspond to the 55 kDa band, 54 kDa to 50 kDa, and 47 kDa to 45 kDa, while the 39 kDa band is unaltered. The 36 kDa band (−DTT) may fragment into 28, 20 and 17 kDa (+DTT). Two bands of 28 and 24 kDa appear under nonreducing conditions in the isolated surface proteins (Fig. 3), while they are not amenable to radio-iodination (Fig. 4, lane 1).

Proteolytic Activity in the Extracts of Surface Antigens and Their Potential Contribution to SDS-PAGE Gel Band Diversity

As it is well known that cystein proteases can be activated by thiols like DTT and inhibited by alkylation with iodacetamide, we checked the possible existence of a copurified cystein protease in the ethanolic extracts of i-AG fractions by different treatments of this fraction *before* the regular SDS-PAGE. While in Fig. 5, lane 1 shows the well known picture of an i-AG fraction in Ag-stained SDS-PAGE, lane 2 was obtained after 1 hr DTT treatment, showing the almost total degradation of the i-AGs. Lane 3 shows the inhibition of this effect by preceding incubation with iodacetamide. Can these results be attributed to a co-isolated protease? To address this question, we repeated the experiments with BSA added to the i-AG fraction. This foreign protein is also degraded by the putative protease contained in the ethanolic extract (Fig. 5, lane 5), under conditions comparable to lane 2 (1 hr DTT), while in lane 4 this is inhibited by sample boiling before 1 hr DTT treatment. In this case, BSA is still visible as an intense band of 66 kDa (Fig. 5, arrowhead). Therefore, we infer cleavage of large MW surface components by an endogenous co-extractable protease which is activated by DTT. Remarkably this may occur within a short time, e.g., during sample preparation for SDS-PAGE, but it is inhibited by alkylation (Fig. 5, lane 3), by boiling (Fig. 5, lane 4), or by adding a specific cystein protease inhibitor E64 (*data not shown*). Lower MW bands are not affected (Fig. 5). The 33 kDa band may represent a cathepsin L like protease released in large amounts to the medium [51] which may be responsible for the large MW shift we see and which may undergo self-digestion in lanes 2 and 5 of Fig. 5.

From these data we conclude that endogenous protease activity can affect protein bands and that this must be counteracted by alkylation with iodacetamide before applying DTT for SDS-PAGE.

Lipophilic Labeling of Cell Membrane Components

Next we tried to see which proteins can be labeled in vivo by lipophilic [^{125}I]-INA (Fig. 6, +DTT). Labeling requires UV irradiation (lane 1) which was combined with the photolabile crosslinking agent, HSAB (lane 2). Cells survive when manipulations are restricted, e.g., to 20 sec. As lane 1 shows, without crosslinking, large MW proteins remain completely unlabeled, labeling being restricted to sizes of $\leq$122 kDa. Under nonreducing conditions, the electrophoretic pattern was the same (*data not shown*). Closer inspection reveals similarities to iodogen-labeled samples (+DTT), as summarized in the Table. These proteins of 55, 50, 45, 39, 20 kDa, therefore, cannot be derived from i-AGs. Some iodogen

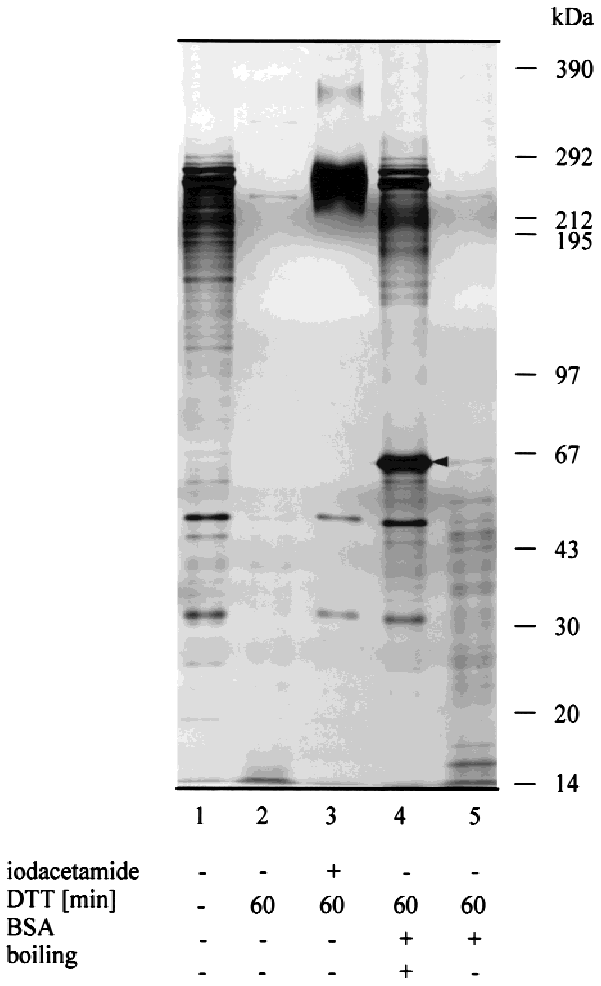


Fig. 5. Silver stained SDS-PAGE of ethanol extracted GPI-anchored surface protein fraction. Evidence of proteolytic activity and disulfide bond dissociation caused by prolongated DTT treatment and its avoidance by iodacetamide or boiling. Top horizontal line marks start of separation gel. Fragmentation may be caused by inherent protease activation by DTT (*see* Discussion) depending on time of DTT (0.5%) treatment (lane 1, 3 min, lanes 2–5, 60 min). Addition of iodacetamide (2%) before DTT inhibits, though not completely, dissociation of fragments from high MW proteins (lane 3). Fragmentation cannot be avoided by boiling since high MW forms of ≥100 kDa are seen in lane 4, in addition to the ≥ 250 kDa forms. BSA (1 μg per lane, arrowhead) has been added to differentiate between proteolysis (lane 5) and S-S linkage fragmentation (lane 4).

labeled bands obtained under nonreducing or reducing conditions, respectively, are only slightly diverging, e.g., 60 *vs.* 55 kDa, 54 *vs.* 50, or 47 *vs.* 45 kDa. However, we did not analyze in any detail whether the smaller size obtained with DTT is due to reduction or to proteolytic effects. Four bands of 122, 104, 94, and 26 kDa are exclusively labeled by [^{125}I]-INA, and, hence, may be integral or internal membrane associated proteins. When the cells are simultaneously crosslinked (lane 2), changes in three protein bands occur, i.e., a band of 104 kDa disappears and two bands of 145 and 270 kDa are formed anew. For potential interpretations, *see* Discussion.

Hydrophilic Labeling of Cell Surface Components and Subsequent Crosslinking

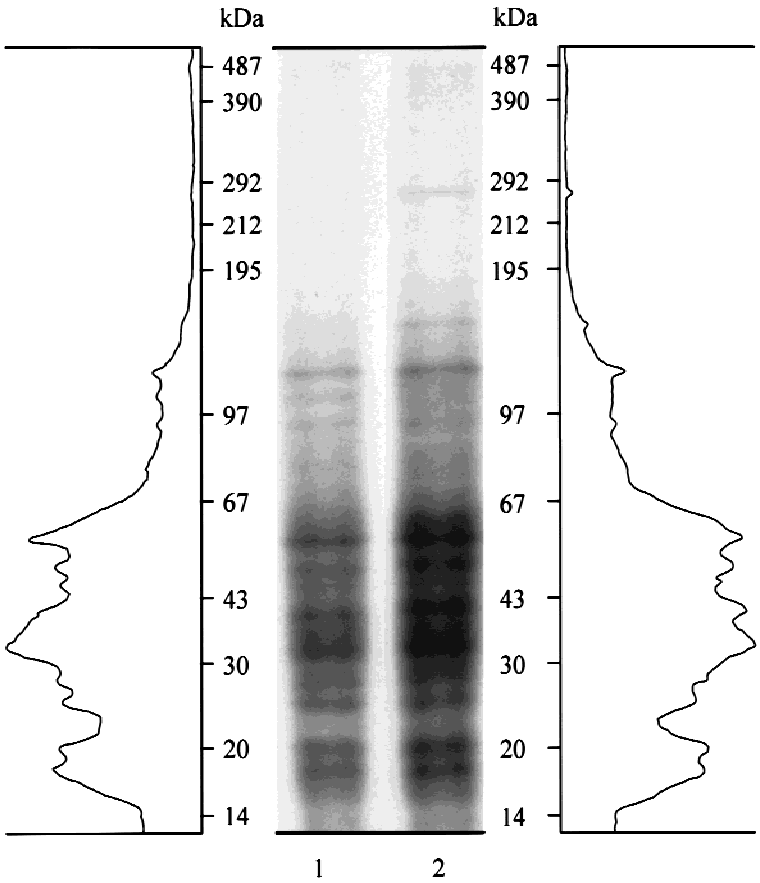
In Fig. 7 we present samples (+ protease inhibitors) labeled by [^{125}I]-iodogen, with or without crosslinking, either by EGS or DFDNB, respectively. The non-crosslinked control is contained in Fig. 2. Both crosslinkers are not easily applicable in vivo and both, particularly EGS, produce very high MW forms (gel 1). Surprisingly, these large MW products can be split by DTT, especially after DFDNB (gel 2). This may be explained as follows. The more extended EGS spacer may crosslink S-S linked chains, which then can be dissociated by DTT, although not to the size of the much smaller bands which we normally see with DTT without crosslinking (*see above*). Other bands appear less affected, e.g., those of 39, 45 or 55 kDa. A new band of 81 kDa is prominent after both crosslinkers (+DTT) in Fig. 7 (gel 2, lanes 1 and 2). Moreover, EGS produces DTT-resistant bands of 369 and >510 kDa (Fig. 7, gel 2, lane 1). For a tentative interpretation, *see* Discussion.

CHAPS Solubilization of Membrane Proteins

CHAPS is known to extract integral membrane proteins [22, 43]. Figure 8 demonstrates extraction of a wide range of proteins from *Paramecium* cells. We compare normal cells with aliquots after massive exocytosis stimulation by AED, since this is known to be accompanied by dispersal of some oligomeric cell membrane proteins (*see* Discussion). For comparison aliquots were also crosslinked by DFDNB after triggering. Numerical data achieved are contained in Fig. 8, and include a change of a variety of bands in the course of exocytosis stimulation. Without crosslinking bands of 285, 73, 61, 40, 38, 24, and 16 kDa disappear, while bands of 131, 102, 100, and 95 kDa reappear. With crosslinking, after AED stimulation, we observe occurrence of 121 and 102 bands, while 285, 141, 90, 70, 42, 39, 24, 18, and 15 kDa bands are seen only in nonstimulated cells. For interpretation, *see below*.

Isolated Cell Membrane Vesicles

Another method to get access to cell membrane proteins is presented in Fig. 9 which shows an SDS-PAGE profile after isolation by phase separation. This fraction is selective for GPI-anchored surface proteins while those occurring in a variety of intracellular compartments [19] are not "seen" by this method. As seen in the Table, all of the bands seen after ethanolic or CHAPS extraction or after radio-iodination by either of the two methods ap-



kDa	lane 1	lane 2
270		+
145		++
122	+	++
104	+	
94	++	++
55	+++	+++
50	++	++
45	+	+
39	++	++
32	++	++
26	++	++
24	++	++
20	++	++
17	++	++

Fig. 6. SDS-PAGE autoradiography of homogenates (+ protease inhbitors) obtained by in vivo labeling with [^{125}I]-INA. Lane 1, without crosslinker, lane 2, with 0.2 mM HSAB. Note absence of labeling in high MW regions and MW shift of some bands, as evidenced by densitometer scans.

plied are contained in the isolated cell membrane fraction.

Discussion

Our goal was to obtain more insight into molecular constituents of *Paramecium* cells beyond the overwhelming content of GPI-anchored proteins. The only method available to isolate cell membranes is based on such components [44] and, therefore, must be highly selective. On the other hand, such components are distributed intracellularly over vast compartments [19] and they are liable to degradation during isolation, thus yielding false signals (this paper). Therefore, we developed a combination of new approaches.

Using SDS-PAGE we have first isolated i-AG fractions (Fig. 3) which we then compared with bands obtained by hydrophilic (Fig. 4) or lipophilic (Fig. 6) radioiodination. Additionally, we controlled degradation of i-AGs which are most sensitive to DTT-activated endogenous proteases (Fig. 5). On this basis, combined with crosslinking experiments, we can obtain more reliable information on "genuine" cell membrane proteins in our system. As to the other dominant protein type, we can exclude any significant contamination of our fractions by the most abundant secretory proteins of dense-core vesicles, the "trichocysts," because of their small size and dimer ↔ monomer transition with DTT [47] which we do not see.

Proteolytic Effects

When unaffected by proteolytic effects, i-AG bands are of 380 and 330 kDa under nonreducing, and of 285 and 262 kDa under reducing conditions, respectively. Minor bands, including a fairly constant 45 kDa band, are also included, as reported previously [1]. Time-dependency of DTT effects clearly indicates proteolysis which cannot easily be counteracted by usual protease inhibitors [10], superimposed by disulfide bond cleavage [21]. The thiol-activated protease originally envisaged by Hansma [21] can possibly be attributed to cathepsin L, a 33 kDa secretory product found in spent *Paramecium* culture

Table. Survey of electrophoretic patterns derived from Figs. 3, 4 and 6* showing that lipophilic [^{125}I]-INA labels medium-sized proteins different from those "seen" by hydrophilic [^{125}I]-iodogen which largely comprise i-AGs.

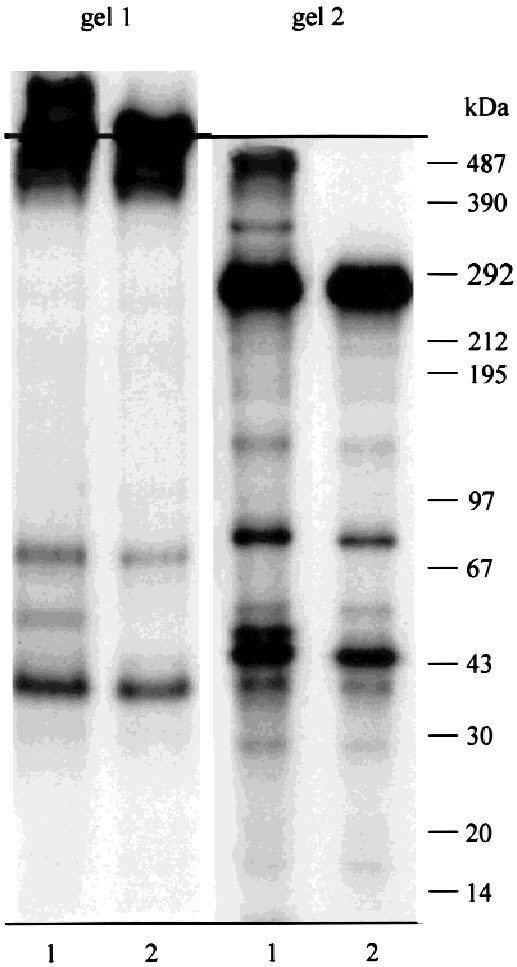
Molecular mass [kDa]	Extracted GPI-anchored surface proteins		In vivo radio-labeling of surface proteins with iodogen		In vivo radio-labeling of membrane proteins with [^{125}I]-INA	Proteins in isolated plasma-membrane vesicles
	Non reduced	Reduced	Non reduced	Reduced	Non reduced/reduced	Reduced
~382	++++		++++			
~330	++++		++++			
285		+++		++		++++
262		++++		++++		++++
150		+		+		+++
125		+		++		++
122					++	+
104					+	++
93					++	++
76				++		++
63		+				++
60			+			+
55		++		+	+++	++++
54			+			
52		+				
50				++	++	++++
47			+			
45		++		+++	++	+++
39			+	++	++	++
36			++			
32		+			++	++
28	+			+		+
26					++	+
24	+				++	+
20				+	++	++
17				+	++	+++

* Labeling intensities: ++++ very strong, +++ strong, ++ medium, + weak. Encased or light-gray marked crosses (+) point to identities of electrophoretic patterns. Dark-gray marked crosses (+) indicate bands selectively labeled with the corresponding approach. Dotted outlines indicate a set of proteins, which undergoes, under nonreducing conditions, a shift to lower MW, probably dure to reduction by DTT (arrows).
Only those protein bands from isolated plasma-membrane vesicles are listed which also occur in any of the other preparations (additional bands indicated in Fig. 9). For further details, see text.

medium [51] which also occurs in Tetrahymena [48]. Concomitantly, in Fig. 5, we see a 33 kDa band which apparently undergoes (auto-?)digestion with increasing DTT exposure time.

Surface Radio-Iodination

Standard methods work with hydrophilic agents like iodogen [40]. Yet in cells with extensive GPI-anchord i-AGs these may practically be the only proteins labeled, as we realize (Fig. 4) and they may by far outnumber any other important components. In contrast, gels from cell membranes isolated from *Paramecium* cells contain a large spectrum of other proteins (Fig. 9). Most importantly, proteins of this type are widely different from fragmentation products of i-AGs (*see* above and Fig. 5). The medium- to small-sized membrane proteins labeled by [^{125}I]-INA cannot be considered as such degradation



	gel 1		gel 2	
kDa	lane 1	lane 2	lane 1	lane 2
> 510	+++	+++	+++	
~369			++	
262			+++	+++
125			++	+
81			+++	++
76	++	+		
55	+		++	+
50			++	
45			+++	+++
39	+++	+++	+++	++
28			++	+
17			+	+

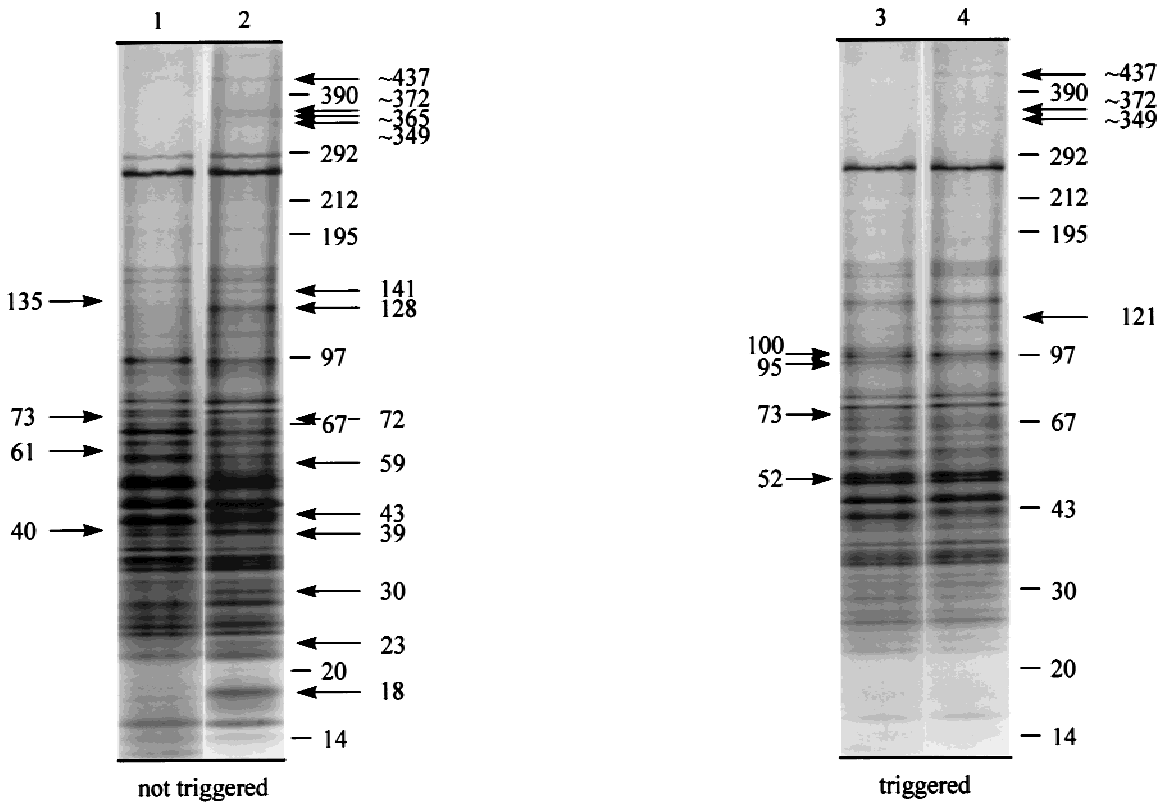
Fig. 7. SDS-PAGE autoradiography of homogenates (+ protease inhibitors) obtained by in vivo labeling with [^{125}I]-iodogen, with or without subsequent crosslinking. Gel 1, lane 1, +EGS, −DTT. Gel 1, lane 2, +DFDNB, −DTT. Gel 2, lane 1, +EGS, +DTT. Gel 2, lane 2, +DFDNB, +DTT. Horizontal line on top marks start of separation gel. Note more pronounced MW size effect by the larger spacer, EGS (±DTT). Noncrosslinked control is identical with Fig. 4.

products, but rather as genuine membrane components, because they are obtained by labeling live cells, because of their size range (*see below*), and because they remain unaltered with DTT (Fig. 6).

Possible Molecular Equivalents

By combining CHAPS extraction of integral proteins with exocytosis stimulation and/or crosslinking (an approach not possible with isolated cell membranes), we hoped to see some changes pertinent to cell membrane structure. As expected, several bands undergo changes (Fig. 8) and some bands coincide with [^{125}I]-INA labeling. On this basis, we can speculate on a few potential candidates.

To our knowledge Schwaller et al. [42] were the only ones applying [^{125}I]-INA labeling to a secretory system, i.e., chromaffin cells. In depolarization-activated cells, INA labeled several proteins, some of which were identified on Western blots as annexins. In *Paramecium*, we had identified binding sites for antibodies (ABs) against annexin-specific peptides, anti-call-15 ABs and anti-B-15-ABs [25]. The first labels trichocyst docking sites and the second labels another exocytosis site, the cytoproct, while on Western blots ABs tag bands of 44.5 to 46 and 51 kDa, respectively. This is compatible with bands labeled by INA (Fig. 6). Calmodulin is also associated with the cell membrane, including exocytosis sites. This has been shown by EM immunocytochemistry [29, 31, 32] and it must be inferred from the presence of a calmodulin-binding domain in the plasmalemmal Ca^{2+}-pump [16, 49]. Considering variation between experiments and possible modifications, these aspects would be compatible with occurrence of 17 or 18 kDa and 121 to 145 kDa bands (Figs. 6 and 8).



1 not crosslinked	2 crosslinked
	~437
	~372
	~365
	~349
	141
135	
	128
73	
	72
61	
	59
	43
40	
	39
	30
	23
	18

3 not crosslinked	4 crosslinked
	~437
	~372
	~349
	121
100	
95	
73	
52	

not crosslinked		crosslinked	
1 not triggered	3 triggered	2 not triggered	4 triggered
285		285	
	131	141	
	102		121
	100		102
	95	90	
73		70	
61		42	
40		39	
38		24	
24		18	
16		15	

Fig. 8. Silver stained SDS-PAGE gel from CHAPS-solubilized membrane proteins. The electrophoretic patterns of membrane proteins (+DTT, +protease inhibitors) from triggered and nontriggered cells, with or without subsequent crosslinking, are compared. Numerical data refer to bands changed when (non-)crosslinked or (non-)triggered pairs are compared. "Triggered" means total exocytosis stimulation with 0.005% AED, "crosslinked" means exposure to 0.3 mM DFDNB (30 min, 4°C), eventually after AED.

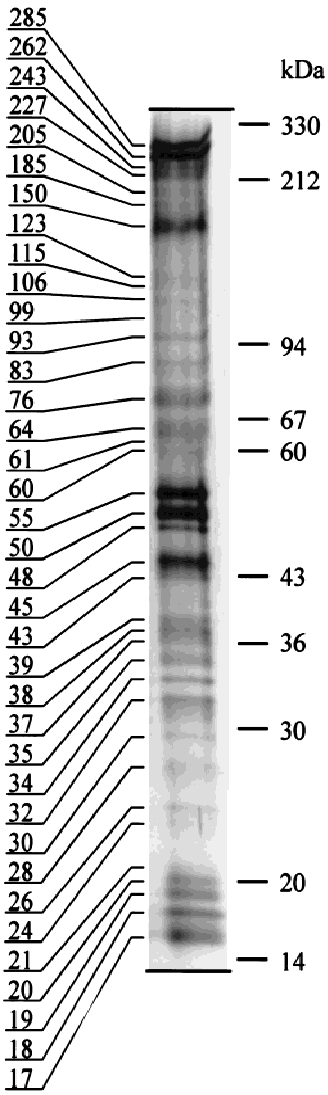


Fig. 9. Silver stained SDS-PAGE gel (7.5 to 15%, +DTT, +protease inhibitors) obtained from isolated cell membrane vesicles.

Another aspect concerns preformed trichocyst exocytosis sites which, in *Paramecium* contain a group of 7 to 8 "rosette" particles [34] identified as integral proteins [50]. On high resolution Ta/W replicas, one such particle measures, with the metal pile-up subtracted, ~12 nm [35]. Assuming a density of 1.2, a global molecular mass of ~650 kDa would result [36]. Since every rosette particle decays into 6 subunits [26], this would yield ~110 kDa subunits. Unfortunately, products from their crosslinking within a rosette particle would no more be recognizable on our SDS-PAGE analysis. Assuming a 1.4 nm^2 area occupied by one membrane-spanning helix in freeze-fracture replicas [17], every subunit of a rosette particle could then accommodate 10 transmembrane helices. This would also be compatible with one Ca^{2+}-pump as a molecular equivalent, since it possesses 10 transmembrane domains in *Paramecium* [16] just as in other systems [30]. Slight deviation from actual MW values could be due to some modifications. Since this has not yet been analyzed in detail with our system, we can only emphasize the compatibility of our data with the few molecular data available.

Conclusions

Inhibition of DTT-activation of proteases can reduce medium-sized protein bands caused by i-AG decay. These may normally predominate in hydrophilic radio-iodination experiments, whereas we show usefulness of in vivo lipophilic radio-iodination which results in totally different, medium-sized bands. These data are now amenable to detailed analysis in *Paramecium* and the method used may be successfully applied to related parasitic protozoa whose GPI-anchored surface variant antigens represent a major medical challenge [15, 20, 28].

We thank Dr. Kurt Rosenheck (Weizmann Institute of Science, Rehovot, Israel) for introducing the INA-labeling method in our laboratory and Dr. J.-D. Wissmann for establishing this method in some preliminary experiments.

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