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Intracellular dynamics of ricin followed by fluorescence microscopy on living cells reveals a rapid accumulation of the dimeric toxin in the Golgi apparatus

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Abstract

The intracellular dynamics of fluorescent conjugates of the toxic lectin ricin was followed by video fluorescence microscopy on living CHO cells, demonstrating that the ricin heterodimer and its isolated B chain, after binding to the plasma membrane receptors, migrate to and accumulate in the Golgi apparatus following internalization. A ricin derivative labelled with fluorescein on the A chain and rhodamine on the B chain did not display significant splitting of the A–B heterodimer during translocation of the toxin to the Golgi; this novel finding provides support for the hypothesis that further processing of ricin takes place in this cellular compartment.

Key words: Endocytosis; Fluorescence microscopy; Golgi; Toxin

1. Introduction

Ricin, the cytotoxic lectin from *Ricinus communis*, is constituted of two polypeptide chains (≃ 30 kDa) called A and B. The A chain is responsible for the toxic activity exerted on the eukaryotic ribosome, where enzymatically removes the Adenine-4324 of the 28 S rRNA of the major subunit [1,2]; the B chain is responsible for the binding to plasma membrane receptors, constituted mainly by the galactose or the *N*-acetylgalactosamine residues of glycoproteins and glycolipids of the cell surface.

Following binding to the cell membrane, ricin is internalized by receptor mediated endocytosis [3,4], but probably also by other mechanisms [5]. The endocytic pathway of ricin is still unclear, but several pieces of evidence indicate that the protein inside the cell is still a heterodimer associated (depending on the incubation time) with coated or non-coated vesicles, endosomes, lysosomes and the Golgi apparatus [6–11], until the active A chain is separated from the B chain and translocated to the cytoplasm.

The intracellular distribution of ricin has been investigated using artificial conjugates prepared for electron microscopy; however the use of electron dense molecules has been suspected to induce additional pathways of internalization dictated by the label as in the case of ricin conjugated with Fe or Au, found in lysosomes but not in the Golgi apparatus [7,12] or a ricin-horse radish peroxidase conjugate, found in Golgi cisternae [13]. Recent experiments have revealed the role of the Golgi stacks and the trans-Golgi network (TGN) in the activation of the protein, and the apparent colocalization of ricin with a membrane glycoprotein in the TGN has been demonstrated by immunocytochemistry [10]. However all these studies have been carried out using either fixed specimens or isolated organelles, and direct evidence on the dynamics of the phenomenon in intact cells is lacking.

The dynamics of internalization of ricin (labelled with FITC and/or TRITC) and its accumulation in the Golgi (visualized with NBD-Pc, a fluorescent derivative of phsphatidylcholine [14]) have been followed by videomicroscopy on living CHO cells; the results show that the intracellular distributions of ricin and NBD-Pc superimpose after about 30 min incubation, indicating that a significant fraction of the toxin is fairly quickly transported to the Golgi. In addition, experiments were carried out with the isolated B chain (labelled with TRITC),

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Abbreviations: FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; NBD-Pc, 2-(6-(7-nitrobenzen-2-oxa-1,3-diazol-4-yl)amino) hexanoyl-1-hexadecanoil-*sn*-glycero-3-phosphocholine; FITC- or TRITC- (-ricin, -A chain, or -B chain) indicate protein labelled with fluorescein or rhodamine.

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and with a doubly labelled heterodimer (containing FITC-A chain and TRITC-B chain); these experiments demonstrate that the two polypeptide chains migrate to the same intracellular localization, and therefore that splitting of the toxin is a late event in the pathway toward its target.

2. Materials and methods

Reagents were of analytical grade. Ricin, fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (TRITC) were purchased from Sigma Chemicals (USA); NBD-Pc was purchased from Molecular Probes (USA).

2.1. Preparation of the fluorescent derivatives of ricin

Ricin was labelled with the fluorescent compounds as described [15]. The two isolated chains [16] were labelled with either FITC (the A chain) or TRITC (the B chain); when mixed in equimolar amounts in the presence of 1 mM DTT and dialized against PBS, the chains reconstitute into a doubly labelled ricin heterodimer (as demonstrated by SDS-PAGE under reducing and non reducing conditions).

2.2. Video microscopy

A Zeiss Axiophot microscope, equipped with a 100-W epiillumination lamp, an electric shutter and the standard filter sets for fluorescence, was used throughout. A solid state CCD camera (Type 558, by I2S, France) was connected to the microscope outlet and an Apple IIGS PC was used to control the camera and the shutter of the microscope. The assembly of the instrument and the necessary electronics is described elsewhere (Guidarini et al., in preparation). Image analysis was carried out on an Apple MacIntosh IIvx using the program 'Image'.

2.3. Cell labelling and images acquisition

CHO cells were grown in D-MEM (Gibco) at 37°C under 5% CO_2 on coverslides, up to an approximate density of 200,000 cells /ml and incubated with a 5 μM solution of NBD phosphatidylcholine as described [14]. NBD labelled cells were transferred to 4°C and incubated with 300 nM labelled ricin and after 5–10 min the coverslides were washed, mounted on microscope slides and transferred to the thermostated plate of the microscope (at 37°C); images were taken at different times after temperature rise using the filter sets for fluorescein and rhodamine. The same procedure was followed in the experiments carried out with three other different derivatives of ricin (i.e. FITC-ricin labelled only on the A chain, TRITC-B chain and doubly labelled ricin reconstituted with FITC-A chain and TRITC-B chain). Some specimens were fixed for 30 min at 4°C with 2% *p*-formaldehyde in PBS and observed with a laser scanning confocal microscope (Phoibos 1000, Sarastro, USA).

3. Results

3.1. Dynamics of internalization of TRITC-ricin

Fig. 1 depicts the result of an experiment in which the time dependence of internalization of TRITC-ricin was followed in vivo on CHO cells. The time evolution of the left images clearly shows how TRITC-ricin migrates from the membrane (heavily stained at time = 0) and gradually converges from tiny spots to larger structures which seem to overlap with the Golgi apparatus. The whole process is complete in about 30 min; after this time (up to 1 h) only minor changes are observed. On the right hand side, the images of the same cell reveal NBD-Pc; this fluorophore, added before ricin, accumulates in the Golgi [14] where it remains trapped during the whole experiment (apart from slight fading). It is concluded that after 30 min incubation, labelled ricin accumulates in the Golgi; this result was reproducibly observed on several cells (see also Fig. 2A).

Some of the samples used for the experiments described above were analyzed with a confocal microscope

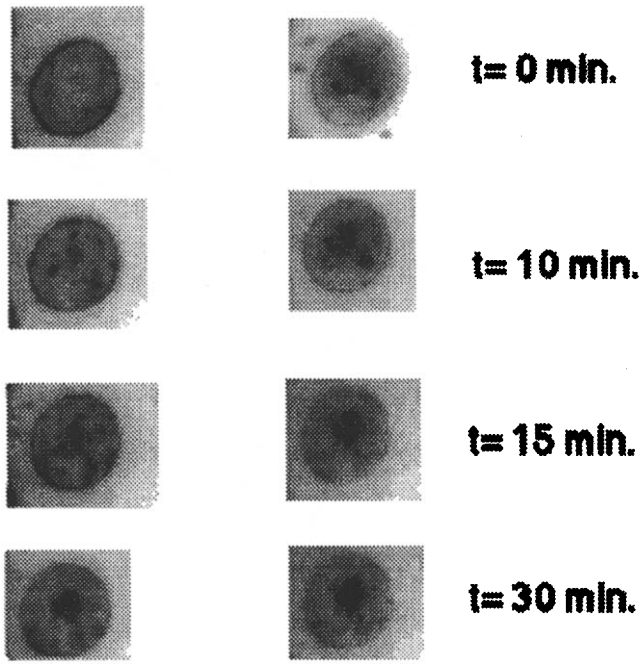

Fig. 1 A set of images of the same cell taken at different times at 32 × (oil immersion objective). CHO cells were exposed in succession to NBD-Pc and TRITC-ricin as described (see section 2). Left images were taken using the filter set for rhodamine and reveal TRITC-ricin; right images were taken with the filter set for fluorescein and reveal the Golgi apparatus stained with NBD-Pc (Sleight and Pagano [14]). Each image is the average of 8 frames accumulated over 9.6 s. A thermostatted microscope plate at 37°C was used throughout.

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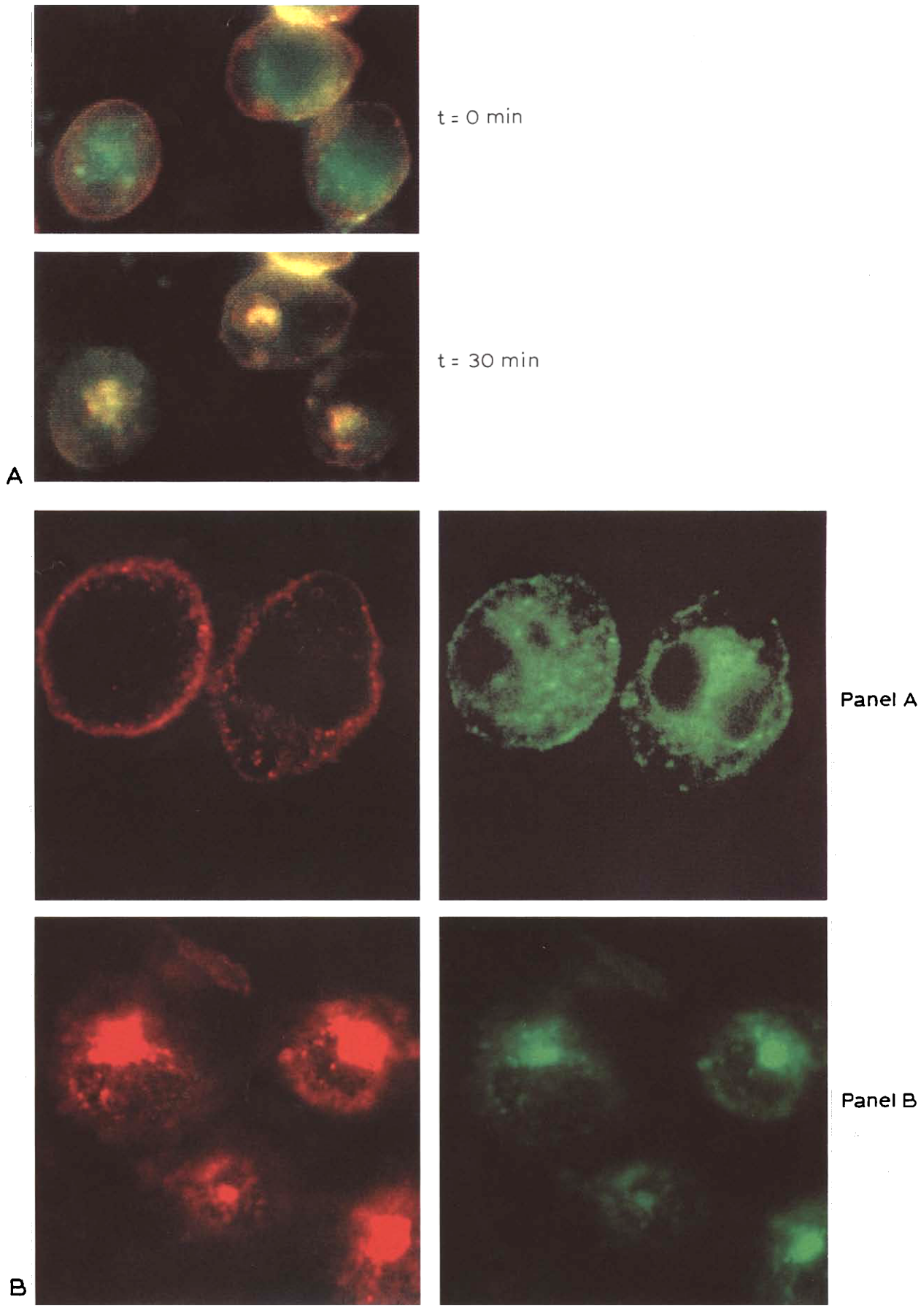
Fig. 2 (A) Computer elaboration of a microscopic field in which CHO cells, treated as in Fig. 1, were observed with the filters for fluorescein (revealing NBD-Pc) or rhodamine (revealing TRITC-ricin) at different incubation times. Each panel (t = 0 and t = 30 min) depicts the two images obtained in succession (within 1 min), one showing NBD-Pc emission in green and the other TRITC-ricin in red; this procedure generates a colour image similar to the view of the field illuminated with both blue and green light. The two emissions have different distributions at t = 0, and coalesce (yellow) in the same region of the cell after 30 min incubation at 37°C. (B) Confocal images (depth of focus approx. 0.2 μm) of CHO cells pre-treated with NBD-Pc and subsequently with TRITC-ricin under the following conditions: (panel A) cells were pre-treated with NBD-Pc and subsequently incubated with TRITC-ricin at 4°C (which hinders endocytosis). Immediately after washing away TRITC-ricin, the cells were fixed at 4°C for 30 min and observed with the confocal microscope; (panel B) cells were treated as in panel A, but after washing TRITC-ricin incubation was continued at 37°C for 30 min. Left images refer to laser line excitation for rhodamine (TRITC-ricin revealed), while right images refer to fluorescein (NBD-Pc revealed). Artficial colors from Molecular Dynamics software. Magnification 60 × (oil immersion objective).


t = 0 min
t = 30 min
A
Panel A
Panel B
B

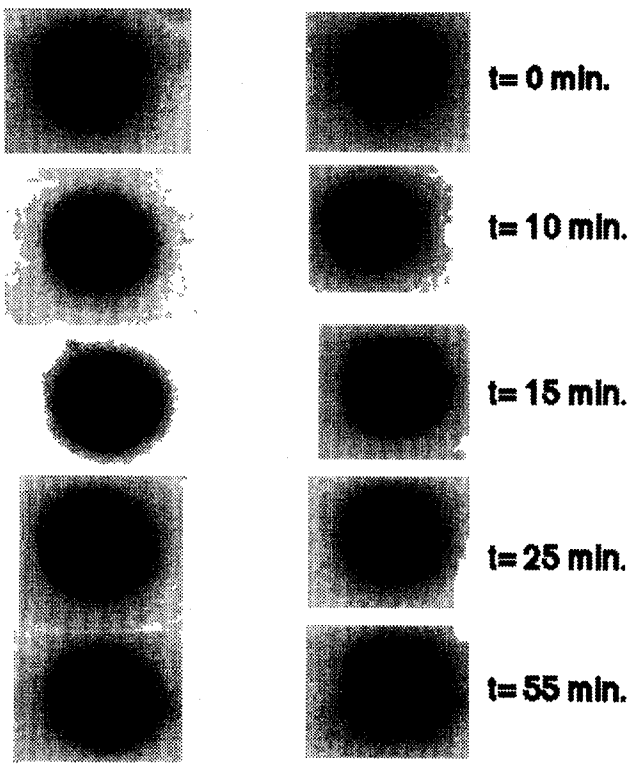


Fig. 3. Internalization of the isolated B chain labelled with TRITC as a function of time. The experiment was made with CHO cells under conditions identical to those in Fig. 1. Left images refer to rhodamine emission (TRITC-B chain revealed), while right ones refer to fluorescein emission (NBD-Pc revealed).

after fixation, as a control of the intracellular localization of the fluorophores. The results of this analysis are reported in Fig. 2B. Comparison of the four images shows clearly that TRITC-ricin is bound to the plasma membrane after incubation at 4°C, but it is internalized only after incubation at 37°C for 30 min (Panel B); no difference in the intracellular distribution of ricin and NBD-Pc is evident at this spatial resolution (0.4 μm).

An experiment identical to that shown in Fig. 1 carried out with FITC-ricin [15], reveals an endocytotic pattern indistinguishable from that seen with TRITC-ricin, indicating that the intracellular dynamics of ricin is not affected by the fluorochrome.

3.2. *Intracellular transport of the isolated B chain of ricin*

Since FITC- and TRITC-ricin are both labelled on the A chain [15], it cannot be excluded a priori that the pattern described above is a property exclusive of this subunit. Therefore we prepared a TRITC labelled isolated B chain and followed the endocytosis of this subunit in the absence of the A chain. The results presented in Fig. 3 show that the B chain alone is internalized (presumably by receptor mediated endocytosis) and converges to the Golgi apparatus, similar to the heterodimeric toxin (Fig. 1).

The time course of internalization of TRITC-ricin and TRITC-B chain was quantitatively assessed by measuring the increase of rhodamine fluorescence in an intracellular area identified from the distribution of NBD-Pc (i.e. the Golgi). The results are satisfactory (Fig. 4); in spite of some (expected) variability among different cells, the time course of the two molecules is essentially the same.

3.3. *A reconstituted ricin heterodimer with two different fluorophores on the A and B chains was used to follow the processing of the toxin*

In order to detect possible splitting (and hence activation) of the ricin heterodimer during transport from the plasma membrane to the Golgi, we prepared and purified a doubly labelled ricin derivative, with fluorescein on the A chain and rhodamine on the B chain. This experiment provides information about the intracellular fate of the bulk of the toxin, nothwithstanding that killing a cell may require only a small number of molecules. Fig. 5 depicts the time course of endocytosis of the doubly labelled ricin; during the first 30 min, both fluorophores migrate from the membrane and gather into the Golgi apparatus with identical kinetics. No evidence was found for the presence of non-overlapping fluorescent intracellular compartments, thus excluding that a significant fraction of splitting of the two chains occurs before the toxin has reached the Golgi.

4. Discussion

In spite of several studies, the intracellular routing of ricin is not yet well defined; evidence collected on living cells in particular is scarce. Our results, obtained with in

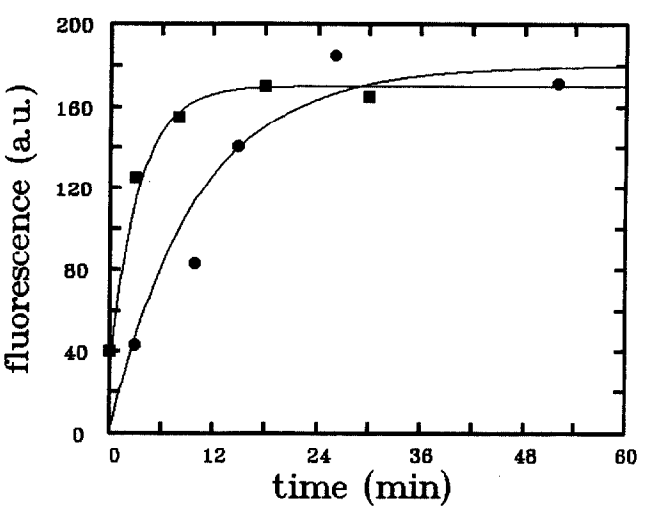


Fig. 4. Time course of internalization of TRITC-ricin (■) and TRITC-B chain (●) followed on single cells from the increase of rhodamine fluorescence intensity (a.u., arbitrary units) in the area delimited by the Golgi apparatus (as revealed by NBD-Pc). Images on several CHO cells were analyzed by the program 'Image' and gave similar results.

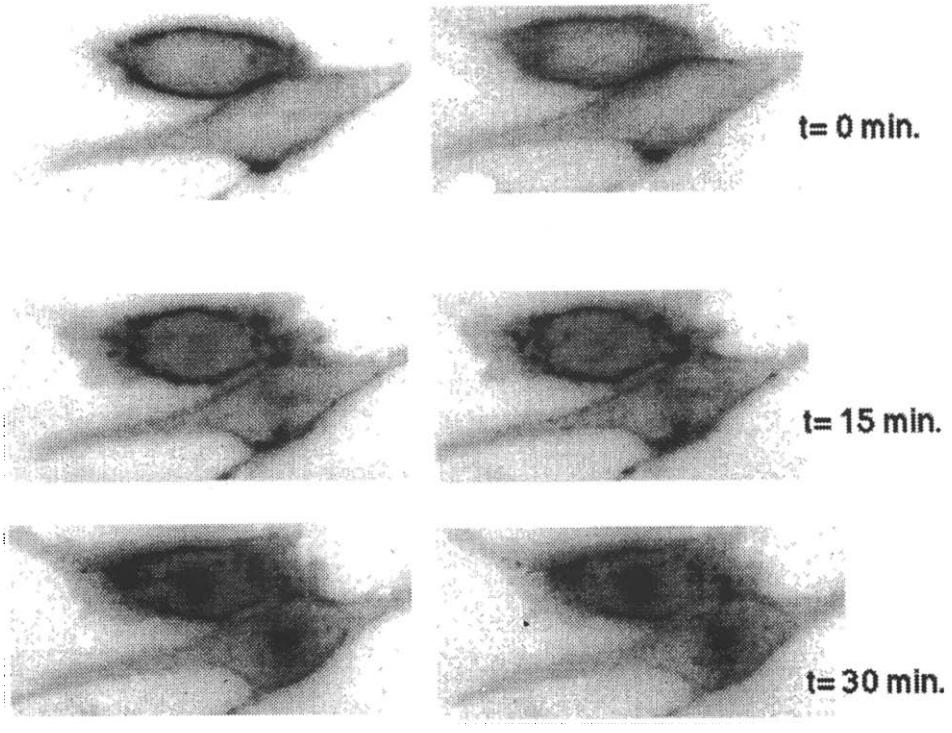


Fig. 5. Time dependence of the endocytosis of doubly labelled ricin by CHO cells. Conditions as in Fig. 1. Left images refer to fluorescein emission (which reveals FITC-A chain), right images to rhodamine emission (which reveals TRITC-B chain).

vivo observations by fluorescence microscopy, indicate that in a relatively short time (~30 min) labelled ricin bound to the cell membrane is internalized and accumulates in the Golgi region, in agreement with independent evidence obtained on fixed cells. According to recent investigations, the final processing of ricin occurs in the Golgi apparatus [10,17]. Since all the data on the time dependence of ricin toxicity on various cell lines indicate a lag time (40–60 min) preceding inhibition of protein synthesis [18], it seems obvious to suggest that this lag corresponds to the time interval necessary for delivering the toxin to the cellular compartment where activation takes place. Our kinetic measurements on accumulation of fluorescent ricin in the area of the Golgi apparatus show that the time necessary for the TRITC- or FITC-ricin to reach the Golgi is comparable to the delay in the onset of inhibition of protein synthesis. Since the catalytic efficiency of ricin is very high, and by extrapolation a single molecule may be sufficient to kill a cell [19], the fraction of the toxin transported to the Golgi represents much more than a lethal dose.

The experiments carried out with TRITC-B chain indicate that this subunit and the ricin heterodimer are both internalized with very similar routing and kinetics, supporting the conclusions that (i) binding and internalization of the toxin from the plasma membrane to the Golgi are driven by its B chain, and (ii) splitting of the two chains is a subsequent event. While the Golgi system is thought to be involved in the activation of ricin, several authors suggested that, at least with some cellular strains, ricin may accumulate in endosomal structures from which the A chain or the intact toxin could escape directly into the cytoplasm [20,21]; some results indicate that activation is also possible in the cytoplasm, via disulfide bond reduction [11] or proteolytic processing [22]. We searched, but did not find evidence for a differential processing of the two subunits of ricin during translocation from the plasma membrane to the Golgi (see Fig. 5). Our results show that the majority of ricin molecules reach this organelle in their native dimeric form and remain trapped there for a relatively long time (40–60 min). This conclusion is also in agreement with the results published by Youle and Colombatti [23] who demonstrated, using hybridoma cells producing anti-ricin antibodies, that whole ricin meets the antibody in the Golgi and is inactivated there. The role of the Golgi apparatus in ricin activation is confirmed by the action of the drug Brefeldin A (BfA) which disorganizes this organelle [24]; treatment of cells with BfA [25] can completely inhibit the toxicity of ricin. Finally it should be pointed out that, nothwithstanding the report [26] indicating escape of ricin from 'non-Golgi' compartments, if splitting of the heterodimer involved only a minority of the labelled toxin molecules, detection may be below the sensitivity of our instrument.

In summary, our results are compatible with the following interpretation: a significant fraction of the ricin bound to the cell membrane is rapidly (20–30 min) trans-

ported to the Golgi apparatus, and the fraction of the dimeric protein which is further processed during transport to the Golgi is very small, if any. It would be of interest to compare the in vivo cellular dynamics of ricin with that of other toxins, such as modeccin [27] or *Pseudomonas*, *Diphtheria* and *Cholera* toxins. These belong to either one of two classes [25], depending on their endocytic pathway: the members of one group, which comprises ricin, are delivered primarily to the Golgi apparatus before being released into the cytoplasm; those of the other, represented by *Diphtheria* toxin, directly escape from acidic vesicles. Quantitative experiments on the in vivo intracellular traffic of these toxins are lacking, and since some of them are used to prepare artificial conjugates (with immunoglobulins or hormones, [28]) or genetically engineered [29] as anti-cancer drugs, clarification of the mechanism of activation and intracellular routing is of great interest and should be pursued.

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