Non-endocytic penetration of core histones into petunia protoplasts and cultured cells: a novel mechanism for the introduction of macromolecules into plant cells

Joseph Rosenbluh[a], Sunil Kumar Singh[b,1], Yedidya Gafni[b],
Adolf Graessmann[c], Abraham Loyter[a,*]

[a] Department of Biological Chemistry, The Alexander Silberman Institute of Life, Sciences, The Hebrew University of Jerusalem,
91904 Jerusalem, Israel
[b] Department of Plant Genetics ARO, The Volcani Center Bet Dagan 50250, Israel
[c] Institut fuer Molekularbiologie und Biochemie, Free University of Berlin, Germany

Abstract

The results of the present work demonstrate that core histones are able to penetrate the plasma membrane of plant cells. Confocal microscopy has revealed that incubation of petunia protoplasts with fluorescently labeled core histones resulted in cell penetration and nuclear import of the externally added histones. Intracellular accumulation was also confirmed by an ELISA-based quantitative method using biotin-labeled histones. Penetration into petunia protoplasts and cultured cells was found to be non-saturable, occurred at room temperature and at 4 °C and was not inhibited by Nocodazole. Furthermore, penetration of the biotinylated histone was neither blocked by the addition of an excess of free biotin molecules, nor by non-biotinylated histone molecules. All these results clearly indicate that the observed uptake is due to direct translocation through the cell plasma membrane and does not occur via endocytosis. Our results also show that the histones H2A and H4 were able to mediate penetration of covalently attached BSA molecules demonstrating the potential of the histones as carriers for the delivery of macromolecules into plant cells. To the best of our knowledge, the findings of the present paper demonstrate, for the first time, the activity of cell penetrating proteins (CPPs) in plant cells.
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1. Introduction

Macromolecules and high molecular weight complexes such as proteins, viruses or oligonucleotide–protein complexes are taken into animal cells mainly via a process of receptor-mediated endocytosis [1–3]. Binding of such ligands to surface receptors promotes a process of microtubule-dependent plasma membrane invagination, and subsequently the formation of intracellular coated vesicles [4]. The process of receptor mediated endocytosis leads, in most cases, to the formation of clathrin-coated vesicles. However, in addition, non-clathrin-dependent endocytosis can direct

the internalization of external molecules. This may include phagocytosis [5], macropinocytosis [6] and the formation of non-clathrin-coated caveolae vesicles [7]. Following ATP dependent uncoating, and intracellular trafficking as well as recycling of membrane receptors, the internalized vesicles are eventually fused with lysosomes resulting in degradation of their content [8]. The use of various labeled markers as well as cell free systems [1] greatly contributed to our present understanding of the molecular mechanism, as well as the proteins involved in the process of endocytosis and vesicle fusion in animal cells.

A plant cell in nature is surrounded by a cell wall, thus, direct plasma membrane invagination is not a natural process in the plant kingdom. Yet, experiments done explanta indicate that a process of plasma membrane invagination with features resembling that of endocytosis in animal occurs also in plant cells [9–11]. However, much

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less is known about its detailed mechanism [9–11]. It was shown that the addition of biotinylated macromolecules such as hemoglobin, BSA or IgG to cultured soybean cells resulted in their intracellular accumulation [12,13]. Promotion of endocytosis by biotin was inferred from experiments showing that the addition of free biotin molecules strongly inhibited the uptake of the biotinylated macromolecules.

Internalization was not observed at 4 °C demonstrating a temperature-dependent process and indicating a requirement for metabolic energy [12,13]. The uptake of the biotinylated macromolecules into the plant cells was a saturable process and was inhibited by the addition of Nocodazole [14], a tubulin-disrupting agent that was shown to block endocytosis in animal cells [15].

During the past few years, it became apparent that certain small molecular weight proteins are able to directly cross the plasma membrane of mammalian cells without the involvement of the endocytic pathway and thus are not susceptible to the intraendosomal enzymes [16]. Due to their ability to translocate across cell plasma membranes independently of transporters or specific receptors, these proteins—as well as active peptides derived from them—have been designated as cell penetrating proteins/peptides (CPP). Translocation across the plasma membrane is a major limiting step for the delivery of macromolecules such as proteins and DNA into living cells. A promising strategy to overcome this problem is the use of CPP as a vehicle following their conjugation to the macromolecule of interest. Indeed, various CPPs have been used to mediate the internalization of a large number of different cargo molecules such as oligonucleotides, peptides, peptide nucleic acids, proteins or even nanoparticles [17–19].

Penetration of CPPs, such as the HIV-1 Tat or the arginine-rich motif (ARM) peptide derived from it [20] or of the Penetratin peptide obtained from the *Drosophila* mastoparan protein [21], occurs at 4 °C, is not saturable and is observed in the presence of endocytosis inhibitors. The mechanism by which these CPPs (and their conjugates) enter cells is not yet elucidated and the involvement of the endocytic pathway in the penetration process is currently under dispute [22–26].

Recently, we have observed that recombinant purified core histones, namely H2A, H2B, H3 and H4, can cross mammalian plasma membranes via a non-endocytic process [27]. Penetration was temperature- as well as energy-independent and was not inhibited by microtubule and microfilament disrupting agent, resembling a process that characterizes penetration of the abovementioned CPPs.

The results described in present work show that core histones are able to cross plasma membranes of plant cells as was demonstrated with petunia protoplasts and cultured cells. Internalization was evident from fluorescence microscopy studies as well as using biotinylated histones and a newly developed quantitative assay system. Since uptake of the core histones was temperature-independent and was neither inhibited by Nocodazole nor by excess of histone

molecules, we suggest a non-endocytic mechanism. Our present results demonstrate, for the first time, the activity of the histones as CPPs in plant cells.

2. Materials and methods

2.1. Petunia cell culture and protoplasts preparation

Petunia hybrida (cell line 3704) was grown by weekly subculturing in UM medium [28] and protoplasts were prepared from rapidly growing culture as described [29]. Cell wall formation was determined using the calcofluor white staining technique [30]. Protoplasts were washed three times in a washing medium (CPW: 9% Mannitol, supplemented with (mg/l) 27.2 KH_2PO_4, 101 KNO_3, 1480 $CaCl_2·2H_2O$, 246 $MgSO_4·7H_2O$, 0.16 KI, 0.025 $CuSO_4·5H_2O$ at pH = 5.8) and resuspended in 10 ml of the same medium to give a final concentration of 1×10^6 cells/ml.

2.2. Preparation and purification of recombinant histone proteins

Core histone expression vectors were a generous gift from Dr. Karolin Luger (Department of Molecular Biology, Scripps Research Institute, 92037, La Jolla, CA, USA) and Dr. T.J. Richmond (Institut fur Molekularbiologie und Biophysik, Eidgenossische Technische Hochschule-Honggerberg, CH-8093 Zurich, Switzerland) Expression and purification of the four core histones (H2A 14 kDa, H2B 13.8 kDa, H3 15.2 kDa, H4 11.2 kDa) was preformed in *E. coli* BL21plysS strain as previously described by Luger et al. [31].

2.3. Biotin labeling of core histones

Purified recombinant core histones were incubated with biotin-maleimide (Sigma) in a molar ratio of histone/biotin of 1:15 as described before [27]. The amount of covalently attached biotin was estimated by using the HABA reagent (Sigma) according to manufacturer's instructions and was found to be, on average, 4 mol biotin/mol histone.

2.4. Fluorescence labeling of core histones

A histone mixture containing all the five histones (Sigma cat no: H5505) as well as the individual recombinant histones (H2A, H2B, H3 and H4) were labeled with Lissamine Rhodamine (Molecular Probes) or covalently attached to fluorescently labeled BSA [32] and the unincorporated Rhodamine was removed by gel filtration (Sephadex G-25, prewashed with PBS, pH 7.4) exactly as described before for labeling of other protein molecules [33]. The labeled histones and the BSA obtained were then dialyzed and solublilized in PBS.

2.5. Chemical conjugation of the core histones to biotinylated BSA

Conjugation of core histones to biotin-labeled BSA (Bb) molecules (Sigma cat. no: 082k9191) was preformed exactly as described previously for conjugation of synthetic peptides to Bb [27,34]. Briefly, a Bb solution (30 mg in PBS) was incubated with 4.3-mg Sulfo-SMCC (Pierce) (in a final volume of 1.2-ml PBS) for 2 h at room temperature to give Bb-Sulfo-SMCC. Unbound, free Sulfo-SMCC was removed using a Sephadex G-25 column. (Pharmacia). The Bb-Sulfo-SMCC was then incubated overnight at 4 °C with the purified core histones in a 1:1 (molar ratio) and the histone–Bb conjugates obtained were concentrated using a VivaScience (Sartorius) at MWCO of 50,000.

2.6. Incubation of Rhodamine-labeled histone with petunia protoplasts: microscopic observations

Petunia protoplasts (200 μl of washed suspension) were incubated for 1 h at room temperature with 30 μM of Rhodamine-labeled histones and following centrifugation (700 rpm) and two washings with the CPW medium, the protoplasts were analyzed by a confocal microscope using an MRC 1024 confocal imaging system (Bio-Rad). The microscope (Axiovert 135 M; Zeiss, Germany, a 63 × objective; Apoplan; NA 1.4) was equipped with an argon ion laser for Rhodamine excitation at 514 nm (emission 580).

2.7. Quantitative determination of histone accumulation within petunia protoplasts and cultured cells using an ELISA-based method

Accumulation of externally added biotinylated molecules within petunia protoplasts and cultured cells was quantitatively determined essentially as described before for accumulation of biotinylated molecules in animal cells [27]. Briefly, biotinylated histones or Bb–histones conjugates were incubated with petunia protoplasts or cells (300 μl of 1×10^6 protoplasts/ml of CPW and the same concentration of petunia cultured cells suspended in UM medium [28]) for 1 h at room temperature. The suspensions obtained were then centrifuged at 700 rpm at room temperature. Surface bound biotin molecules were neutralized by resuspending the pellet obtained in 200 μl of 2 mg/ml avidin (Sigma) (in CPW). Following incubation at room temperature for 30 min, free avidin molecules were neutralized by the addition of 200 μl of biocytin (Sigma) (5 mg/ml in CPW) followed by an additional incubation for 15 min. Suspensions containing either petunia protoplasts or cultured cells were then centrifuged at 700 rpm, and the protoplasts/cells in the pellet were lysed with 200 μl of 1% Triton X-100. The suspension containing the petunia cultured cells was further sonicated (three rounds of 30 s each). A volume of 10 μl of the protoplast/cell extract

obtained was then added to maxisorp® plate's (NUNC) wells, each of which contained 190 μl of Triton–gelatin solution (0.2% Triton + 0.05% gelatin (Sigma) in PBS). When Bb–histone conjugates were used, the maxisorp® plates were pre-coated with anti-BSA antibodies as described before [27,35]. Following overnight (about 12–14 h) incubation at 4 °C, plates were washed three times with PBS, and incubated for 5 min in 37 °C with 200 μl of the Triton–gelatin solution. After another three washes with PBS, the plates were incubated for 1 h in 37 °C with 200 μl of avidin–POD (Roche Diagnostics) in Tween gelatin solution (0.2% tween + 0.1% gelatin in PBS) 1:5000 (v/v). Following additional three washes with PBS and incubation for 5 min with 200 μl of the Triton–gelatin solution and another three washes with PBS, avidin–POD was determined by estimation of fluorescence emission at 490 nm exactly as described before [27,36].

2.8. Determining protein concentration

Protein concentration was determined using a DC-protein assay kit (Bio-Rad cat. no: 500-0116).

3. Results

3.1. Penetration of core histones into petunia protoplasts: fluorescence microscopy observations

The results in Fig. 1 show that incubation of petunia protoplasts with fluorescently labeled core histones resulted in fluorescent staining of the protoplasts. The intracellular and nuclear presence of the externally added histones is evident from observing fluorescence staining of both cytosol and nuclei in two microscopic sections of the same cell (Fig. 1a,a′ –d,d′). Plant protoplasts, which lack a cell wall, are extremely fragile and easily disintegrate whenever their plasma membrane becomes leaky. The fact that no fluorescence staining was observed following incubation of the petunia protoplasts with Rhodamine-labeled BSA molecules (Fig. 1e) indicates the intactness of the plasma membrane as well as the viability of the protoplasts. Essentially the same picture, namely very little fluorescence, was observed following the addition of free Rhodamine (not shown).

The accumulation of labeled histones within the cytoplasm and nuclei of the petunia protoplasts was highly reproducible, however, as is evident from Fig. 1, a diverse degree of the cytosol, but not of the nuclei, staining was observed with the different labeled core histones. This may result from a slight difference in the degree of the fluorescence labeling of the histones and not necessarily due to diversity in the penetration extent of the core histone. Thus, in order to achieve a more accurate estimation of the penetration process and to study its mechanism, an ELISA-based system was used to quantify the amount of the intracellular histones.
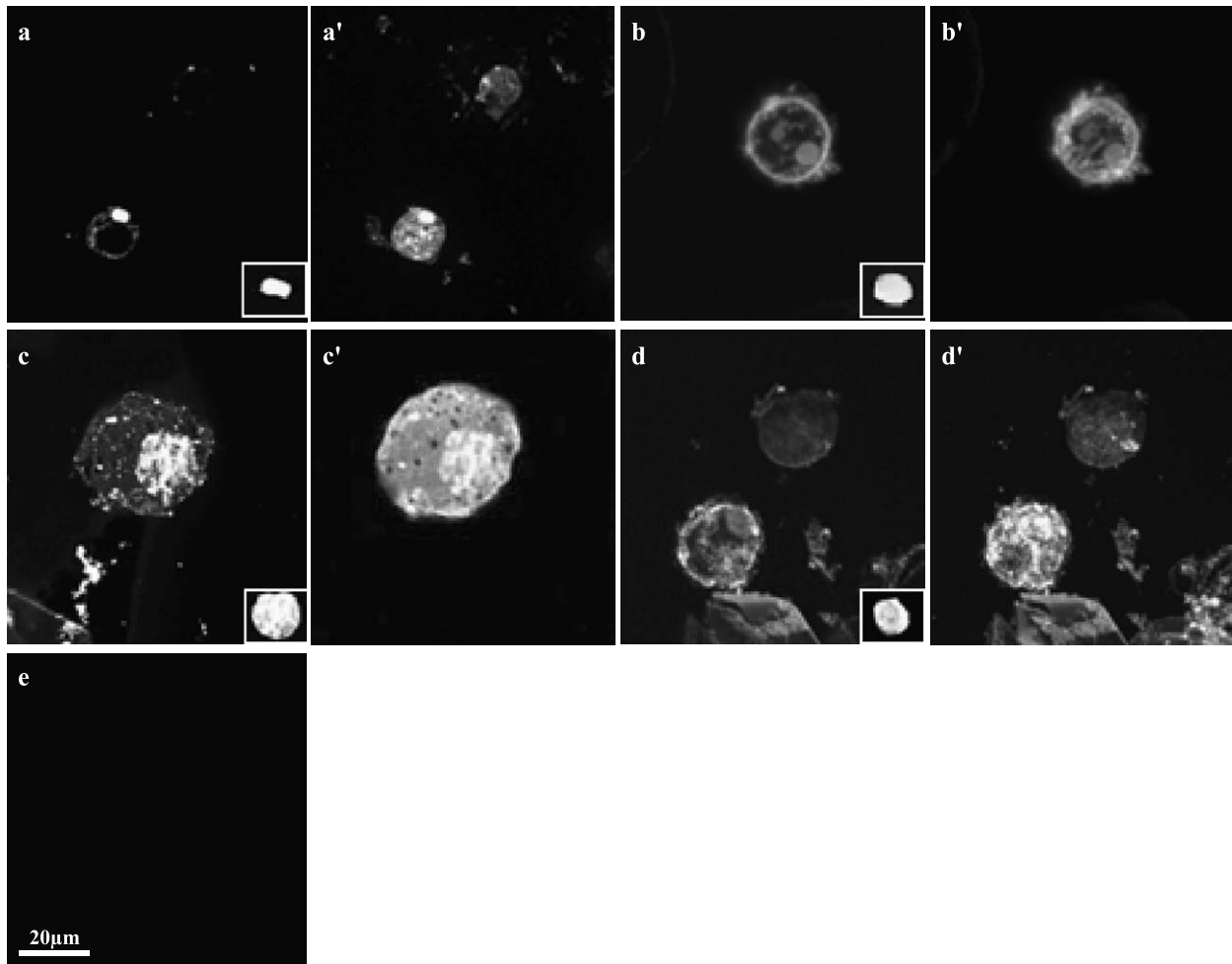
Fig. 1. Cell penetration and nuclear import of core histones into petunia protoplasts as visualized by scanning laser confocal microscopy. Protoplasts were incubated for 1 h at room temperature in the presence of 30 μM of Rhodamine-labeled core histones or BSA. For each of the individual core histones, two microscopic sections are presented. a,a′ : H2A; b,b′ : H2B; c,c′ : H3; d,d′ : H4; e: BSA. Large images of stained cell nuclei are shown at the right corner of a–d.

3.2. Penetration of core histones into petunia protoplasts is a non-endocytic process: quantitative estimation

The ELISA system used for estimation of the intracellular accumulation of histones was based on the following principles: (a) Histone molecules are labeled with biotin and these conjugates are incubated with the petunia protoplasts or cultured cells. (b) Any external, membrane—associated, not penetrated, free or histone-bound, biotin molecule—are neutralized by the addition of avidin. (c) Following the neutralization step, the protoplasts or cells are lysed by the addition of triton and the biotin conjugates present in the lysate are estimated. Only biotin molecules that were detected following the neutralization and the lysis step were considered to represent histone molecules accumulated within the intracellular space of the protoplasts or cultured cells (Ref. [27] and see Materials and methods).

The results in Fig. 2(A–E, c) reveal that incubation of externally added biotinylated core histones with petunia protoplasts resulted in intracellular accumulation of the histone molecules. Following neutralization of externally

present biotin molecules, only intracellular and not surface-bound histone molecules are estimated. Indeed, under the present experimental conditions, all the externally membrane-bound biotin molecules could be neutralized by the added avidin (Fig. 2A–E compare a to b; in a, both surface bound and intracellular biotin were determined, while in b, biotin molecules were neutralized before they were added to the protoplasts). Furthermore, as should be inferred by comparing the results in Fig. 2A–E(c) to those in Fig. 2A–E(d), very little, if any, biotin molecules could be detected following neutralization of surface-bound biotinylated histones, unless cells were lysed by the triton treatment. Our quantitative estimation indeed reveals that all the four core histones, namely H2A, H2B, H3 and H4, were able to penetrate into the petunia protoplast with slightly different degrees (Fig. 2A–E(c)) indicating that the variation obtained using the microscopic assay is not significant. It appears that the internalization process was temperature-independent, because it did not decrease by incubation at 4 °C; on the contrary, it was significantly stimulated at this low temperature probably indicating a non-endocytic pro-

A - D
a= un-neutralized
b= pre-neuralized
c= intracellular
d= intracellular - triton
e= 4°C
f= + 1 mM biotin
g= + 10 μM Nocadazole

E
a= un-neutralized
b= pre-neuralized
c= intracellular
d= intracellular - triton
e= + 1 mM biotin
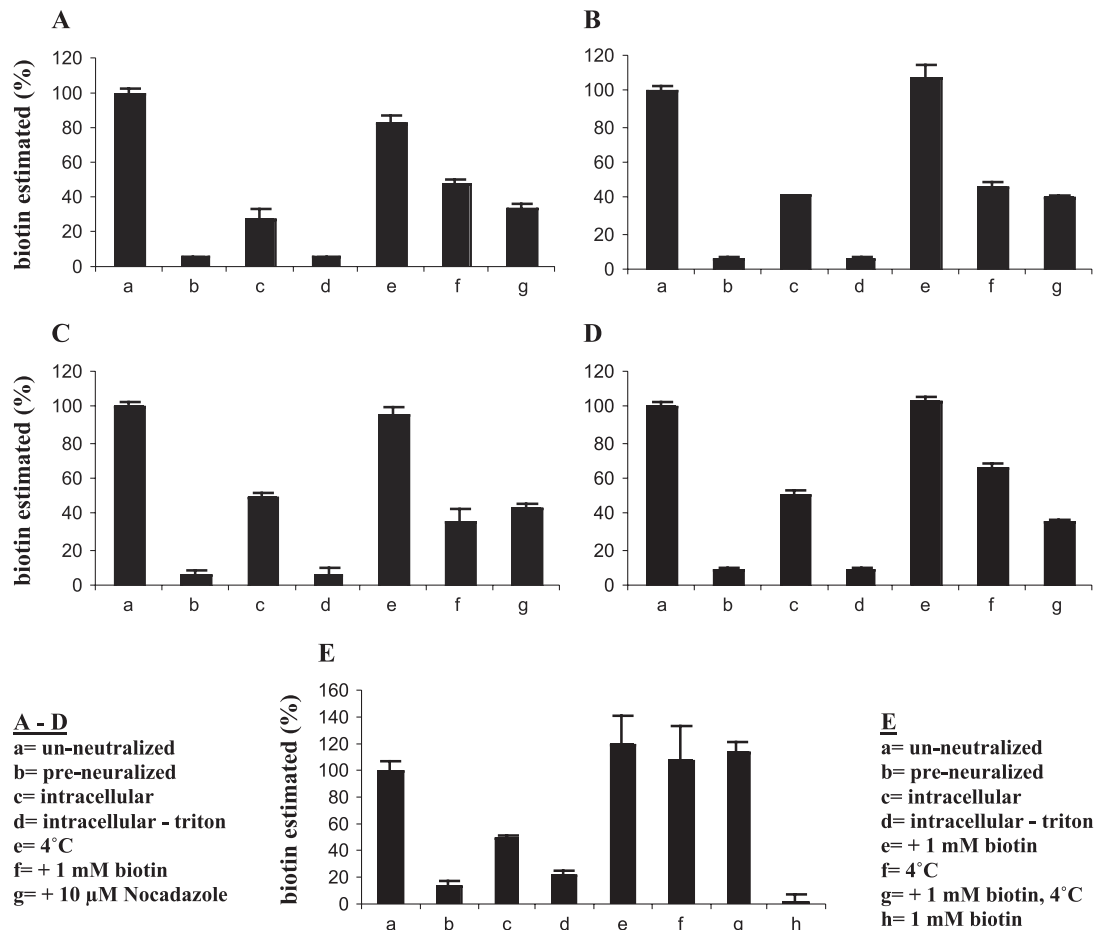f= 4°C
g= + 1 mM biotin, 4°C
h= 1 mM biotin

Fig. 2. Penetration of biotinylated core histones into petunia protoplasts: quantitative estimation. Protoplasts were incubated for 1 h at room temperature with 1.3 μM biotinylated core histones and the presence of biotin molecules was estimated as described in Materials and methods. (A) H2A; (B) H2B; (C) H3; (D and E) H4. (a) Unneutralized histones. Experimental conditions were as described in Materials and methods except that the step of the avidin addition (neutralization step) was omitted. The total amount of the biotin molecules (100%) represent the amount of biotin estimated in washed and lysed protoplasts (membrane-bound + penetrated). (b) Pre-neutralized histones. The biotinylated core histones were neutralized by avidin before they were added to the protoplasts. (c) Intracellular-accumulated histones. Incubation with the biotinylated histones, neutralization of surface bound biotin molecules by avidin and estimation of intracellular biotin following the lysis step as described in Materials and methods. (d) As in c but protoplasts were not treated with triton following incubation with the biotinylated histones. [e−g (in A−D)] As in c but with following modifications. (e) As in c but incubation at 4 °C. (f) In the presence of 1 mM biotin. (g) Protoplasts were pre-incubated with 10 μM Nocodazole. e−h (in E). (e) As in c, in the presence of 1 mM biotin. (f) As in c but incubation was performed at 4 °C. (g) As in f but in the presence of 1 mM biotin. (h) 1 mM biotin was added in the absence of H4. Total amount of biotinylated histone (membrane-bound + penetrated, 100%) was estimated to be 0.23 nmol histone/mg lysate.

cess (compare results of Fig. 2A−D(e) to Fig. 2A−D(c)). Moreover, Nocodazole, a well-established inhibitor of endocytosis in plant cells [14], did not inhibit the intracellular accumulation of the core histones (Fig. 2A−D(g)). In our experiments (Fig. 2A−D(f) and E) also the addition of excess free biotin molecules (1 mM, biotin: biotinylated histone = 770 mole/mole) did not have any effect on the penetration of the biotinylated histones, indicating that the internalization observed was not mediated by the covalently attached biotin molecules. Furthermore, the results in Fig. 2E(e−h) show that even incubation of the protoplasts with the histones H4 with 1 mM biotin in the cold did not have any effect on the penetration. The same results were essentially obtained with H2A, H2B and H3 (not shown), consistent with a non-endocytic process.

The results in Fig. 3A show that the penetration process, as is demonstrated with H2A, was not saturable, indicating receptor-independent process. The same results were in the presence of Nocodazole (Fig. 3A) as well as with H2B, H3 and H4 (not shown). Kinetic studies revealed that the intracellular accumulation of H2A was time-dependent reaching a maximum degree at about 25−30 min of incubation at room temperature (Fig. 3B). Very similar results were obtained with H2B, H3 and H4 (not shown).

3.3. Core histones are able to penetrate petunia cultured cells

The results in Fig. 4(A−D) demonstrate that the four core histones are able to penetrate petunia cultured cells, which,
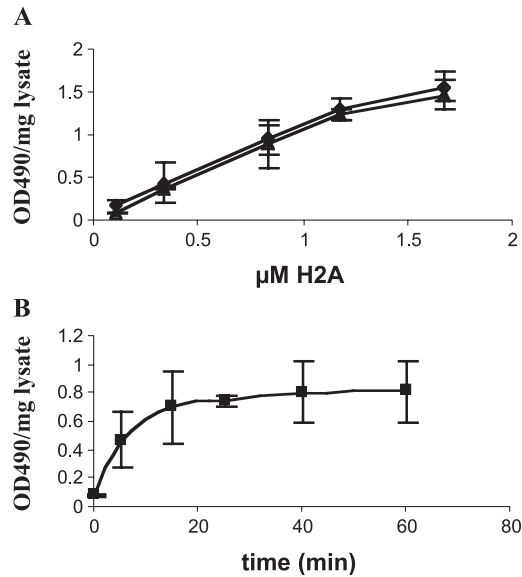
Fig. 3. Penetration of H2A into petunia protoplasts. (A) Protoplasts were incubated at room temperature with increasing concentration of H2A and its penetration was studied in the absence (■) or the presence (▲) of Nocodazole. (B) Histone H2A (1.3 μM) was incubated at room temperature with the petunia protoplasts and its penetration was estimated as a function of the incubation period. All other experimental conditions as described in Materials and methods.

as opposed to protoplasts, are surrounded by a cell wall, by a mechanism that is consistent with a non-endocytic process. Similar to the results obtained with the protoplasts (see Fig. 2), the externally added biotinylated histones could be neutralized by the addition of avidin (Fig. 4A–D(a and b)). Estimation of only intracellular biotin molecules should be inferred from the results showing that only a low amount of biotin molecules was detected unless the cells were sonicated and lysed (Fig. 4A–D(c) and (d)). Penetration into the culture cells was not inhibited either by excess of biotin (1 mM) or by pre-incubation with Nocodazole, again indicating a non-endocytic process (compare results in Fig. 4AD(f) and A–D(e) with A–D(c)). As is evident from the results in Fig. 4A–D(g) and A–D(h), the penetration degree of free biotin or of biotinylated BSA (Bb) molecules did not exceed that of the background penetration, namely the amount of biotin molecules estimated following pre-neutralization of the biotinylated histones (Fig. 4A–D (b)).

3.4. BSA molecules covalently attached to histones can be translocated into petunia protoplasts but not into petunia cultured cells

The results in Fig. 5A and D show that the histones H2A and H4 were able to mediate the penetration of covalently

A-D
a= un-neutralized   b= pre-neutralized
e= + 1 mM biotin   f= + 10 μM Nocadazole
c= intracellular   d= intracellular - triton
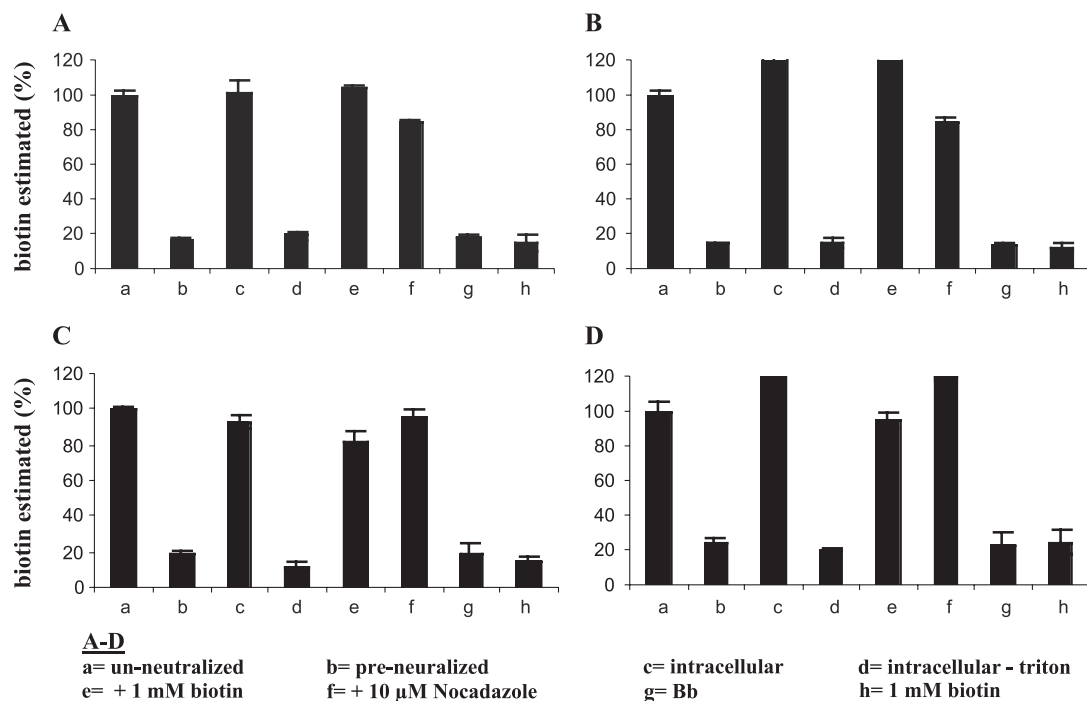g= Bb   h= 1 mM biotin

Fig. 4. Penetration of biotinylated core histones into petunia cultured cells. Plant cells (300 μl of 1×10^6 cells/ml of UM medium) were incubated for 1 h at room temperature with 1.3 μM biotinylated histones and biotin molecules were estimated as described in Materials and methods. (A) H2A; (B) H2B; (C) H3; (D) H4. (The experimental conditions in a–c are identical to those described in a–c in the legend to Fig. 2). (a) Unneutralized histones. (b) Pre-neutralized histones. (c) Intracellular-accumulated histones. (d–g) As in c with the following modifications. (d) Following incubation with biotinylated histones and neutralization of membrane-bound histone molecules, the cells were not treated with triton. (e) As in c in the presence of 1 mM biotin. (f) Cells were pre-incubated for 2 h with 10 μM Nocodazole. (g) Cells were incubated with biotinylated BSA molecules and not with biotinylated histones. (h) Cells were incubated with 1 mM biotin in the absence of biotinylated histones. Total amount of biotinylated histone (membrane-bound + penetrated, 100%) was estimated to be 0.5 nmol histone/mg lysate.

A-D
a= un-neutralized   b= pre-neuralized   c= intracellular   d= intracellular - triton
e= 4°C   f= + 1 mM biotin   g= + 10 µM Nocadazole   h= + unlabeled histone   i= Bb
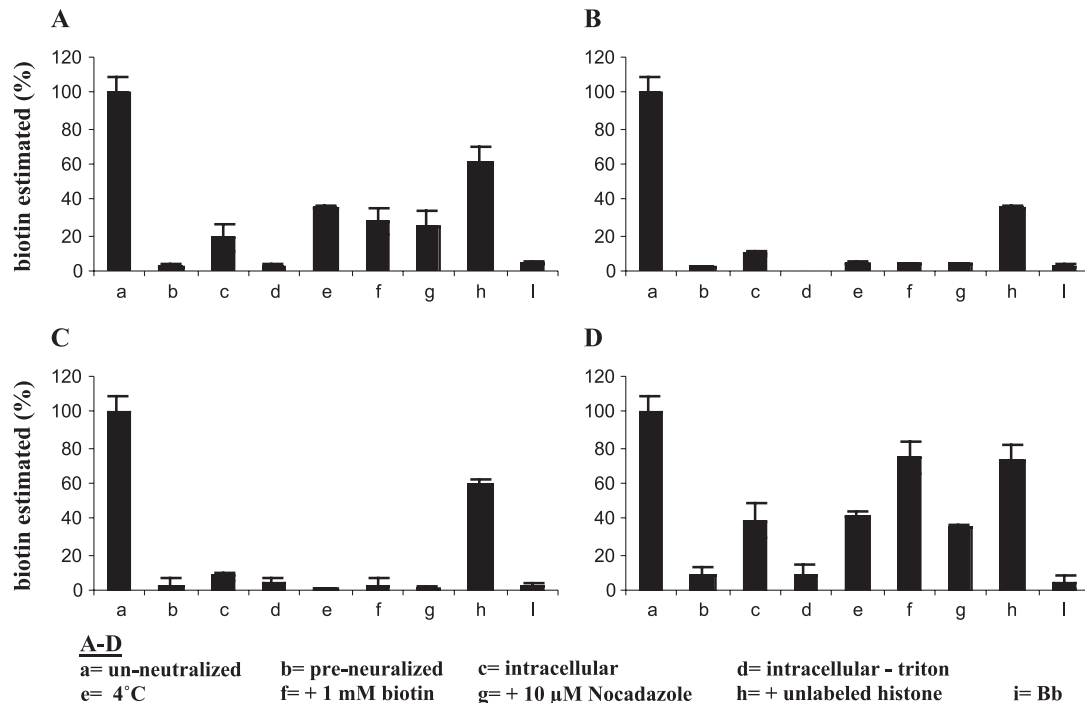
Fig. 5. Histone-mediated penetration of BSA molecules into petunia protoplasts. All the experimental conditions of incubation with the biotinylated labeled BSA (Bb)−core histone conjugates with petunia protoplasts and determination of Biotin molecules as described in Materials and methods and in the legend to Fig. 2(a−c). (A) H2A−Bb; (B) H2B−Bb; (C) H3−Bb; (D) H4−Bb. Protoplasts were incubated for 1 h with 40 nM of Bb−histones. (a) Unneutralized Bb−histones. (b) Pre-neutralized Bb−histones. (c) Intracellular Bb−histones. (d−i) As in c with the following modifications: (d) After the incubation with the Bb−histones, the protoplasts were not treated with triton. (e) Incubation was performed at 4 °C. (f) In the presence of 1 mM biotin. (g) In the presence 10 µM Nocodazole. (h) With an excess of × 200 (mole/mole) of unlabeled histone. (i) Incubation with Bb alone instead of Bb−histones. Total amount of biotinylated histone (membrane-bound + penetrated, 100%) was estimated to be 0.446 pmol Bb−histone/mg lysate.

attached biotin-labelled BSA (Bb) molecules into petunia protoplasts. A very low degree of intracellular accumulation was observed when Bb−H2B and Bb−H3 were incubated with the protoplasts (Fig. 5B(c) and C(c)). It is noteworthy that within the BSA−core histone conjugates only the BSA molecules are biotinylated, thus the detection of intracellular biotin molecules indicates penetration and accumulation of BSA. Intracellular Bb−core histone molecules could be detected only following lysis of the protoplasts (Fig. 5A−

D(d)). The results in Fig. 5A−D(a) and A−D(b) clearly show that neutralization of surface-bound biotin molecules was efficient. Penetration of Bb−H2A and Bb−H4 was neither inhibited by excess of biotin (1 mM) nor by pre-incubation with Nocodazole or by incubation at low temperature (4 °C) again indicating a non-endocytic process (compare results in Fig. 5A and D (f) and A and D (e) with Fig. 5A and D (g)). Evidently, under the present experimental conditions, the penetration degree of free Bb mole-
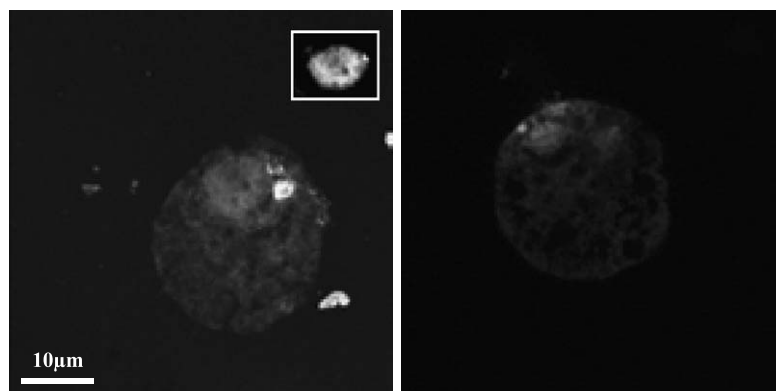

Fig. 6. Confocal microscopy observations of histone−BSA conjugate penetration into petunia protoplasts. 30 µM of a mixture of histone molecules conjugated to BSA-Rho was incubated with petunia protoplasts for 1 h; two microscopic sections are presented. At the right corner a large image of the stained nuclei is shown. Experimental conditions as described in Materials and methods.

cules did not exceed that found following pre-neutralization of the biotinylated histones, namely of background penetration (compare Fig. 5A and D (i) and A and D (b)), indicating the intactness of the protoplasts and the requirement histone molecules for the penetration of the Bb–histone conjugates. Penetration of all the Bb–core histones was strongly stimulated when an excess of the same unlabelled histone was added (Fig. 5A–D (h)). Together, these results demonstrate that the histone molecules can mediate the translocation of large exogenous molecules, such as BSA, into protoplasts.

Confocal laser scanning microscopy observations have confirmed penetration of core histone–BSA conjugates into petunia protoplasts (Fig. 6). From the microscopic studies it appears that penetration of the histone–BSA conjugate is lower than that of the histone themselves (compare the intracellular fluorescent staining in Fig. 6 to that in Fig. 1). From our microscopic studies it also appears that following their penetration, the histone–BSA conjugates are translocated into the nuclei (Fig. 6) as was observed with the histone molecules themselves (Fig. 1). In spite of their ability to penetrate into protoplasts, none of the Bb–core histone conjugates were internalized into cultured plant petunia cells (not shown).

4. Discussion

Based on these results, we suggest that core histones, similar to their penetration into mammalian cells [27], can penetrate plant protoplasts and cultured cells via direct translocation through the plasma membrane. Intracellular presence of the fluorescently labelled histones is evident from the confocal microscopic studies. It also appears that following their penetration and accumulation within the petunia cytoplasm, the core histones are imported into the nuclei probably via a process mediated by their nuclear localization signal (NLS) motifs [37,38].

In addition to the microscopic observations, an ELISA-based quantitative method further established penetration and intracellular accumulation of biotinylated core histones in petunia protoplasts and even into cultured cells, indicating that the low molecular weight histones can move through the cell walls. Our results demonstrate that histones H2A and H4 were able to mediate penetration of covalently attached BSA molecules into the petunia protoplasts, but not into petunia cultured cells. This may indicate the presence of an unbreached cell wall, since it has been well established that macromolecules above 25 kDa are unable to traverse the plant cell wall and, thus, their interaction with the cell plasma membrane is prevented [39]. Biotinylated (or fluorescently labelled) BSA molecules alone failed to penetrate the petunia protoplasts or the petunia cultured cells. It is our view that these negative results further strengthen the reliability and the constancy of the quantitative assay system used in the present work. Histone internalization was rapid as it could be observed after 10 min of incubation, and it

was not saturable, occurred at 4 °C and was not inhibited neither by Nocodazole nor by excess of free histone or biotin molecules. All these results suggest a receptor-independent, non-endocytic process. Previously, biotinylated molecules were shown to be taken into plant protoplasts and cells via a process that was inhibited by free biotin or Nocodazole, did not occur at 4 °C and thus was postulated to be due to receptor mediated endocytosis [12–14]. However, endocytosis was observed with much higher concentration of externally added protein and required much long incubation periods than those used in the present work [14]. Here penetration was observed following the addition of 1 μg of histone or Bb–histone molecules and it reached maximum value after 10 min of incubation. On the other hand, induction of endocytosis was observed only after the addition of 200 μg of Bb and reached its maximum value following 3–4 h of incubation [14]. Interestingly, in our experiments incubation at 4 °C as well as in the presence of excess of unlabeled histone significantly increased the degree of penetration, further indicating that endocytosis is not involved. Stimulation in the cold may indicate direct involvement of the membrane phospholipids, which undergo a process of phase transition and lateral separation at relatively low temperatures [40]. In spite of extensive work during the last few years, the mechanism by which CPPs (and their conjugates) enter cells has not been elucidated. Inverted phospholipid micelles, whose structure may be stabilized at low temperature, were proposed as a possible membrane configuration that may allow translocation of the CPPs through the plasma membrane [41].

The stimulation of penetration observed by excess of histones appears to be a more complicated phenomenon. It is noteworthy that the same results were observed following incubation of histone or histone–BSA conjugates with animal cultured cells [27] or with phospholipid liposomes (manuscript in preparation). It is possible that the binding of relatively large quantities of positively charged molecules such as histones may promote destabilisation of the cell's phospholipid bilayer. Indeed, polymers, particularly positively charged polymers, have been shown to have different effects on lipid bilayers, including induction of membrane fusion [42], selective membrane permeabilization [43], formation of laterally phase-separated membrane domains [44], and phase transformations to micelles or lamellar gels [45]. In many cases, these effects may be dependent on variables such as pH or temperature. It certainly will be of interest to find whether other positively charged polymers, in addition to histones, will stimulate penetration of histone or histone–BSA conjugates, or by themselves will penetrate.

The results of the present work demonstrate, for the first time, the ability of histone molecules to act as CPPs in plant cells, namely by penetrating directly through petunia protoplasts and cultured cells plasma membranes. Direct protein translocation through cell plasma membranes was first demonstrated for the HIV-1 Tat protein which was shown to

enter mammalian cells when added to the surrounding media [46,47] Moreover, following its penetration, the Tat protein could be imported into the nuclei and then trans-activate the viral long terminal repeat (LTR) promoter, demonstrating preservation of its biological activity in the intracellular environment. Several other proteins with trans-ducing capabilities have been identified [21,48,49]. The ability to directly cross the cell plasma membranes was attributed to specific regions within the CPPs called protein transduction domains (PTDs).

Recently, however, the observed penetration of the Tat protein or of its peptide derivatives was suggested to be an artifact caused by the use of fixed cells [50,51]. It has been shown that the Tat protein, as well as other CPPs, binds strongly to the plasma membrane and remains associated with cells even after repeated washings. Fixation was claimed to promote redistribution of the surface-bound Tat, resulting in what appears, by microscopic observations, as cell penetration and nuclear import. It is our view that an artifact of cell fixation cannot explain the results obtained in the present work because the present studies, both the microscopic observations as well as the ELISA quantitative determinations, were performed with unfixed protoplasts and cultured cells. Furthermore, the possibility that mem-brane-bound histone molecules were estimated was exclud-ed, as only biotinylated molecules that were exposed following neutralization of surface bound biotin and deter-gent disruption of the protoplasts or cells were considered as intracellular molecules.

In the past decade, the development of gene transfer technology into plant cells has focused in part on the design of new methods for gene delivery into cells. The major challenge resides in the design of vectors that can overcome the low permeability of the cell membrane for nucleic acids and improve intracellular trafficking and nuclear delivery of genes into target cells with minimal toxicity. Indeed, the development of new tools for introducing foreign genes into plants, combined with the growing knowledge and technol-ogy related to gene identification and isolation, has revolu-tionized the field of plant biotechnology. Since the discovery of *Agrobacterium*'s natural ability to transfer DNA fragments to plant cells [52], other biological and mechanical means for transforming plant species have been developed [53–56]. However, there is still a need for the development of new and efficient, not expensive, methods for gene transfer, which is still the main limiting step in obtaining transgenic plants from many species and espe-cially for the monocotyledonous and conifers plants. Un-fortunately, transformation protocols available for these plants rely mainly on techniques that are expensive on one hand (due to the cost of the equipment involved) or less efficient than the methods used for dicotyledonous plants.

Undoubtedly, histone molecules can serve as a vehicle to deliver small molecules and proteins into protoplast's and plant cell's mitochondria and chloroplasts following chem-

ical attachment of the appropriate leader signals [57,58]. Histone molecules may also be instrumental as a DNA delivery system into protoplasts and possibly into plant cells. The ability of histone–DNA complexes to transfect protoplasts and especially whole plant cells is under inves-tigation in our laboratory.

Recent studies have demonstrated that histones such as H2A [59,60], H3 and H4 [61] are effective mediators for transferring DNA molecules into several mammalian cell lines. The potential of histones to serve as delivery systems is due to their ability to mediate the entry of DNA mole-cules, not only into the cytoplasm, but also into the nuclei via their NLS [38]. In eukaryotes, the nucleosome is a complex composed of the histone octamer, consisting of two molecules each of histone H2A, H2B, H3, and H4, and the chromosomal DNA [62]. This packaging has significant effects on DNA metabolism and gene regulation [63]. Thus, the use of histone as a gene carrier in plant cells may open new possibilities for obtaining transgenic plants. Recently a 37-aa peptide derived from histone H2A has been identified and shown to be effective in gene transfer in mammalian cultured cells [59]. Current experiments are being conducted in our laboratory to study the efficiency of this peptide as a DNA vector in plant protoplasts and cells.

Acknowledgements

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