J. Biochem. **135**, 713–719 (2004)
DOI: 10.1093/jb/mvh087

Low pH Facilitates Uptake of Proteins by Cells through a Non-Endocytic Pathway

Mitsuyoshi Motizuki[1], Toshiaki Takei[4], Kachio Tasaka[2], Sadaki Yokota[3], Shuichi Kojima[4], Tatsuya Haga[4] and Kunio Tsurugi[*,1]

[1]Department of Biochemistry 2, [2]Department of Immunology, and [3]Biological Laboratory, Faculty of Medicine, University of Yamanashi, Nakakoma, Yamanashi 409-3898; and [4]the Institute for Biomolecular Science, Gakushuin University, Tokyo 171-8588

Received September 24, 2003; accepted April 14, 2004

We previously noted that bovine apolipoprotein A-II (apoA-II) had a bactericidal effect causing morphological changes in the cytoplasm. To determine whether and how apoA-II and apoA-I, which have acidic isoelectric points (pIs), enter cells, we determined the rates of uptake of FITC-labeled proteins by fibroblast cells and found that they entered cells more easily at low pH than at neutral pH under conditions where endocytosis was inhibited. The enhanced uptake of proteins at low pH was also observed for other proteins examined regardless of the molecular weight (M_r) or pI in a time-dependent manner, although the efficiency of uptake varied among the proteins. Furthermore, a pH gradient was shown to be the main driving force for the translocation. As cells were viable above pH 4 for 2 h at 4°C and internalized ß-galactosidase was active under these conditions, we suggest that this procedure is applicable to the injection of proteins into cells without the use of an apparatus such as a microinjector.

Key words: apolipoprotein A-II, endocytosis, low pH, membrane.

Abbreviations: ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II; DPBSG, Dulbecco's phosphate-buffered saline supplemented with glucose; FHV, flock house virus; FITC, fluorescein isothiocyanate; HDL, high-density lipoprotein; HIV-1, human immunodeficiency virus 1; HRP, horseradish peroxidase; HSV-1, herpes simplex virus 1; PC, L-dimyristoylglycero-phosphocholine; TFE, trifluoroethanol.

It is generally accepted that the plasma membrane of cells is impermeable to proteins and peptides. Although cells have developed a complex system for exporting secretory proteins, little transfer of proteins from the extracellular environment to the cytosol is thought to occur (*1*). Adsorptive or fluid-phase endocytosis (pinocytosis) has been thought to be a typical mechanism of cellular internalization for many proteins (reviewed in Refs. *2–4*). In general, the internalized proteins in endocytic vesicles are delivered to lysosomes for degradation. However, there have been some reports showing the uptake of proteins through a non-endocytic pathway. It has been reported that a low pH is sufficient for diphtheria toxin to enter Vero or V79 cells (*5*, *6*). Translocation of diphtheria toxin to the cytosol is normally carried out by acidic endosomes following receptor-mediated endocytosis (reviewed in Ref. *7*). However, direct translocation of the catalytic A-fragment of the toxin can be induced when cells with surface-bound toxin are exposed to low pH, which mimics the conditions in the endosomes. Furthermore, it has been reported that basic proteins or peptides like Tat protein from human immunodeficiency virus 1 (HIV-1), homeobox peptide of *Drosophila* Antennapedia protein, a capsid protein VP22 from herpes simplex virus 1 (HSV-1), and the RNA-binding peptides from HIV-1 Rev and flock house virus (FHV) coat protein are translocated across the cell membrane at neutral pH (*8–12*) and even at 4°C in a non-endocytic manner (*11–14*). The domains responsible for this translocation contain basic amino acid clusters (*11–14*).

Apolipoprotein A-I and A-II (apoA-I and apoA-II) are the major protein constituents of high-density lipoprotein (HDL). ApoA-I is responsible for a major part of the anti-atherogenic activity of HDL, and apoA-II is thought to be an antagonist as to the anti-atherogenic property of apoA-I (reviewed in Refs. *15* and *16*). We have reported that bovine apoA-II shows anti-*Escherichia coli* activity (*17*), and that its C-terminal region from residue 49 to C-terminal 76 is critical for the activity (*18*). The target site of the C-terminal fragment, named peptide 49–76, was shown to exist in the cytoplasm because electron micrographs of *E. coli* treated with peptide 49–76 revealed morphological changes only occur in the cytoplasm (*18*). This finding also raised the possibility that bovine apoA-II can be translocated across the cell membrane, although it has an acidic isoelectric point (pI) (5.0) and contains no obvious clusters of over three residues of basic amino acids.

In this study, to determine whether apolipoproteins enter cells through endocytosis, we measured the uptake of FITC-labeled proteins by fibroblast cells. We demonstrate that fibroblast cells take up apolipoproteins and other proteins examined with different efficiencies through a non-endocytic pathway at low pH regardless of the pI or molecular weight (M_r), and that a pH gradient is the main driving force for the translocation.

[*]To whom correspondence should be addressed. Tel/Fax: +81-55-273-6784, E-mail: Ktsurugi@yamanashi.ac.jp

Table 1. **Certain properties of the proteins used in this study and the quantities of cell-associated protein calculated from the data in Fig. 3.**

Protein	M_r (kDa)	pI[a]	Uptake (% of administered load per 10^5 cells per hour) pH 7	pH 5	(fold)
ApoA-I	27	5.7	0.2	2.8	(14)
ApoA-II	8.6	5.0	0.3	3.1	(10)
BSA	66	5.7	0.1	16	(160)
Histone[b]	11–15	10–11	0.3	13	(43)
α-Lactalbumin	14	4.9	0.2	3.4	(17)
Ovalbumin	45	5.2	<0.1	1.1	(>11)
IgG	150	6.6–10	<0.1	0.5	(>5)
HRP[c]	43	7.1	<0.1	0.4	(>4)

[a]The isoelectric point (pI) was calculated from the amino acid sequence of each protein or protein mixture. [b]This preparation of histone contains few H1 species. [c]The molecular weight (M_r) and pI of HRP are the averages for the C1 and C2 species.

MATERIALS AND METHODS

Proteins—Bovine apoA-I and apoA-II were isolated from HDL as described previously (*17*). Bovine serum albumin (BSA, lipid-free), *E. coli* β-galactosidase, chicken ovalbumin, bovine α-lactalbumin, horseradish peroxidase (HRP), and FITC-labeled goat immunoglobulin G (IgG) were purchased from Sigma Chemical (St. Louis, MO). Certain properties of these proteins are summarized in Table 1. Fluorescein isothiocyanate (FITC, Sigma) was covalently conjugated to the proteins by constant stirring in 0.5 M Na_2CO_3 (pH 9) at 4°C for 3 h. The FITC-labeled proteins were purified by gel-permeation chromatography (Sephadex G-25; Amersham Biosciences, Piscataway, NJ) with 10 mM Tris buffer (pH 7), and reverse-phase HPLC (Resource RPC; Amersham Biosciences) with a linear gradient of H_2O and acetonitrile.

Cell Culture—Mouse fibroblasts (BALB/3T3) were cultured in 24-well plates at 37°C in a humidified 5% CO_2 in air environment in minimal essential medium (RPMI 1640, Sigma) supplemented with 100 mg/liter of kanamycin, 300 mg/liter glutamine and 10% fetal calf serum (FCS; EQUITEC-BIO, Ingram, TX). After 4 days without replacement of the medium, the cells (2×10^5 cells/well) were used for the uptake experiments.

Uptake Experiment—After removal of the medium, the cells were rinsed twice with Dulbecco's phosphate-buffered saline (DPBS) supplemented with glucose (DPBSG; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1 g/liter D-glucose) at 4°C and subsequently exposed to 500 μl of an each FITC-labeled protein solution (0.5 μM) in DPBSG for the indicated periods at the indicated temperature. At the end of the incubation, the cells were washed twice with ice-cold PBS/2% FCS and then once with PBS, and finally lysed with 10 mM Tris (pH 8) containing 1% Triton X-100 for 1 h at 37°C. The resulting lysate was used for measuring the fluorescence with a Hitachi F-4000 fluorescence spectrophotometer and for determination of the protein content according to the method of Lowry *et al*. (*19*). The average protein content determined with this method was 250 μg per 10^6 cells. In some experiments, the pH of DPBSG was lowered with H_3PO_4. The cells were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed thoroughly with 10mM Tris/0.1M NaCl (pH 7), and then exposed to an each FITC-labeled protein solution in DPBSG. After incubation at 25°C, the cells were lysed with 10 mM Tris (pH 8) containing 1% Triton X-100 and 0.025% trypsin.

Cell Viability—The cell viability at various pHs was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method, which is based on reduction of tetrazolium salts by mitochondrial dehydrogenases of living cells (*20*). After incubation in DPBSG (pH 8–3) for 2 h, the cells were incubated with cell culture medium containing 0.5 mg/ml of MTT.

To determine whether or not cells exposed to acidic pH retain their proliferative activity, cells culutured in DPBSG (pH 8–3) for 2 h were incubated with cell culture medium for 24 h and then the cell number was determined.

Confocal Laser Scanning Microscopy—Cells were grown on coverslips in 6-well plates. After the cells had reached semiconfluence, the coverslips were washed twice with DPBSG (pH 5), and then treated with an each FITC-labeled protein solution in DPBSG (pH 5) at 4°C. After 30 min, the cells were washed three-times with ice-cold PBS containing 2% FCS, fixed with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 6.8) for 1 h at 4°C, and then washed with PBS. Individual coverslips were mounted cell-side down on fresh glass slides. The cells were viewed under a Leica TCS4D confocal laser scanning microscope.

Histological Staining—The cells grown on glass coverslips were washed twice with DPBSG, and then treated with β-galactosidase (100 μg/ml) in DPBSG (pH 7 or pH 5) at 4°C. After 1 h, the cells were washed with ice-cold PBS/2% FCS, fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.8) for 30 min at 4°C, and then washed with 10 mM Tris (pH 7), 0.1 M NaCl. The fixed cells were incubated in 1 mg of X-Gal (5-bromo-4-chloro-3-indolyl β-galactoside) per ml, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$. After 30 min, the reaction was terminated by washing with distilled water.

Circular Dichroism (CD) Measurements—CD spectra of the proteins were recorded with a J-720 spectropolarimeter (JASCO) using a 1 mm optical path cuvette. The CD spectra of the proteins in 10 mM sodium phosphate buffer (pH 7.4) or 10 mM sodium acetate buffer (pH 5), or in a 1:1 (v/v) mixture of trifluoroethanol (TFE) and buffer were recorded at room temperature in the 200–250 nm wavelength range. CD spectra were also measured in the presence of phospholipid vesicles (peptide/lipid molar ratio, 1:30). The phospholipid vesicles (liposome) were

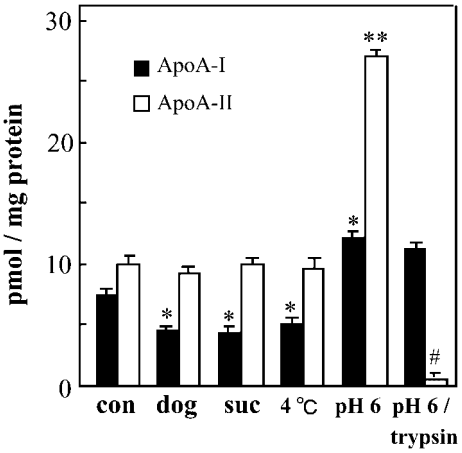

Fig. 1. **Cellular uptake of apoA-I or apoA-II by fibroblast cells under various conditions.** In the first four experiments, fibroblast cells were incubated with 0.5 μM FITC-labeled apoA-I or apoA-II for 30 min in DPBSG at pH 7 at 25°C (con), and then endocytosis was inhibited with 25 mM 2-deoxyglucose/10 mM Na-azide in place of glucose (dog), with 450 mM sucrose (suc), or at 4°C (4°C). In the last two experiments, cells were incubated at pH 6.0 (pH 6) and, after the incubation, treated with trypsin (pH 6/trypsin). Statistical analysis with the *t*-test (n = 3) indicated: $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control; $^{\#}P < 0.01$ *versus* pH 6.

prepared by sonication of multilamellar vesicles composed of L-dimyristoylglycero-phosphocholine (PC, Sigma). CD data are expressed in terms of mean residue ellipticity at 222 nm ($[\theta]_{222}$). The helicity was estimated with the following equation: Percentage α-helix ={–($[\theta]_{222}$ + 2340)/30300} ×100 (*21*).

RESULTS

Effect of Inhibition of Endocytosis on Uptake of Apolipoproteins—To determine whether or not mammalian cells take up apolipoproteins, we incubated fibroblast cells (BALB/3T3) with fluorescence-labeled apoA-I or apoA-II at 25°C for 30 min (Fig. 1). In a control experiment, about 0.2% of the administrated load per 10^5 cells for both proteins was taken up during the incubation. When endocytosis of the cells was inhibited by either depleting ATP with 2-deoxyglucose/Na-azide, increasing the osmotic pressure with 450 mM sucrose, or lowering the temperature to 4°C, the uptake of apoA-I was reduced to 40–60% of the control level (Fig. 1). The results suggested that apoA-I was taken up mostly through endocytosis because a reduction of the uptake under such conditions is characteristic of endocytic uptake (*2–4*). In contrast, the uptake of apoA-II was not affected under any of these experimental conditions suggesting that apoA-II was taken up through a non-endocytic pathway.

The plasma membrane is thought to have a receptor for apoA-I, which promotes secretion of lipids from cells through an active transport pathway (*22–24*). So, we examined whether or not the uptake of apoA-I is receptor-mediated by means of incubation at acidic pH, which is known to enhance receptor-ligand dissociation (*3*). However, on incubation at pH 6, the uptake of apoA-I and apoA-II increased 1.7- and 2.7-fold, respectively, compared with on incubation at pH 7 (Fig. 1), raising the possibility that apoA-I is also taken up through a non-endocytic pathway, at least at acidic pH.

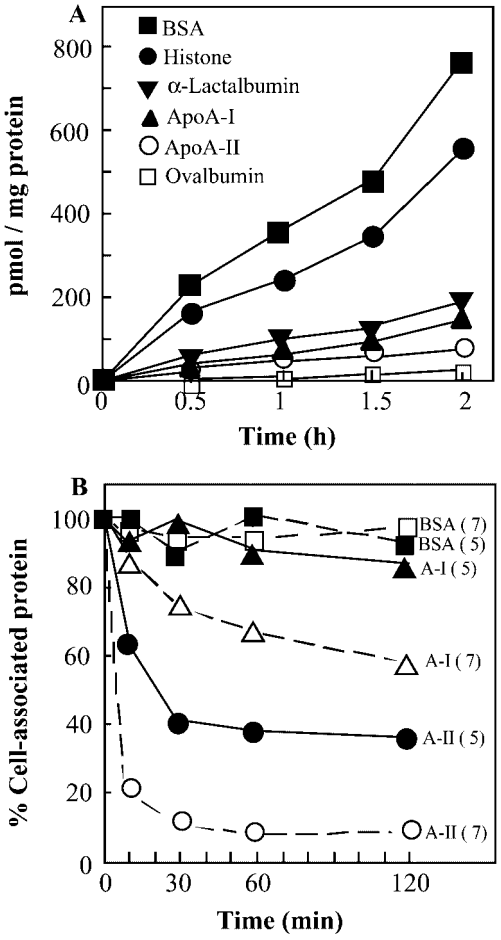

Fig. 2. **Time courses of protein uptake into or protein release from fibroblast cells.** (A) Fibroblast cells were incubated with 0.5 μM FITC-labeled protein in DPBSG at pH 5 and 4°C for the indicated time periods. Cells were isolated and lysed, and then the quantities of the proteins in the cell lysates were determined by fluorescence spectrometry. (B) The cells, preloaded with FITC-labeled proteins, were incubated at pH 7 or 5 in DPBSG at 4°C. The results shown are representative of two independent experiments.

As it is also possible that apolipoproteins are associated with cells on their outside, cells loaded with apoA-I or apoA-II at pH 6 were treated with trypsin. The great majority of the cell-associated apoA-I was insensitive to trypsinization (Fig. 1), suggesting that it was inside the cells. In contrast, trypsinization reduced cell-associated apoA-II to less than 5% of the control level (Fig. 1), suggesting that most of the apoA-II was bound to the surface of the cells, although it remains possible that apoA-II rapidly exited the cells during the trypsinization.

Time Dependency of Protein Translocation—To further characterize the mode of translocation of apolipoproteins across the cell membrane, the time courses of protein uptake were determined at 4°C and compared with those of other proteins listed in Table 1. The results showed that all the proteins examined except for apoA-II were taken up in an approximately linear manner for at least 2 h (Fig. 2A), eliminating the possibility that the process is an artificial one like adsorption. Exceptionally, however,

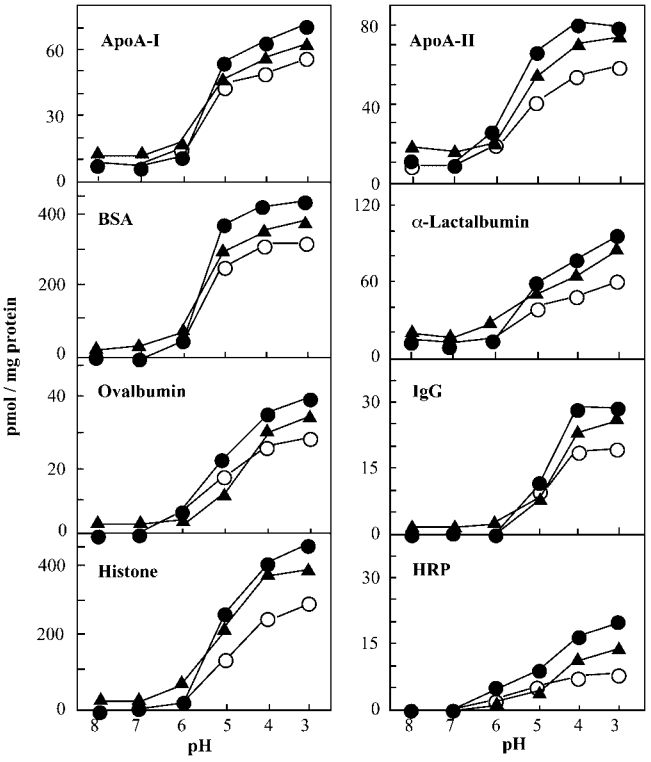

Fig. 3. **Quantities of proteins taken up by fibroblast cells at different pHs.** Fibroblast cells with (solid triangles) and without fixation with 1% glutaraldehyde (solid circles, open circles) were exposed to 0.5 μM FITC-labeled protein in DPBSG at the indicated pHs ranging from 8 to 3 for 30 min at 25°C (solid circles, solid triangles) or 4°C (open circles). Cells were isolated and lysed, and then the quantities of the proteins in the cell lysates were determined by fluorescence spectrometry. The results shown are representative of two independent experiments.

the uptake of apoA-II reached a plateau after 60 min incubation. This indicated two possibilities, namely that apoA-II is adsorbed on the cell surface or that it rapidly exits the cells, an equilibration between protein import and export being reached. To examine these possibilities, we determined the rates of release of the proteins from cells (Fig. 2B). The results showed that, among the proteins examined, apoA-II was most rapidly released from the cells, followed by apoA-I, and this process occurred more rapidly at pH 7 than at pH 5, while BSA (Fig. 2B) and other proteins like histone, ovalbumin, and α-lactalbumin (data not shown) were not significantly released in 2 h at either pH. About 90% and 60% of apoA-II was released from the cells within 30 min at pH 7 and pH 5, respectively. ApoA-I was released at a slow rate (25% in the first 30 min) at pH 7 and not significantly released at pH 5 (Fig. 2B). Thus, it is likely that all the proteins examined were taken up by the cells in a time-dependent manner at low pH, although apoA-II and, to a lesser extent, apoA-I readily exited cells.

Protein Uptake at Low pH—To provide an insight into the mode of protein uptake at low pH, we investigated the uptake of fluorescence-labeled apolipoproteins and other proteins at various pHs ranging from 8 to 3. The uptake of all the proteins was significantly stimulated when the pH of the medium was lowered below 5 (Fig. 3). When the amounts of proteins taken up by the cells at pH 7 were compared with those at pH 5 (Table 1), the efficiencies of protein uptake at low pHs significantly differed among the proteins independent of the pI or Mr. BSA showed the highest, and IgG and HRP the lowest efficiencies among the proteins. On the other hand, the uptake of the proteins by chilled (at 4°C) or glutaraldehyde-fixed cells was inhibited only partially, with greater inhibition for chilled cells than for fixed cells (Fig. 3). Furthermore, trypsinization of chilled cells loaded with the proteins at pH 5, except for those with apoA-II, did not decrease the fluorescence intensities (data not shown), indicating that proteins were present inside the cells. To determine the cellular localization of apoA-II, chilled cells incubated at pH 5 with FITC-labeled apoA-II were observed under a confocal laser scanning microscope. The micrographs clearly showed the diffuse distribution of the fluorescence in the cytoplasm indicating the internalization of apoA-II (Fig. 4). Similar patterns of distribution were found for all the other proteins on fluorescence microscopy (data not shown). These results suggested that proteins enter cells through an endocytosis-independent pathway at low pH.

To rule out the possibility that the internalization of proteins at low pH is caused by a passive process like increased leakiness of the lipid bilayer itself or a conformational change of some membrane spanning proteins, we examined the leakage of cytoplasmic proteins during incubation at low pH. After incubation of 10^7 cells (2.5 mg protein) in 1 ml of an acidic medium (pH 4 or 5) at 4°C for 2 h, 10 μl of the medium was analyzed by SDS/PAGE. No bands were seen on silver-stained gel (data not shown), suggesting that the internalization of proteins is not a passive process caused by damage to the plasma membrane.

Cell Viability at Low pH—We checked cell viability after the incubation of cells for 2 h at various pHs using the MTT test (Fig. 5). While the cell viability was little decreased above pH 5, it was severely decreased at pH 3, being completely lost at 25°C and partly (<40%) at 4°C. At pH 4, about two-thirds of the incubated cells were viable at 4°C, although over half were dead at 25°C. Thus, the increased uptake of proteins at low pH, as shown in Fig. 3, was not, at least not completely, due to death at least above pH 4 for the chilled cells and above pH 5 for the cells incubated at 25°C. Furthermore, we examined the proliferation activity after the incubation of cells for 2 h at acidic pHs at 4°C by measuring the increase in the cell number afterwards. The proliferation activity was little decreased above pH 5 compared with that of cells incubated at neutral pH, although it was partly (<20%) decreased at pH 4 (data not shown), suggesting that the cells exposed to an acidic medium underwent the normal process of cell division afterwards.

Enzymatic Staining of Internalized β-Galactosidase—To determine whether or not a protein taken up at low pH through a non-endocytic pathway still possesses enzymatic activity, β-galactosidase was incubated with cells at pH 5 and 4°C for 1 h. The cytoplasm of the cells was stained blue after incubation with X-Gal as a substrate, indicating that the enzyme, if not completely, was catalytically active (Fig. 6). Thus, it is suggested that proteins recover their native conformations after translocation

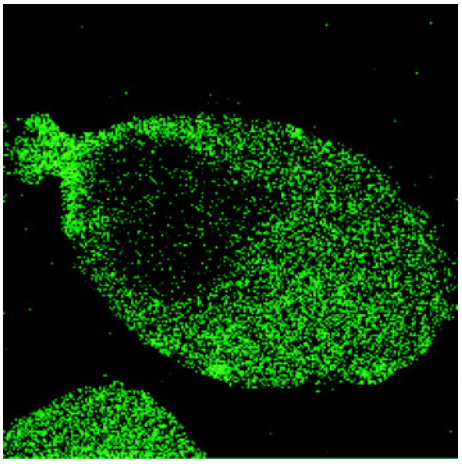

Fig. 4. **Uptake of FITC-labeled apoA-II by fibroblast cells.** Confocal laser scanning micrograph of cells incubated with FITC-apoA-II (4 μg/ml) for 30 min at 4°C. A representative photomicrograph is shown.

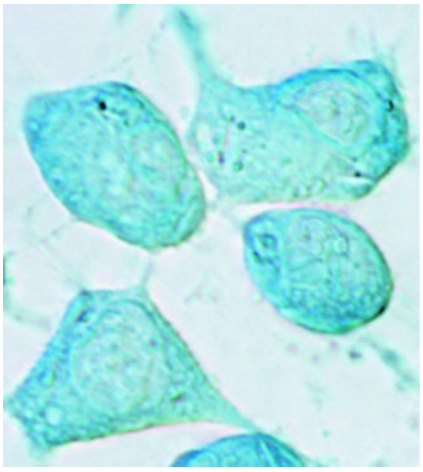

Fig. 6. Cytochemical staining of internalized β-galactosidase. Fibroblast cells were incubated for 1 h at 4°C at pH 5 with β-galactosidase (0.1 mg/ml), washed, and then stained for enzyme activity with X-Gal as the substrate. A representative photomicrograph is shown.

into cells at low pH. It should be added that cells incubated with β-galactosidase at neutral pH and 4°C were not stained with X-Gal (data not shown), suggesting that the protein was not taken up at neutral pH, similar to other proteins (Fig. 3).

CD Measurements—As human apoA-I and apoA-II have been reported to exhibit increased α-helicity on binding to PC liposomes at neutral pH (*25*), we examined whether or not the secondary structures of bovine apolipoproteins change at low pH by CD spectrometry (Table 2). Both apoA-I and apoA-II exhibited increased α-helicity in the presence of liposomes at pH 7 up to the level induced in organic solvent TFE, in which proteins are known to exhibit the maximal α-helicity. The pH shift from 7 to 5 did not affect the α-helicity, suggesting that the proteins were still associated with liposomes at pH 5 and that α-helix formation was required for binding to the cell surface. On the other hand, we observed a decrease in the helical content of BSA from 53% to 47% on the pH shift from 7 to 5 in the presence of PC liposomes (Table 2), indicating that the transition from the normal (N) to fast (F) form (*26–28*) occurred even in the presence of liposomes. Thus, it seems possible that specific changes in the secondary structure are not required for binding to liposomes at low pH.

Driving Force for the Low pH–Induced Entry—It has been proposed that some kind of energy input is required for protein translocation across the membranes of cells and organella (Reviewed in Ref. *29*). The energy can be provided by either ATP, a membrane potential (Δφ), a pH gradient (ΔpH), or a combination of these factors. To determine what sort of energy input is required for protein translocation across the plasma membrane at low pH, we first studied the effect of ATP by depleting it with 2-deoxyglucose and Na-azide (*30*). We found that ATP depletion did not significantly inhibit the uptake of apoA-I, apoA-II, BSA or histone (data not shown).

We next studied the effect of plasma membrane depolarization on protein uptake using gramicidin D, a pep-

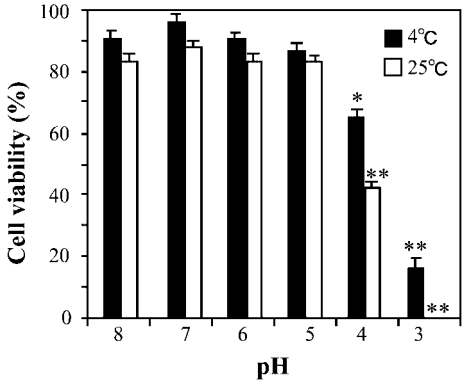

Fig. 5. **Effect of pH on cell viability.** The viability of fibroblast cells incubated at different pHs for 2 h at 25°C or 4°C was assayed by means of the MTT test. Relative activity (mean ±SD, $n = 3$) is presented taking the activity detected in the culture medium as 100%. *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

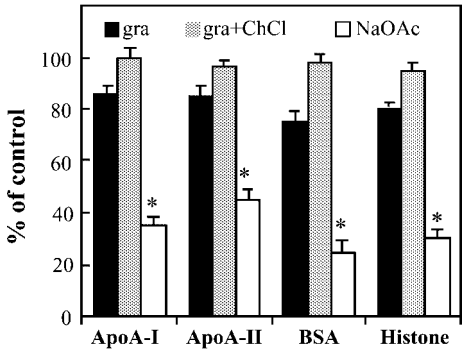

Fig. 7. **Effects of electrical depolarization and cytosolic acidification on cellular uptake of proteins.** Fibroblast cells were incubated for 30 min at 25°C and pH 5 with 0.5 μM FITC-labeled proteins in DPBSG in the absence (control) or presence of either 10 μg/ml gramicidine D (gra), 10 μg/ml gramicidine D in a choline chloride solution (gra + ChCl), or 20 mM sodium acetate (NaOAc). The results (mean ±SD, $n = 3$) are presented as percentages of the control level. *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 2. **Ellipticity of apoA-I, apoA-II, and BSA.**

		Mean residue ellipticity ($[\theta]_{222}$, in degree × cm^2 × $dmol^{-1}$)		
		Protein		
		ApoA-I	ApoA-II	BSA
Aqueous	pH7	–19,600 (57)	–6,470 (14)	–17,600 (50)
	pH 5	–18,300 (53)	–9,150 (22)	–16,100 (45)
With TFE	pH 7	–24,500 (73)	–20,700 (61)	–18,200 (52)
	pH 5	–24,900 (74)	–21,500 (63)	–18,800 (54)
With PC	pH 7	–24,100 (72)	–21,100 (62)	–18,500 (53)
	pH 5	–26,100 (78)	–20,400 (60)	–16,600 (47)

Values in parentheses indicate percentages of α-helix.

tidic channel-forming ionophore (*31*). As shown in Fig. 7, gramicidin D slightly decreased the uptake of all the proteins examined. Replacement of extracellular NaCl by choline chloride, a nondiffusive cation that can prevent the depolarization provoked by gramicidin D (*32*), restored the uptake of the proteins to the control levels. This suggested that the protein uptake at low pH was driven only partly, if at all, by the energy of a membrane potential.

Lastly, to determine if a pH gradient across the plasma membrane is required for protein translocation, we acidified the cytosol by preincubating cells with acetic acid, which penetrates the cell membranes in a protonated form and decreases the pH in the cytosol (*33*, *34*). As shown in Fig. 7, incubation of cells with 20 mM acetic acid (*35*) significantly prevented the cellular uptake of the proteins. This suggested that a pH gradient is the main driving force for the translocation of proteins across the plasma membrane at low pH.

DISCUSSION

In this paper, we show that various proteins including ones with acidic pIs and Mrs above 100 kDa are efficiently taken up by fibroblast cells when the extracellular pH is lowered. At low pH, proteins entered the cells apparently through a non-endocytic pathway because substantial uptake was observed under all the endocytosis-inhibited conditions tested (Fig. 3). Furthermore, the cells were viable for at least 2 h incubation above pH 4 at 4°C and the enzyme β-galactosidase retains its enzymatic activity after translocation into cells. Thus, we suggest that these procedures could be applicable to the injection of proteins into cells without the use of an apparatus such as a microinjector.

Although the precise mechanism by which hydrophilic proteins traverse a membrane at low pH remains unclear, the interaction of proteins with the cell membrane seems to be important for the cellular entry of the proteins. We showed that bovine apoA-I and apoA-II bound to phospholipids like human homologues even at low pH, judging from the helical contents (Table 2). Furthermore, the addition of PC liposomes significantly inhibited the cellular entry of BSA or histone (data not shown), suggesting the proteins bind to phospholipids of both the cell membrane and liposomes. Thus, it is likely that proteins first bind to membrane phospholipids at low pH, this being facilitated by both positively charged amino acids and increased hydrophobicity due to neutralization of carboxyl groups in the proteins. Then, a pH gradient may provide the energy necessary for the proteins to cross the cell membrane.

Recently, it was reported that basic proteins or peptides like Tat protein from HIV-1, homeobox peptide from *Drosophila* Antennapedia protein, a capsid protein, VP22, from HSV-1, and the RNA-binding peptides from HSV-1 Rev and FHV coat protein are translocated across the cell membrane at neutral pH (*8–12*) in a non-endocytic manner (*11–14*). The domains responsible for this translocation contain basic amino acid clusters, such as RKKRR and RRR for HIV-1 Tat, RR and KK for Antennapedia peptide and HSV VP22, and RRRR for HIV-1 Rev and FHV coat protein (*11–14*). A study on Antennapedia peptide has confirmed that positively charged amino acids are necessary for the interaction with negatively charged phospholipids (reviewed in Ref. *36*). In this regard, the proteins used in this study do not contain any clusters of over three residues of basic amino acids except histone H2B, but contain some dimers of them like KK, KR, or RR, most of which are scattered throughout the proteins. Possibly, a dimer of basic amino acids is important for efficient binding of proteins to cells at low pH.

In this report, we showed, for the first time, that proteins efficiently enter cells through a non-endocytic pathway when added at low pH. These results will facilitate evaluation of the nonclassical pathway by which proteins are translocated across membranes.

REFERENCES

1. Hapala, I. (1997) Breaking the barrier: Methods for reversible permeabilization of cellular membranes. *Crit. Rev. Biotech.* **17**, 105–122
2. Silverstein, S.C., Steinman, R.M., and Cohn, Z.A. (1977) Endocytosis. *Annu. Rev. Biochem.* **46**, 669–722
3. Steinman, R.M., Mellman, I.S., Muller, W.A., and Cohn, Z.A. (1983) Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* **96**, 1–27
4. Mellman, I. (1996) Endocytosis and molecular sorting. *Annu. Rev. Cell. Dev. Biol.* **12**, 575–625
5. Sandvig, K. and Olsnes, S. (1980) Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell Biol.* **87**, 828–832
6. Draper, R.K. and Simon, M.I. (1980) The entry of diphtheria toxin into the mammalian cell cytoplasm: Evidence for lysosomal involvement. *J. Cell Biol.* **87**, 849–854
7. Falnes, P.Ø. and Sandvig, K. (2000) Penetration of protein toxins into cells. *Curr. Opin. Cell Biol.* **12**, 407–413
8. Frankel, A.D. and Pabo, C.O. (1988) Cellular uptake of the Tat protein from human immunodeficiency virus. *Cell* **55**, 1189–1193
9. Green, M. and Loewenstein, P.M. (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus Tat trans-activator protein. *Cell* **55**, 1179–1188

10. Joliot, A., Pernelle, C., Deagostini-Bazin, H., and Prochiantz, A. (1991) Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Natl Acad. Sci. USA* **88**, 1864–1868
11. Elliott, G. and O'Hare, P. (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**, 223–233
12. Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* **276**, 5836–5840
13. Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G., and Prochiantz, A. (1996) Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* **271**, 18188–18193
14. Vivès, E., Brodin, P., and Lebleu, B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* **272**, 16010–16017
15. Blanco-Vaca, F., Escolà-Gil, J.C., Martín-Campos, J.M., and Julve, J. (2001) Role of apoA-II in lipid metabolism and atherosclerosis: Advances in the study of an enigmatic protein. *J. Lipid Res.* **42**, 1727–1739
16. Forte, T.M. and McCall, M.R. (1994) The role of apolipoprotein A-I-containing lipoproteins in atherosclerosis. *Curr. Opin. Lipid.* **5**, 354–364
17. Motizuki, M., Itoh, T., Yamada, M., Shimamura, S., and Tsurugi, K. (1998) Purification, primary structure, and antimicrobial activities of bovine apolipoprotein A-II. *J. Biochem.* **123**, 675–679
18. Motizuki, M., Itoh, T., Satoh, T., Yokota, S., Yamada, M., Shimamura, S., Samejima, T., and Tsurugi, K. (1999) Lipid-binding and antimicrobial properties of synthetic peptide of bovine apolipoprotein A-II. *Biochem. J.* **342**, 215–221
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
20. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63
21. Yang, J.T., Wu, C.-S.C., and Martinez, H.M. (1986) Calculation of protein conformation from circular dichroism. *Methods Enzymol.* **130**, 208–269
22. Oram, J.F. and Yokoyama, S. (1996) Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* **37**, 2473–2491
23. Oram, J.F., Lawn, R.M., Garvin, M.R., and Wade, D.P. (2000) ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J. Biol. Chem.* **275**, 34508–34511
24. Lin, G. (2002) Insights of high-density lipoprotein apolipoprotein-mediated lipid efflux from cells. *Biochem. Biophys. Res. Commun.* **291**, 727–731
25. Lux, S.E., Hirz, R., Shrager, R.I., and Gotto, A.M. (1972) The influence of lipid on the conformation of human plasma high-density apolipoproteins. *J. Biol. Chem.* **247**, 2598–2606
26. Sadler, P.J. and Tucker, A. (1993) pH-induced structural transitions of bovine serum albumin. Histidine pKa values and unfolding of the N-terminus during the N to F transition. *Eur. J. Biochem.* **212**, 811–817
27. Peters, T. Jr. (1985) Serum albumin. *Adv. Prot. Chem.* **37**, 161–245
28. Foster, J.F. (1977) *Albumin structure*, *Function and Uses*, pp. 53–84, Pergamon. Press, Oxford
29. Agarraberes, F.A. and Dice, J.F. (2001) Protein translocation across membranes. *Biochim. Biophys. Acta* **1513**, 1–24
30. Steinman, R.M., Silver, J.M., and Cohn, Z.A. (1974) Pinocytosis in fibroblasts. *J. Cell Biol.* **63**, 949–969
31. Aidley, D.J. and Stanfield, P.R. (1996) Ion channel in *Molecules in Action*, pp. 115–116, Cambridge Univ. Press, Cambridge, UK
32. Chifflet, S., Hernandez, J.A., Grasso, S., and Cirillo, A. (2003) Nonspecific depolarization of the plasma membrane potential induces cytoskeletal modifications of bovine corneal endothelial cells in culture. *Exp. Cell Res.* **282**, 1–13
33. Rogers, J., Hesketh, T.R., Smith, G.A., Beaven, M.A., Metcalfe, J.C., Johnson, P., and Garland, P.B. (1983) Intracellular pH and free calcium changes in single cells using quene 1 and quin 2 probes and fluorescence microscopy. *FEBS Lett.* **161**, 21–27
34. Rogers, J., Hesketh, T.R., Smith, G.A., and Metcalfe, J.C. (1983) Intracellular pH of stimulated thymocytes measured with a new fluorescent indicator. *J. Biol. Chem.* **258**, 5994–5997
35. Sandvig, K., Tønnessen, T.I., Sand, O., and Olsnes, S. (1986) Requirement of a transmembrane pH gradient for the entry of diphtheria toxin into cells at low pH. *J. Biol. Chem.* **261**, 11639–11644
36. Prochiantz, A. (2000) Messenger proteins: Homeoproteins, tat and others. *Curr. Opin. Cell Biol.* **12**, 400–406