ATP Is Required for Receptor-mediated Endocytosis in Intact Cells

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Abstract. We have demonstrated a requirement for cellular ATP in the receptor-mediated endocytosis of transferrin. This has been accomplished using a novel assay for endocytosis based on acquisition of resistance to the membrane impermeable reducing agent, glutathione (GSH). Diferric-transferrin was conjugated to biotin via a cleavable disulfide bond and iodinated. Internalization of ^{125}I-biotin-S-S-transferrin (^{125}I-BSST) was quantitated by adsorption to avidin-Sepharose after treatment of cells with GSH. Receptor-mediated endocytosis of ^{125}I-BSST was severely inhibited in ATP-depleted cells. Similar results were obtained when ATP was depleted by incubation of cells either under a N_2-atmosphere or in the presence of NaN_3 and NaF. The latter treatment, alone, also resulted in a loss of surface transferrin receptors which could not be correlated to reductions in cellular ATP. In contrast to the acquisition of GSH resistance, the apparent internalization of ^{125}I-BSST as assessed by inaccessibility to antitransferrin antibodies reached control levels in ATP-depleted cells. Our biochemical and morphological data suggested that, although ATP is required for receptor-mediated endocytosis, in ATP-depleted cells ligands can become efficiently sequestered into deeply invaginated pits that are inaccessible to large probes such as antibodies, but remain accessible to small molecules such as GSH.

MUCH is known at the structural level about the early events in receptor-mediated endocytosis, involving the internalization of receptors via coated pits and coated vesicles. Morphological studies have shown that receptors are first clustered into coated pit regions of the cell surface which invaginate and pinch-off forming coated vesicles carrying receptors and their bound ligands into the cell (reviewed by Goldstein et al., 1985; Brodsky, 1988). Genetic studies have demonstrated that specific signals in the cytoplasmic domains of cell surface receptors efficiently direct them into coated pits (Davis et al., 1986; Lazarovits and Roth, 1988; Iaocopetta et al., 1988). Biochemical studies have demonstrated that the major constituents of the coat are clathrin, its associated light chains, and assembly proteins (more recently referred to as "adaptins") (reviewed by Pearse and Crowther, 1987; Brodsky, 1988; Morris et al., 1989). Recently, it has been shown that assembly proteins, which mediate the spontaneous assembly of purified clathrin into cages closely resembling those on intact coated vesicles (Zaremba and Keen, 1983; Keen, 1987), also mediate the interaction of clathrin with the membrane (Virshup and Bennett, 1988), perhaps through direct association with the cytoplasmic tails of receptors (Pearse, 1988; Glickman et al., 1989). Despite this apparent wealth of structural and biochemical information on the components involved in receptor-mediated endocytosis, very little mechanistic information is available as to how these proteins function to mediate coated pit formation and coated vesicle budding.

One important and basic question concerning the mechanism of receptor-mediated endocytosis that remains controversial is when and where energy is required to drive these processes. It is clear from a number of studies both in intact cells and in vitro that vesicular transport along the exocytic pathway is exquisitely sensitive to cellular ATP levels (Balch et al., 1986; Persson et al., 1988). In contrast, although it has been well-established that fluid-phase pinocytosis is inhibited in cells depleted of ATP (Silverstein et al., 1977), studies by others (Clarke and Weigel, 1985; Larkin et al., 1985) have suggested that a single round of receptor-mediated endocytosis can occur in intact cells depleted of ATP. It has been suggested from these studies that spontaneous clathrin assembly provides sufficient energy to drive coated pit assembly, invagination, and the subsequent membrane fission event which leads to coated vesicle budding (McKinley, 1983; Brodsky, 1988). Results from other groups, using different cells and different ligands, have, however, demonstrated an ATP requirement for receptor-mediated endocytosis (Haigler et al., 1980; Ciechanover et al., 1983; Hertel et al., 1986). How can these conflicting findings be resolved?

The unambiguous identification of when, where, and how energy is required for the complex processes involved in receptor-mediated endocytosis will clearly depend on the use of cell-free assays which will enable exploration of these processes in enzymological detail. The results from one such newly developed assay provide a possible explanation for conflicting results on endocytosis in intact cells depleted of ATP. Smythe et al. (1989) have recently developed an assay that measures the internalization of receptor-bound transferrin into semi-intact A431 cells. Internalization is assessed in

a biochemical assay that measures the acquisition of resistance to immuneprecipitation of cell surface transferrin with antitransferrin antibodies. Sequestration of transferrin into an antibody-inaccessible compartment was found to require cytosol and was stimulated two- to threefold in the presence of ATP. While the formation of bona fide coated vesicles, as determined by serial section analysis, was absolutely dependent on ATP, their morphological data suggested that the significant ATP-independent signal observed biochemically might have been derived from the sequestration of transferrin into deeply invaginated coated pits which formed efficiently even in the absence of ATP. Thus, it was possible that the sequestration of ligands into deeply invaginated pits could account for the apparent "internalization" of ligands during a "single round" of endocytosis in intact ATP-depleted cells.

To test this hypothesis and to examine the role of ATP in receptor-mediated endocytosis, we have employed a novel assay for endocytosis that assesses internalization by the acquisition of latency to a small membrane impermeable agent, glutathione (GSH,[1] ~300 mol wt) as opposed to accessibility to or release of large antibodies or protein ligands. This was accomplished by conjugating diferric transferrin to biotin via a cleavable (GSH-sensitive) disulfide bond. The rate and extent of ^{125}I-biotinylated-transferrin internalization into control and ATP-depleted cells was then measured either by inaccessibility to antibodies or resistance to GSH. We found that although prebound transferrin became inaccessible to antibodies in ATP-depleted cells, both the rate and extent of acquisition of GSH resistance were severely inhibited. These results help to explain previous inconsistent findings and demonstrate that ATP is in fact required to drive receptor-mediated endocytosis.

Materials and Methods

Cell Lines and Antibodies

Sheep antitransferrin antiserum was a gift from the Scottish Antibody Production Unit. K562 cells from Dr. Graham Warren (Imperial Cancer Research Fund, London) were maintained in suspension in α-MEM containing Pen/Strep and 5% FCS. HeLa cells were obtained from Dr. Clare McGowan (Research Institute of Scripps Clinic) and maintained in suspension in Joklik's media containing 10% horse serum, Pen/Strep.

Preparation of ^{125}I-BSST

Diferric-transferrin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was dialyzed into 50 mM NaP_i, pH 7.2, 100 mM NaCl and stored at 5 mg/ml (as determined spectroscopically 1% E_{465} = 0.57) in aliquots at −70°C. A stock solution of NHS-SS Biotin (23 μg/ml, Pierce Chemical Co., Rockford, IL) was prepared in DMSO just before use. For biotinylation, 1 μl of NHS-SS-Biotin (3.75×10^{-2} μmol) was added to 60 μl of transferrin (300 μg, 3.75×10^{-3} μmol) and incubated for 60 min at room temperature. Unconjugated NHS-SS-Biotin was removed by gel filtration on a G25 spin-desalt column and the biotinylated transferrin was iodinated in a 12 × 75 glass tube coated with 10 μg iodogen (as described by Pierce Chemical Co.) using 1 mCi Na^{125}I (Amersham Corp., Arlington Heights, IL). Free ^{125}I was removed by chromatography on Dowex AG-1X8 resin (Sigma Chemical Co., St. Louis, MO) preequilibrated with dPBS containing 0.2% BSA. To minimize loss of biotin residues due to disulfide exchange and/or spontaneous reduction, the ^{125}I-biotin-S-S-transferrin (^{125}I-BSST) was aliquoted and stored at −70°C. We estimate that this procedure results in the addition of ~5–8 biotins/transferrin molecule. 75–90% of the total ^{125}I-BSST could be specifically adsorbed to avidin-Sepharose.

1. *Abbreviations used in this paper*: GSH, glutathione; BSST, biotin-S-S-transferrin.

Assays for Internalization of ^{125}I-BSST

Cells were harvested, washed twice with α-MEM containing 20 mM Hepes, pH 7.4, and 0.2% BSA, and preincubated at 37°C for 30 min to remove endogenous transferrin. The cells were then washed into dPBS^{++} (or subjected to an ATP depletion protocol). dPBS^{++} is Dulbecco's PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and for all assays, 0.2% BSA. Assays contained ~10^6 cells (in 50 μl) and were generally performed in 1.5-ml eppendorf tubes (or glass vials). ^{125}I-BSST (5 μl, 20–30 μg/ml) was added to each aliquot of cells and allowed to bind for 15 min at 4°C. These nearly limiting concentrations of ^{125}I-BSST were chosen so as to approximate measurement of a single-round of internalization. The cells were then shifted to 32°C for the indicated times before their return to ice and processed using one of three methods to determine the extent of ^{125}I-BSST internalization.

GSH Resistance Assay

Cells were pelleted for 20 s in a refrigerated eppendorf (model 5402; Beckman Instruments, Inc., Palo Alto, CA), the supernatants aspirated, and the pellets resuspended in 50 μl of 50 mM glutathione (Sigma Chemical Co.) in 50 mM Tris, pH 8.6, 100 mM NaCl (the pH was adjusted to 8.6 using 10 N NaOH). Cells were incubated for 30 min at 4°C with gentle mixing. A second bolus of GSH and was added (12.5 μl of a 250-mM stock, prepared just before addition and pH adjusted using 10 N NaOH) and the cells were incubated 30 min followed by addition of a third bolus of GSH (16 μl of a 250-mM stock) and incubation for an additional 30 min at 4°C. Excess GSH was quenched by addition of 50 μl of 500 mM iodoacetamide (IAA; Sigma Chemical Co.). After a 10-min incubation, the cells were solubilized by addition of 100 μl 2% TX-100 in dPBS containing 0.5% BSA and the protected, unreduced ^{125}I-BSST was absorbed by addition of 50 μl of a 50% suspension of avidin-Sepharose (Pierce Chemical Co.). After a 45–60-min incubation, the bound ^{125}I-BSST was collected by centrifugation and the avidin-Sepharose beads were washed three times with dPBS/1% TX-100/0.5% BSA. The washed beads were then counted on a gamma counter (Beckman Instruments, Inc.). Total surface-bound ^{125}I-BSST was determined using parallel samples incubated at 4°C with ^{125}I-BSST, pelleted, and resuspended directly into 50 mM IAA before solubilization and absorption to avidin-Sepharose. Backgrounds, which corresponded to GSH-resistant ^{125}I-BSST bound to cells at 4°C, varied between 4–10% of total surface bound. Backgrounds were subtracted from the results which were then expressed as the percentage of total surface-bound ^{125}I-BSST that was internalized (i.e., became GSH resistant) after incubation at 32°C.

Ab Inaccessibility

This assay was performed exactly as described by Smythe et al. (1989). Identical results were obtained with either ^{125}I-BSST or ^{125}I-Tfn as ligand. Briefly, after internalization at 32°C and return to ice, cells were pelleted, resuspended in 50 μl dPBS, and incubated for 90 min at 4°C in the presence of 3 μl of sheep antitransferrin serum. The cells were then washed by addition of 1 ml dPBS, pelleted, and resuspended in 100 μl lysis buffer (100 mM Tris, pH 8, 100 mM NaCl, 1% TX-100, 1 mg/ml BSA) containing 5 μg/ml unlabeled transferrin. 10 μl of a 10% suspension of Staph A cells ("Omnisorb", Calbiochem-Behring Corp., San Diego, CA) precoated with rabbit antisheep antibody was then added and the lysates were incubated for 30–60 min at 37°C. ^{125}I-BSST-Ab immunecomplexes adsorbed to the Staph A cells were pelleted and the supernatant counted on a gamma counter (Beckman Instruments, Inc.). The total surface-bound transferrin was determined by counting the Staph A pellets of samples incubated at 4°C during the course of the experiment. Backgrounds, which corresponded to Ab-inaccessible ^{125}I-BSST bound to cells at 4°C, varied between 4–8% of total and were subtracted from the results. As above, results were expressed as the percentage of total surface-bound ^{125}I-BSST internalized (i.e., Ab inaccessible).

Acid/Neutral Wash

After internalization at 32°C and return to ice, samples were washed twice for 5 min in the presence of 50 mM NaAcetate, pH 4.5, 100 mM NaCl containing desferoxamine ("desferal", CIBA-Geigy, Basel, Switzerland) at 4°C followed by pelleting for 30 s in a refrigerated microfuge. Cell pellets were then washed twice for 5 min in the presence of dPBS containing 0.2% BSA and 50 μM desferoxamine. The resulting cell pellets were counted using a Beckman Gamma counter. The total surface-bound transferrin was deter-

mined by counting cells that had been incubated at 4°C with ^{125}I-BSST and washed once with dPBS/BSA. Backgrounds corresponding to cell-associated ^{125}I-BSST resistant to an acid/neutral resistant after binding at 4°C, varied between 5–10% of total and were subtracted from the results shown. As above, results are expressed as the fraction of total surface-bound ^{125}I-BSST internalized (i.e., resistant to the acid/neutral washes).

Depletion of Cellular ATP

Method 1. Cellular ATP was depleted by incubating cells in a N_2 atmosphere essentially as described by Balch et al. (1986). 5-ml glass vials (cat. No. 9717-T27; Thomas Scientific, Swedesboro, NJ) fitted with a gas-tight rubber septum (cat. No. 1780-J22; Thomas Scientific) were flushed with N_2 (high purity grade, Linde Gas Products, San Diego, CA) for 30–45' using two 6-port Mini-vap manifolds (cat. No. C5438-12; Baxter Scientific, San Diego, CA) fitted with 20-gauge needles. A vent was provided by a second 20-gauge needle which was removed immediately before removal of the N_2 gas line. Within 20 min of their preparation a 50 μl suspension of ~10^6 cells in dPBS^{++} containing 5 mM 2-deoxyglucose (dGlc) was introduced into the sealed vials on ice by using a 2.5 ml gas-tight syringe (Hamilton Industries, Two Rivers, WI) fitted with a repeating dispenser. The vials were transferred to 37°C for 10–15 min to allow ATP depletion, and then returned to ice.

Method 2. Cells were washed and resuspended (2×10^7 cells/ml) at 4°C in dPBS^{++} containing 10 mM NaN_3 and 2 mM NaF and then transferred (50 μl/assay tube) to 37°C for 10–15 min to deplete cellular ATP before returning to ice. ATP depletion at 4°C was very much slower and less efficient.

Measurement of Cellular ATP

Cells were injected into 9 vol of boiling 20 mM Tris, 20 mM $MgSO_4$, pH 7.76, and incubated for 5 min at 100°C (as described by Balch et al., 1986). The precipitate was removed by centrifugation in a microfuge (Beckman Instruments, Inc.) and the supernatants were assayed for ATP. ATP was assayed using a modification of the luciferin/luciferase assay essentially as described by Weigel and Englund (1975). All of the reagents were obtained from Analytical Luminescence Lab (San Diego, CA) and used as they described in accompanying protocols. Briefly, samples to be assayed were diluted ~10^{-2}–10^{-3} into Tris/$MgSO_4$ buffer and 5–25 μl aliquots were dispensed into 20 ml glass scintillation vials containing "firelight buffer": total assay volume was 150 μl. After equilibration to room temperature, individual assays were initiated by addition of 25 μl luciferin/luciferase to the open vials. The solutions were swirled to mix and rapidly transferred to the moving belt of a scintillation counter (model L5800; Beckman Instruments, Inc.) with the coincidence circuit turned off and windows at maximum opening. Counting was initiated within 3 s of addition of the luciferin/luciferase. The samples were counted for two sequential 0.1-min intervals. Buffer and enzyme only backgrounds were determined and subtracted from those containing known amounts of an ATP standard in order to construct a standard curve. The second 0.1-min count was used, backgrounds were generally 20,000–30,000 cpm and the assay was linear from 0.01–0.8 pmol ATP (generally 10,000–4 × 10^6 cpm over background). Cellular ATP levels were determined and expressed as nmol ATP/mg cellular protein.

Determination of Cellular Protein

For each ATP determination, a parallel sample of cells was solubilized in 0.1 N NaOH and cellular protein was determined using the BCA protein assay kit (as described by Pierce Chemical Co.) with BSA as a standard curve.

Preparation of samples for EM

Cells were treated exactly as in the experimental protocols described above. ATP was depleted by a 10-min incubation at 37°C either in a N_2-atmosphere, in the presence of 10 mM NaN_3 and 2 mM NaF or in dPBS^{++} containing 5 mM glucose (for control cells). The cells were shifted to ice for 15 min (during which time parallel samples were allowed to bind ^{125}I-BSST) and then returned to 32°C for 10 min. The cells were shifted to ice and fixed with 1% glutaraldehyde, 3% paraformaldehyde in 0.1 M Cacodylate buffer, pH 7.2 (modified Karnovsky), for 1 h at room temperature. Pellets were postfixed with 1% OsO_4 in 0.1 M Cacodylate buffer, pH 7.2, embedded in epon, and sectioned for microscopy by Dr. Cheng-Ming Chang of the RISC Electron Microscopy Laboratory (Research Institute of Scripps Clinic, La Jolla, CA). Biochemical analysis of parallel samples indicated a reduction of cellular ATP and rates of internalization by 90 and 70% in the N_2-treated sample and 90 and 80% in the NaN_3/NaF treated sample, respectively. For quantitation of the total number of coated pits/mm cell surface, micrographs were taken at random at 9,600× magnification, without examination for visible coated pits so as to maximize the amount of surface visible per micrograph. Quantitation of the percentage of "sealed" vs "open" pits was performed at the microscope by examining sections at 18,000× magnification and counting and scoring individual recognizable coated structures at the cell surface.

Results

A Novel Assay for Receptor-mediated Endocytosis of Transferrin

Assays for receptor-mediated endocytosis are based on the ability to distinguish intracellular ligands from those remaining on the cell surface. This can be accomplished by washing away surface-bound ligands using either low pH buffers, chelators (if the binding is cation-dependent), or an excess of competing ligand (if the ligand off-rate is sufficient). Alternatively, internalization can be measured by assessing loss of the ligand's susceptibility to either proteases or to immuneprecipitation. Since the existence of deeply invaginated coated pits that are inaccessible to Con A-HRP (mol wt >150,000) but accessible to Ruthenium red (mol wt 786) has been demonstrated (Willingham et al., 1981), it was possible that ligands might also be sequestered in these structures. If this were the case, procedures routinely used for endocytosis assays might be limited in their ability to distinguish between bona fide internalized ligands and those sequestered into deeply invaginated coated pits. The inability to make this distinction would in turn influence the interpretation of results for the requirements of ATP for endocytosis in intact cells.

To distinguish internalized ligands from those that might be sequestered in deeply invaginated pits, we have developed an assay based on their accessibility to the small (mol wt 307) membrane-impermeant reducing agent, GSH. The efficacy of a GSH resistance assay for internalization of surface proteins was recently described by Brestcher and colleagues who have developed a reagent for the modification of surface proteins with iodotyrosine via a cleavable disulfide bond (Bretscher and Lutter, 1988; Bretscher, 1989). Our approach differs from that described by Bretscher in that we have directly conjugated biotin to a radiolabeled ligand via a cleavable (GSH-sensitive) disulfide bond so as to enable use of a GSH resistance assay for internalization. This assay is generally applicable to studying endocytosis of any ligand or antibody. Thus, diferric-transferrin was conjugated to biotin using the cleavable biotinylating reagent, NHS-S-S-biotin (Pierce Chemical Co.). The biotinylated diferric-transferrin (referred to as BSST) was then iodinated to high specific activity and bound to cells at 4°C. ^{125}I-BSST binding to transferrin receptors on K562 cells was indistinguishable from ^{125}I-diferric-transferrin binding and could be competed by excess unlabeled diferric-transferrin (data not shown). After internalization of ^{125}I-BSST at 32°C, cells were treated with GSH at 4°C in order to reduce any accessible disulfide linkages. GSH was then quenched by addition of excess iodoacetamine, the cells were lysed with detergent and GSH-resistant ^{125}I-BSST could be quantitated after adsorption to avidin-Sepharose (see Materials and Methods).

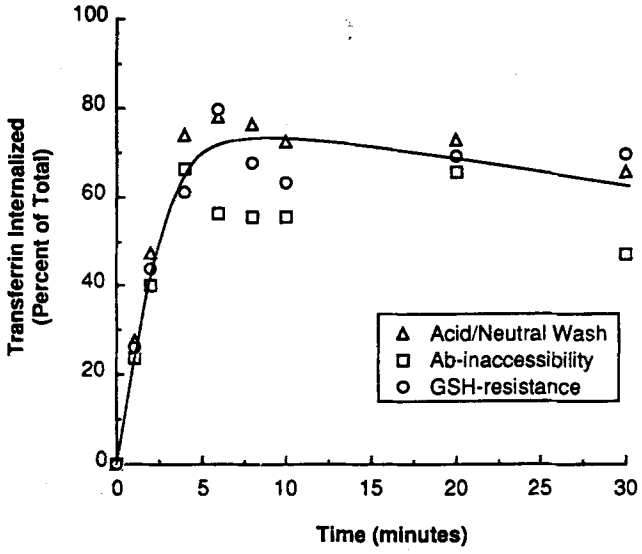

Figure 1. The kinetics of internalization of ^{125}I-BSST are the same as assessed by three different endocytosis assays. K562 cells (~2 × 10^7 cells/ml) were incubated at 4°C in dPBS^{++} containing 5 mM glc, 0.2% BSA, and 2 μg/ml ^{125}I-BSST for 15 min to allow for binding and were then transferred to 32°C for the indicated times to allow for internalization. The cells were returned to ice, split into three equal aliquots (~10^6 cells) and the extent of internalization was analyzed using one of the following three assays, as described in Materials and Methods: (△) sequential acid/neutral washes in the presence of desferoxamine, (□) Ab inaccessibility, or (○) GSH resistance.

To demonstrate that the acquisition of GSH resistance was a valid assay for internalization, the rates of receptor-mediated endocytosis of ^{125}I-BSST as measured by three different assays were directly compared. K562 cells were incubated for 15 min at 4°C in the presence of ^{125}I-BSST (2 μg/ml) to allow binding. Cells were then warmed to 32°C to allow internalization of ^{125}I-BSST (uptake of prebound ^{125}I-BSST at 37°C occurred too rapidly for kinetic studies). After transfer to ice the cells were split into three aliquots and assayed for internalized ^{125}I-BSST using one of the following assays (see Materials and Methods): (*a*) GSH resistance, as described above; or (*b*) Ab inaccessibility, as described by Smythe et al. (1989); or (*c*) sequential acid/neutral washes. The results in Fig. 1 show that the kinetics for internalization of ^{125}I-BSST as assessed by GSH resistance were indistinguishable from those obtained using either the conventional acid/neutral wash in the presence of the iron chelator desferoxamine or by Ab inaccessibility. The apparent saturation reflects establishment of an equilibrium between internalized and recycled transferrin. In most experiments transferrin recycling is not detected since due to the ATP depletion protocols which follow, unbound ^{125}I-BSST could not be removed before warming the cells. We do not believe that much more than a single round of endocytosis occurred since the total amount of internalized transferrin rarely exceeded the total amount bound at 4°C. (As indicated in Materials and Methods, the internalized transferrin was expressed as the percentage of total bound at 4°C.)

Although this assay was developed for measuring the receptor-mediated internalization of transferrin, this same procedure could easily be applied to measure endocytosis of any ligand (or antibody). It requires only that the ligand has free amine groups for conjugation with NHS-S-S-biotin. The degree of biotinylation achieved should be optimized so as to maximize the efficiency of complete reduction of exposed disulfides while maintaining sufficient levels of biotinylation to allow efficient adsorption to avidin-Sepharose. We have used this approach to measure internalization of both ^{125}I-BSS-ricin and ^{125}I-BSS-EGF (data not shown).

Internalization of ^{125}I-BSST into K562 Cells Requires ATP

Given that the acquisition of GSH resistance appears to be a valid assay for the receptor-mediated endocytosis of ^{125}I-BSST, the ATP requirements for this process were next examined by measuring transferrin internalization in intact cells after depletion of ATP. Cellular ATP levels were depleted to within 10–15% of control values (which were 26.4 ± 9.8 (n = 35) nmol ATP/mg cell protein) by incubating cells at 37°C for 10–15 min in a N_2-atmosphere. This was accomplished by injecting 50 μl of K562 cells in suspension using a gas-tight syringe through a rubber septum into sealed 5-ml glass vials that had been pregassed with N_2. (Balch et al., 1986; see Materials and Methods for details). Parallel samples were used to determine cellular ATP levels before and after ATP depletion and at the end of the experimental protocol. ATP levels did not change during sub-

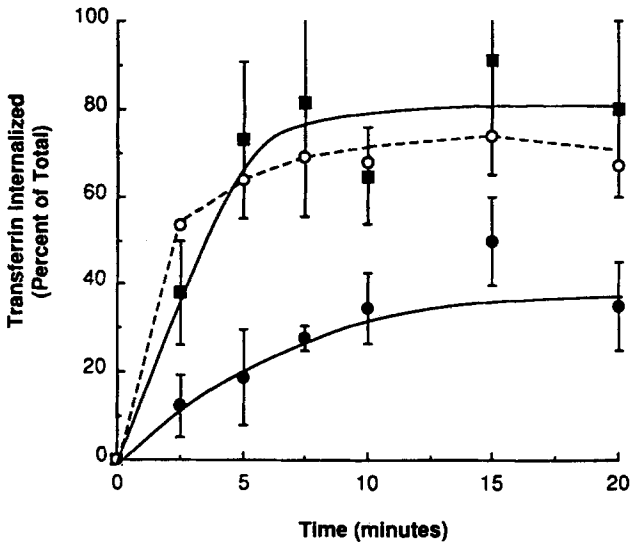

Figure 2. ^{125}I-BSST internalization as assessed by GSH resistance but not Ab inaccessibility is severely inhibited in K562 cells depleted of ATP by N_2. K562 cells (~2 × 10^7 cells/ml in dPBS^{++}) were depleted of ATP (*closed symbols*) by incubation under a N_2-atmosphere in the presence of 5 mM 2-dGlc for 15 min at 37°C. Control cells (*open symbols*) were incubated exposed to air, in the presence of 5 mM Glc for the same time. Cells were then returned to ice and ^{125}I-BSST (2 μg/ml final concentration) was added and bound for 15 min. The cells were then shifted to 32°C for the indicated times, returned to ice temperature, and the extent of internalization was assessed by either Ab inaccessibility or GSH resistance. (○) Control cells, average of results from Ab inaccessibility and GSH resistance assays; (■) Ab inaccessibility assay; (●) GSH resistance assay. Results shown are the averages (± SD) of three experiments.

Table I. The Effects of ATP Depletion on Surface Binding and on the Rate and Extent of Internalization of ^{125}I-BSST in K562 Cells

Assay	Cellular ATP (control)	Rate of uptake (control)	Extent of uptake (control‡)	Surface receptors (control)
	%	%	%	%
A. ATP depleted in N_2-atmosphere				
Ab inaccessibility	15 ± 4*	53 ± 11	94 ± 5	89 ± 7
GSH resistance	12 ± 3	19 ± 8	52 ± 9	92 ± 9
B. ATP depleted in NaN_3/NaF				
Ab inaccessibility	9	51	100	52
GSH resistance	9	10	30	65
Acid/neutral wash	9	20	60	48

* n = 3.
‡ After 15 min at 32°C.

sequent incubations on ice or at 32°C. After ATP depletion, cells were shifted to ice and ^{125}I-BSST (5 μl at 20 μg/ml) was injected into each vial and allowed to bind for 15 min before incubation at 32°C for internalization. The cells were then returned to ice and the extent of internalization of ^{125}I-BSST was determined either by GSH resistance or Ab inaccessibility. The results are shown in Fig. 2 and quantitated in Table 1.

The extent of receptor-mediated endocytosis of transferrin as assessed by inaccessibility to immuneprecipitation by antitransferrin antibodies (Fig. 2, *closed squares*) was unaffected in cells depleted of ATP by incubation in N_2 (94% of control values, *open circles*). There was, however, a significant reduction in rate (53% of control values). Except for the observed reduction in rate, these results were consistent with those reported for internalization of asialoorosomucoid by ATP-depleted hepatocytes (Clarke and Weigel, 1985) and for internalization of LDL by ATP-depleted fibroblasts (Larkin et al., 1985). In contrast, both the rate and extent of internalization of ^{125}I-BSST as assessed by GSH resistance were severely inhibited (to 19 and 52% of controls, respectively) in ATP-depleted cells (Fig. 2, *closed circles*). The very slow rate of ^{125}I-BSST internalization detectable by GSH resistance could be accounted for by residual cellular ATP levels (~10–15% of controls).

To ensure that the inhibition observed was not due to decreased cell viability, we confirmed that the effect of incubation of cells in a N_2-atmosphere was reversible. Cells suspended in dPBS^{++}-containing 5 mM 2-dGlc were depleted of ATP by incubation under N_2 for 10 min at 37°C, returned to ice, removed from the vials, exposed to air, washed free of 2-dGlc, and resuspended in dPBS^{++}-containing 10 mM Glc (t = 0′ recovery). The cells were then returned to 37°C to allow for recovery of cellular ATP levels. After the indicated times (0–30 min) the cells were returned to ice, an aliquot was removed for ATP determinations and ^{125}I-BSST was bound for 15 min at 4°C. The extent of internalization of prebound ^{125}I-BSST during a subsequent 5-min incubation at 32°C was then determined using the GSH resistance assay. The data in Fig. 3 show that cellular ATP rapidly (within 5 min) returns to near-normal levels after cells are reexposed to the air and washed free of 2-deoxyglucose. The ability to internalize ^{125}I-BSST is also rapidly and completely restored.

These results suggested that under conditions of reduced cellular ATP levels, ^{125}I-transferrin is sequestered into structures inaccessible to large antibodies yet still accessible to small molecules. However, to rule out possible artifacts due to the method of ATP depletion, the conditions employed by Clarke and Weigel (1985; 10 mM NaN_3 and 2 mM NaF) were used to deplete cellular ATP levels before again measuring ^{125}I-BSST internalization by antibody accessibility or GSH resistance. Cells were washed, resuspended, and incubated for 10–15 min at 37°C in dPBS^{++}-containing 10 mM NaN_3 and 2 mM NaF to deplete cellular ATP. As before the cells were shifted to 4°C and ^{125}I-BSST

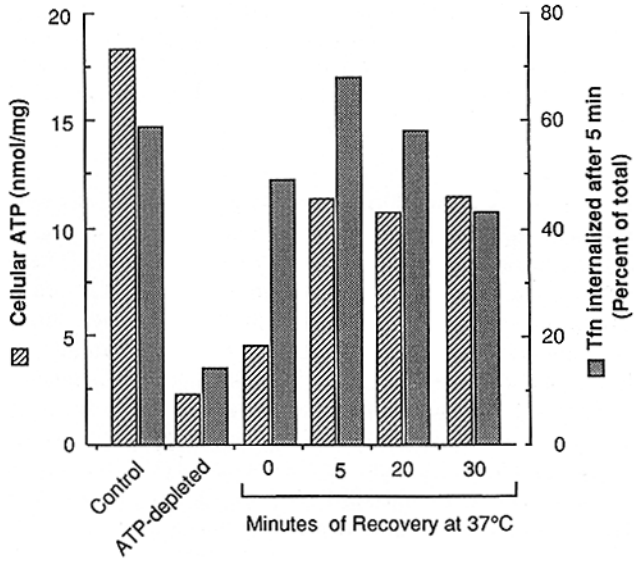

Figure 3. ATP depletion under N_2 is reversible. K562 cells were depleted of cellular ATP by incubation in a N_2-atmosphere for 10 min at 37°C in the dPBS^{++}-containing 5 mM-2-dGlc. Cells were removed to ice, exposed to air, washed into dPBS^{++}-containing 5 mM Glc and shifted to 37°C for the indicated times to enable recovery. The cells were then incubated for 15 min on ice in the presence of 2 μg/ml ^{125}I-BSST to allow binding and then shifted to 32°C for 5 min to allow endocytosis of bound ^{125}I-BSST. Control cells were treated identically except that they were first incubated at 37°C in dPBS^{++}-containing 5 mM Glc, exposed to air. ATP-depleted cells were neither washed nor exposed to air. Internalization (as assessed by GSH resistance) was restored to control levels within 5 min and ATP returned to 70% of control levels within 5 min.

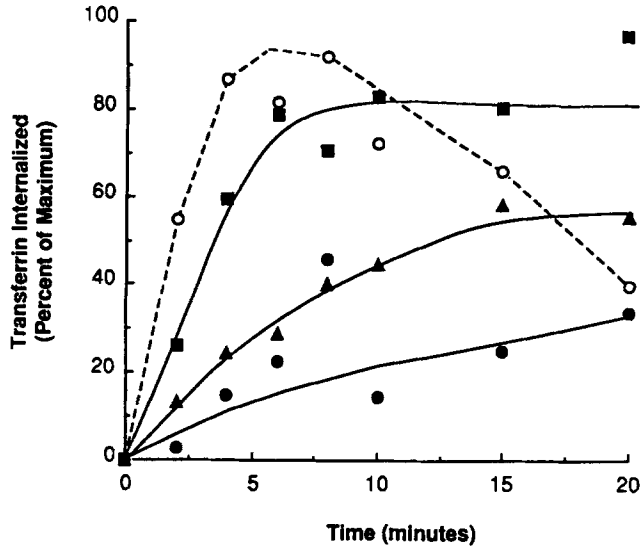

Figure 4. ^{125}I-BSST internalization as assessed by GSH resistance but not Ab inaccessibility is severely inhibited in K562 cells depleted of ATP by metabolic poisons. K562 cells (2×10^7 cells/ml) were depleted of ATP (*closed symbols*) by incubation in the presence of 1 mM NaN_3, 2 mM NaF in $dPBS^{++}$ for 15 min at 37°C. Control cells (*open circles*) were incubated for the same time, in $dPBS^{++}$ containing 5 mM Glc. Cells were then returned to ice and ^{125}I-BSST (2 μg/ml final concentration) was added and bound for 15 min. The cells were then shifted to 32°C for the indicated times, returned to ice temperature, and the extent of internalization was assessed by either Ab inaccessibility (■); GSH resistance (●); or an acid/neutral wash (▲). The values for control cells (○) represent an average of results using the three assays. The results are expressed as Percent of Maximum so that three experiments could be combined. Actual values for the maximum internalized in individual experiments were 120, 130, and 70% of total for acid neutral washes, GSH resistance, and Ab inaccessibility, respectively.

was added and allowed to bind for 15 min at 4°C. Cells were returned to 32°C for the indicated times before assaying for internalization using either Ab accessibility or GSH resistance. After ATP depletion, parallel samples were used to determine cellular ATP levels which did not change during the remainder of the experimental protocol. As can be seen in Fig. 4 and Table I, the results obtained using this method of ATP depletion were similar to those obtained for cells incubated in N_2. Bound ^{125}I-BSST became inaccessible to antibody with reduced kinetics (52% of control) but to the same extent as untreated cells (100% of control). In contrast, when GSH resistance was measured, both the rate and extent of ^{125}I-BSST internalization were severely impaired in the ATP-depleted cells (10 and 30% of control levels, respectively). This greater inhibition of internalization could reflect the greater extent of ATP depletion (<10% of controls) obtained using metabolic poisons. Transferrin internalization was also measured by the more conventional method of resistance to release by sequential acid and neutral washes in the presence of the iron chelator desferoxamine (Fig. 4, *closed triangles*). Inhibition of internalization as measured by this assay was intermediate between that observed using the Ab accessibility and GSH resistance assays (80% inhibition of rate and 40% inhibition of extent, Table I). This may reflect either partial opening of deeply invaginated pits during the low pH conditions of the wash allowing the escape of released ligand or the fact that transferrin (90 kD, R_s = 39 Å) is ~30% smaller than an antibody (156 kD, R_s = 53Å), or both.

Although not investigated directly, the data in Fig. 4 also suggests that transferrin recycling is inhibited in ATP-depleted cells. As for other experimental protocols, although excess, unbound ^{125}I-BSST was not removed, the amount of ^{125}I-BSST appeared to be limiting in this case, so that recycling could be detected in untreated cells as a decline in cell associated ^{125}I-BSST at later time points (Fig. 4, *a–c, open circles*). This decline was not detected in ATP-depleted cells, even when apparent internalization as detected by Ab inaccessibility reached control levels (Fig. 4 *a, closed circles*).

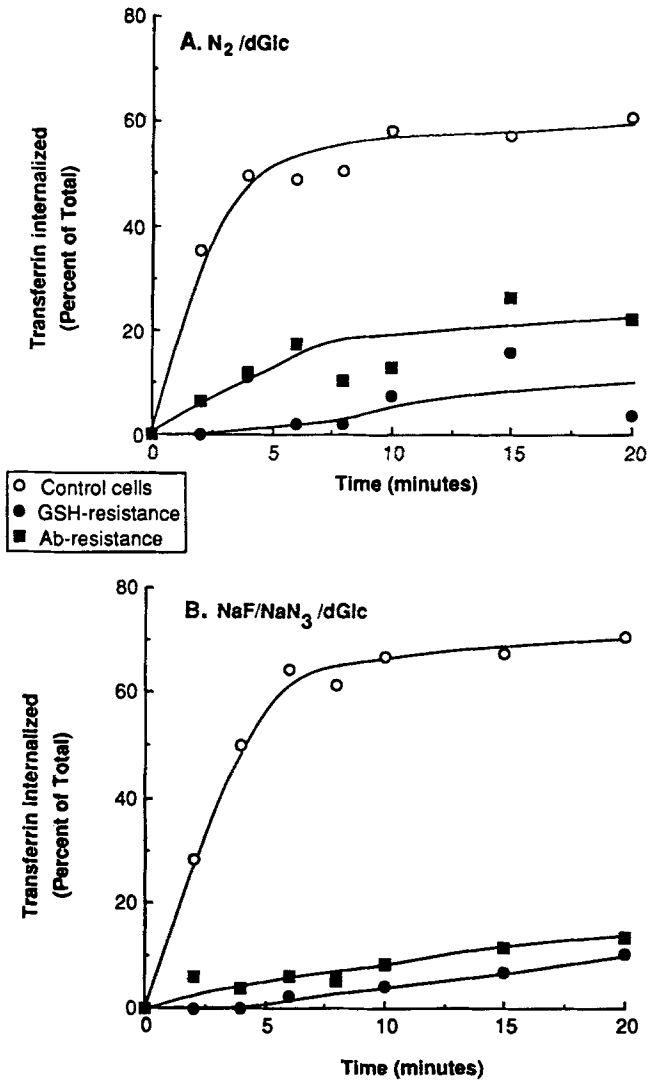

Figure 5. Receptor-mediated endocytosis of ^{125}I-BSST is severely inhibited in HeLa cells after ATP depletion. HeLa cells (2×10^7 cells/ml) were depleted of ATP either by incubation under a N_2-atmosphere in the presence of 5 mM 2-dGlc (*A*) or in the presence of 5 mM d-Glc, 2 mM NaF, and 10 mM NaN_3 (*B*). Internalization of prebound ^{125}I-BSST was assessed as described in Figs. 2 and 4 and in Materials and Methods. (○) Untreated cells, average of GSH resistance and Ab inaccessibility assays; (■) ATP-depleted cells, Ab inaccessibility assay; (●) ATP-depleted cells, GSH resistance assay.

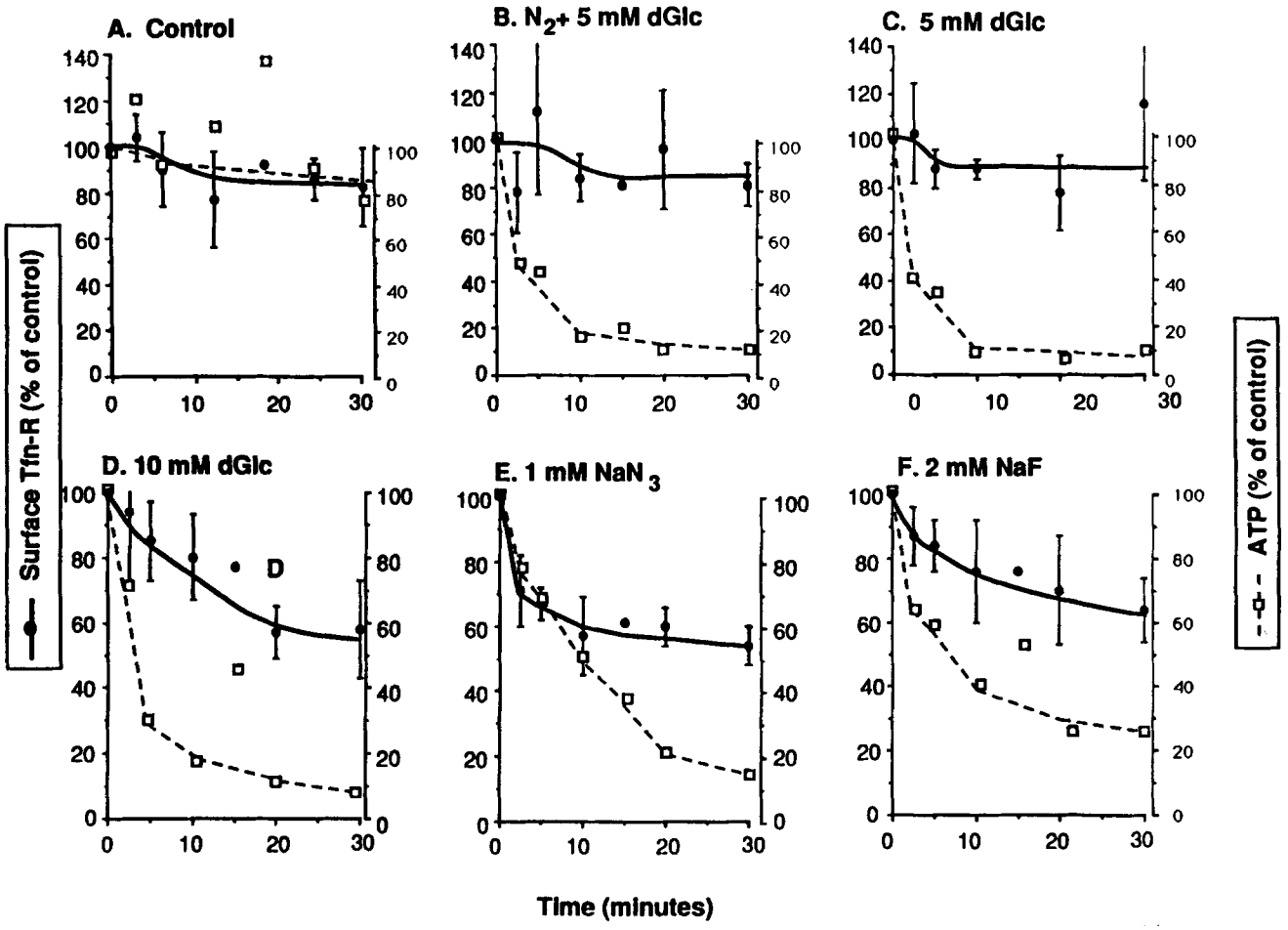

Figure 6. Loss of transferrin receptors from the cell surface cannot be correlated to reduced cellular ATP levels in K562 cells. K562 cells were depleted of cellular ATP as indicated (see Figs. 2 and 4 and Materials and Methods) and returned to ice. An aliquot was assayed for cellular ATP content and ^{125}I-Tfn (2 μg/ml) was bound for 60 min at 4°C. Surface-bound Tfn was counted after washing cells twice at 4°C. The binding data shown are averages ± SD (n = 3); ATP values varied by <5%.

Internalization of ^{125}I-BSST into HeLa Cells Requires ATP

Our results suggest that ATP is required for receptor-mediated endocytosis in intact cells. They further suggest an explanation for the discrepancy between our results and those obtained by others (Clarke and Weigel, 1985; Larkin et al., 1985) that a single round of endocytosis can occur at reduced cellular ATP levels. It is possible that the deeply invaginated coated pits which were found to form efficiently in the absence of ATP in vitro (Smythe et al., 1989) also form in vivo and that these structures sequester ligands making them resistant to wash conditions employed resulting in the false conclusion that they have been internalized. Another possibility existed that the ATP requirement for endocytosis is specific to the K562 erythroleukemic cell line used in the present studies. Although it is unlikely that the basic mechanisms of endocytosis are cell-type specific, this possibility was made less likely when an even stronger ATP dependence was observed using HeLa cells, an endothelial-like cell line. The data in Fig. 5, *a* and *b* show that in HeLa cells receptor-mediated endocytosis of transferrin as measured by both Ab inaccessibility and GSH resistance were severely inhibited in ATP-depleted cells. When ATP was depleted by incubating cells in a N_2-atmosphere, the rate of ^{125}I-BSST internalization as detected by Ab inaccessibility was inhibited 80%, whereas the acquisition of GSH resistance was inhibited by >95% (Fig. 5 *a*). The recovery of HeLa cells from ATP depletion by incubation in N_2 was as rapid and efficient as was that shown for K562 cells (data not shown). The greater extent of inhibition of endocytosis in HeLa cells may be a reflection of the greater extent of ATP depletion obtained in these cells (routinely >95% of controls using N_2). Alternately, or in addition, these results might reflect some cell type heterogeneity in their sensitivity to decreased cellular ATP levels. When metabolic poisons were used to deplete cellular ATP to similar levels (<5% of controls) internalization as assessed both by Ab inaccessibility and GSH resistance was essentially blocked (<5–10% of control rates) (Fig. 5 *b*). As very high concentrations of NaN_3, NaF, and 2-dGlc were found to be required to reduce cellular ATP levels in HeLa cells (see Fig. 5 *b*), it could not be ruled out that other nonspecific effects on cell function and viability accounted for this severe inhibition.

Loss of Cell Surface Receptors Is Not Related to Overall Cellular ATP Levels

For all recycling receptors, the concentration of cell surface receptors at equilibrium is dependent on the relative rates of

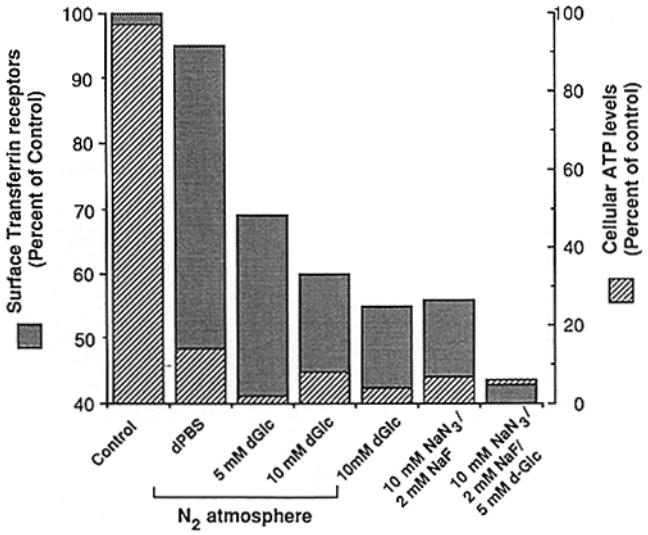

Figure 7. Loss of transferrin receptors from the cell surface cannot be correlated to reduced cellular ATP levels in HeLa cells. HeLa cells were depleted of cellular ATP under the indicated conditions (see Materials and Methods) for 30 min at 37°C and returned to ice. An aliquot was assayed for cellular ATP content and ^{125}I-Tfn (2 μg/ml) was bound for 60 min at 4°C. Surface-bound Tfn was counted after washing cells twice at 4°C. (*shaded bars*) ^{125}I-transferrin binding; (*striped bars*) cellular ATP levels.

endocytosis and recycling. Therefore, an additional finding by Clarke and Weigel (1985) which supported their result that a single round of endocytosis could occur in cells depleted of ATP was the loss of surface receptors from ATP-depleted cells. This could best be explained if internalization continued while receptor recycling was blocked. The results in Table I indicate that the total number of cell surface receptors on K562 cells was not affected by ATP depletion under a N_2-atmosphere (~90% of control). A reduction in cell surface receptors was observed, however, when cells were depleted of ATP using NaN_3 and NaF (50–65% of control).

These results suggested that the loss of surface receptors might not be related to the reduction of overall cellular ATP levels but might instead be due to unrelated effects of either or both metabolic poisons. Therefore, to further explore the relationship between cellular ATP levels and/or drug treatment and the surface expression of transferrin receptors, the effect of ATP depletion by various methods on the number of surface binding sites for ^{125}I-Tfn was determined (Fig. 6). Cells were incubated under the indicated conditions for increasing times at 37°C. The cells were removed to ice, an aliquot was taken for ATP determinations, and ^{125}I-Tfn (2 μg/ml) was added to the remaining cells and allowed to bind for 60 min at 4°C. Total cell-associated ^{125}I-Tfn was determined after washing cells to remove unbound ^{125}I-Tfn. Although each treatment had comparable effects on cellular ATP levels, their effects on the number of cell surface ^{125}I-Tfn binding sites differed greatly. As seen in Table I, incubation of cells in N_2-atmosphere, in the presence of 2-dGlc (Fig. 6 *b*) did not result in a significant loss of cell surface transferrin receptors relative to control cells incubated in dPBS^{++} containing 5 mM Glc (Fig. 6 *a*). Approximately 80% of cell surface binding sites were retained on average after each of these treatments. In contrast, incubation of cells in either 1 mM NaN_3 or 2 mM NaF resulted in the rapid and marked reduction in cell surface transferrin receptors by 40–50% (Fig. 6, *e* and *f*). Thus, these metabolic poisons appear to alter cell surface expression of transferrin receptors by some effect not directly related to cellular ATP levels. The K562 cell line appears to rely heavily on glycolysis as a source of cellular ATP (perhaps due to its erythroleukemic origins) so that ATP levels were substantially reduced by incubation in the presence of 2-dGlc alone (Fig. 6, *c* and *d*). Despite a >90% reduction in ATP levels, cells incubated in 5 mM 2-dGlc retained 90% of their cell surface receptors after 30 min at 37°C (Fig. 6 *c*). Increasing the concentration of 2-dGlc to 10 mM, however, resulted in the loss of surface transferrin receptors (Fig. 6 *e*). Thus, at sufficient concentrations, this metabolic poison also affects the surface expression of transferrin receptors, which cannot be correlated with reduced ATP levels alone. The results in Fig. 7 demonstrate that this phenomenon, as well, was not cell-type specific since similar results were obtained when HeLa cells were incubated under the various conditions for 15 min at 37°C. Again the loss of cell surface receptors could not be correlated with cellular ATP levels and was not seen when ATP was depleted by incubation in a N_2-atmosphere alone. Surface expression of transferrin receptors appeared to be more sensitive to the effects of 2-deoxyglucose in HeLa cells as compared to K562 cells.

Cells Depleted of ATP Have Increased Numbers of Deeply Invaginated Cell Surface Coated Pits

The data so far indicates that ATP is in fact required for receptor-mediated endocytosis and suggests that in the absence of ATP deeply invaginated pits can form which exclude bulky ligands or probes. This speculation was supported by examination of the morphology of coated pits in K562 cells which had been depleted of cellular ATP (Fig. 8). Although the number of coated pits (~60–70 coated pits/mm cell surface) did not significantly differ between control (Fig. 8 *a*) and ATP-depleted cells (Fig. 8, *b* and *c*), the morphology of the coated pits appeared significantly different. These results are quantitated in Table II. Only clearly distinguishable coated structures were counted. Coated pits were scored as being "sealed" when they were completely spherical structures located less than two profile diameters from the cell surface (Sandvig et al., 1987) (see for example, Fig. 8, *b* and *c*, *closed arrowheads*). Coated pits with any visible connection to the cell surface were scored as "open" (Fig. 8 *a*, *open arrowheads*). Closed structures less than two profile diameters from the plasma membrane were assumed to be coated vesicles (an example is circled in Fig. 8 *c*) and were not counted. By these criteria, ATP depletion resulted in a twofold increase in the proportion of deeply invaginated coated pits (~60% of total) as compared to control cells (36% of total). Although serial section analysis would be needed to more accurately assess these morphological distinctions, this data is nonetheless consistent with our interpretation that in ATP-depleted cells ligands become inaccessible to bulky but not small probes due to sequestration into deeply invaginated coated pits. It was also our impression that although the general morphology of ATP-depleted cells incubated under N_2 was similar to control cells, cells incubated with metabolic poisons exhibited altered cytoplasmic,

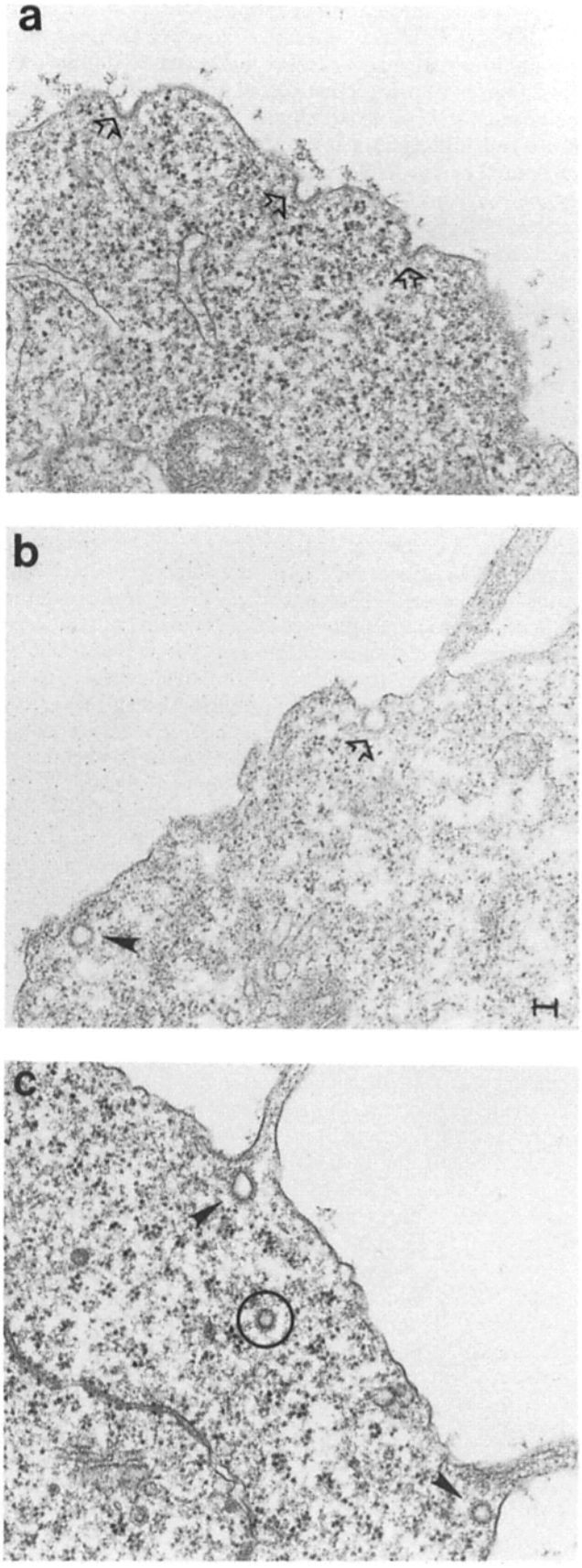

Figure 8. Electron micrographs showing coated pit morphology in control and ATP-depleted cells. K562 cells were depleted of cellular ATP, fixed, embedded, and sectioned as described in Materials and Methods. (*a*) Control cells; (*b*) NaF/NaN_3-treated cells; (*c*) N^2-treated cells. Closed arrowheads indicate "deep" or "sealed" coated pits, open arrowheads indicate "open" coated pits. A coated vesicle is circled in *c*. Bar, 100 nm.

Table II. Effect of ATP Depletion on the Number and Morphology of Coated Pits on the Surface of K562 Cells

Treatment	Number of coated pits per mm surface (μm counted)	Deep ("sealed") coated pits* % of total (number counted)
Control	69 (524)	36 (n = 122)
N_2-atmosphere	68 (427)	62 (n = 121)
NaN_3/NaF	57 (404)	60 (n = 147)

* ATP-depleted samples are significantly different from control samples (α = 0.005, χ^2-test) but not from each other.

membrane, and nuclear morphology (data not shown, but see Fig. 8).

Discussion

These results demonstrate that ATP is required for receptor-mediated endocytosis of transferrin in intact cells. This has been shown using two different methods of ATP depletion and two different cell types. In addition, our results suggest a possible explanation for other studies which have generated conflicting results that a single round of endocytosis can occur in ATP-depleted cells. As others had found (Clarke and Weigel, 1985; Larkin et al., 1985) we measured an apparent rapid and efficient internalization of transferrin in ATP-depleted cells, but only when a bulky probe was used to assess transferrin internalization. In contrast, when a smaller probe was used to assess internalization, both the rate and extent of transferrin uptake were severely inhibited under identical conditions of ATP depletion. The simplest explanation for the discrepancy between these two assays is that deeply invaginated pits which sequester ligand from bulky probes can form in the absence of ATP leading to the false conclusion that ligands have been internalized. This explanation is supported by morphological data which shows an increase in the number of deeply invaginated coated pits at the surface of ATP-depleted cells. Similar observations were made earlier by Merisko et al. (1986) when they examined pancreatic lobules incubated in N_2-atmosphere and found a significant increase in coated pits at both the basolateral and apical cell surfaces. These results suggest that the efficient formation of deeply invaginated pits observed by Smythe et al. (1989) in the absence of ATP in vitro also occurs in intact cells and tissues.

The finding that transferrin internalization is inhibited in ATP-depleted cells is entirely consistent with studies on transferrin internalization into HepG2 cells by Ceichanover et al. (1983). Why then, did some authors observe ATP-independent endocytosis and others not? One possibility is that these differences reflect different energy requirements for different ligands. For example, ligands that are normally preclustered in coated pits may not require ATP for internalization whereas energy might be required to form new coated pits and engulf diffusely distributed receptors. Support for this might be found in the consistent results of several groups (Haigler et al., 1980; King et al., 1980; Hertel et al., 1986)

that endocytosis of EGF, whose receptor is not normally clustered in coated pits, is blocked in ATP-depleted cells. However, we think this explanation is unlikely for the following reasons. First, if receptors already in coated pits could be efficiently internalized we would have detected two different populations of transferrin receptors in our kinetic analysis. Since ~50% of transferrin receptors on the surface of K562 cells are in coated pits (Watts, 1985), we would have observed the rapid and efficient internalization of ~50% of bound transferrin while the remaining would stay on the cell surface. No evidence for this heterogeneous behavior was observed. Secondly, internalization and recycling of transferrin receptors in K562 cells occurs even in the absence of ligand (Watts, 1985) so that the number of transferrin receptors on the cell surface reflects an equilibrium distribution dependent on the relative rates of endocytosis and recycling. Should a single round of internalization occur while receptor recycling was blocked one would expect a loss of at least 50% of cell surface receptors. Therefore, the result that the number of transferrin binding sites on the cell surface was not substantially reduced in anoxic cells depleted of ATP suggests that endocytosis does not proceed in the absence of receptor recycling.

Although the total number of transferrin binding sites was unaffected during ATP depletion under N_2, incubation of cells in the presence of metabolic poisons resulted in a rapid loss of ~50% of cell surface transferrin binding sites. This loss of cell surface receptors did not directly correlate with either overall cellular ATP levels or with the rate of ATP depletion. These results suggest the possibility that metabolic poisons have other effects, unrelated to overall cellular ATP depletion, which alter the relative rates of endocytosis and recycling in a noncoordinate fashion. In this regard, it is noted that neither NaF nor NaN_3 are specific inhibitors of metabolic enzymes alone. NaF, at millimolar concentrations, not only inhibits nucleotide-binding proteins, including a variety of ATPases as well as serine and threonine phosphatases (Ballou and Fischer, 1986), but is also known to activate adenylate cyclases. This latter effect occurs in the presence of trace, commonly contaminating, quantities of aluminum and is due to permanent activation of GTP-binding proteins (recently reviewed by Chabre, 1990). Likewise, the inhibition of metalloenzymes by NaN_3 is not restricted to those involved in energy production (Dawson et al., 1986). Thus, interpretation of the effects of these metabolic poisons only with regard to their reduction of cellular ATP levels might be misleading. Our results suggest that one or more of these other inhibitory and/or stimulatory effects of metabolic poisons is somehow responsible for the observed loss of surface Tfn binding sites. However, it is also possible that the different methods of ATP depletion differentially affect the utilization of remaining ATP pools so as to more or less favor receptor internalization over recycling. Since the fraction of receptors lost corresponds to those localized in coated pits (50%), these effects might be restricted to that population of Tfn receptors.

Our data suggests that surface-bound transferrin becomes efficiently sequestered into deeply invaginated pits when ATP-depleted cells are incubated at 32°C. If so, why didn't surface transferrin receptors become inaccessible to transferrin during the 10–15 min-incubations under N_2 required to deplete cellular ATP? As discussed above, sequestration might be expected to result in an apparent loss of transferrin binding sites from the cell surface, however, there are three possibilities that might explain this apparent discrepancy. First, our data suggests that coated pits which exclude antibody remain at least partially accessible to transferrin. A second possibility is that the total number of cell surface receptors, in fact, increases while the apparent number remains constant due to the sequestration of some receptors in deeply invaginated pits. Such a trend toward increasing numbers of cell surface transferrin receptors was often observed especially at early time points when cellular ATP was depleted without the use of high concentrations of metabolic poisons. Although variable from experiment to experiment, Tfn binding to as high as 130% of control levels was often observed as reflected in the sizable error bars in Fig. 6, *b* and *c*. The possibility that the budding of coated vesicles from the cell surface might be more sensitive to cellular ATP levels than the delivery of recycling vesicles to the cell surface is consistent with findings on transport along the exocytic pathway that demonstrated the formation of transport vesicles from both the ER and Golgi to be more sensitive to reductions in cellular ATP than their subsequent delivery to target compartments (Balch et al., 1986). A third possibility is that transferrin receptors can diffuse in and out of coated pits more readily than receptor–ligand complexes. Thus, we detect near normal transferrin binding to unoccupied receptors under conditions that receptor–ligand complexes are apparently sequestered. It is possible that each of these factors contribute to this apparent inconsistency in our results.

Studies in which a "single round" of endocytosis was observed in ATP-depleted cells could reflect the different wash conditions and ligand sizes used which would, in turn, affect the extent of sequestration observed. ATP-independent internalization was observed by both Clarke and Weigel (1985) who were studying internalization of asialoorosomucoid (50,000 mol wt) mediated by the galactose receptor on isolated hepatocytes and by Larkin et al. (1985) who were studying internalization of LDL (a large particle) by human fibroblasts. Both groups used gentle wash conditions (low salt, neutral pH) to displace surface-bound ligand since binding was Ca^{2+} dependent and competing ligands could be used. In contrast, the consistent observations of ATP-dependent EGF internalization (Haigler et al., 1980; King et al., 1980; Hertel et al., 1986) might be explained by the combined situation that EGF is a small polypeptide (6,000 mol wt) that requires harsh acid wash conditions for dissociation from its receptor. An advantage of the approach described here is that any ligand could readily be derivatized using NHS-S-S-biotin allowing internalization to be assessed by GSH resistance. Thus, the validity of these explanations could be tested using GSH resistance as a more definitive criteria for internalization.

Where is ATP required during the complex processes involved in receptor-mediated endocytosis? A clear answer to this question will require the use of cell-free assays which facilitate biochemical analysis of the mechanisms involved. Cell-free assays which reconstitute events in receptor-mediated endocytosis have recently been developed (reviewed by Gruenberg and Howell, 1989). For example, the recycling of clathrin from coated vesicles for subsequent rounds of coated pit formation, appears to be an ATP-dependent reaction mediated by hsc70, the uncoating ATPase (Schlossman

et al., 1984; Rothman and Schmid, 1986). A number of investigators have also demonstrated an ATP requirement for fusion of endocytic vesicles (Gruenberg and Howell, 1986; Braell, 1987; Diaz et al., 1988; Woodman and Warren, 1988). However, a requirement for ATP in coated pit and coated vesicle formation has yet to be clearly resolved. Recently, Anderson and colleagues (Moore et al., 1987; Mahaffey et al., 1989; Moore and Anderson, 1989) have developed an assay that measures clathrin reassembly onto planar membranes from which the bulk of clathrin has been stripped by a high pH wash. As was observed for cage reassembly, clathrin will spontaneously reassemble onto these surfaces forming coated pits that are morphologically similar to those present on unstripped membranes. Reassembly occurs in the absence of ATP, at reduced temperatures (4°C) and appears not to require additional cytosolic components. These results suggest that ATP is not required for clathrin assembly into coated pits. However, since a significant fraction of clathrin remains on the "stripped" membranes (30–40%), it cannot be ruled out that these studies are instead measuring the spontaneous reassembly of clathrin onto itself. This possibility is supported by results of Mahaffey et al. (1989) that demonstrate that the optimum pH and ionic strength requirements for clathrin assembly in this system are nonphysiological and mirror those for spontaneous cage formation.

ATP might instead be required for de novo coated pit formation, perhaps during some nucleation event. A requirement for ATP in de novo coated pit formation was reported by Smythe et al. (1989) in a cell-free assay system involving internalization of HRP-conjugated transferrin into scraped, "semi-intact" A431 cells. Using EM cytochemistry, these authors observed a two- to threefold increase in the number of labeled coated pits formed when cells were incubated at 32°C in the presence of both cytosol and ATP. No such increase was observed in the absence of either cytosol or ATP, or at 4°C. Similarly, our observation that the total number of coated pits/mm cell surface did not increase during ATP depletion might provide additional indirect evidence to support a requirement for ATP in de novo coated pit formation although it cannot be ruled out that potential nucleation sites for coated pit assembly are instead limiting.

ATP appears also to be required in later stages of receptor-mediated endocytosis for coated vesicle budding. When untreated membrane sheets prepared by Moore et al. (1987; Moore and Anderson, 1989) were incubated in a defined buffer at elevated temperatures, clathrin was lost from the membranes. The addition of large amounts of apyrase prevented this loss. Although there was no evidence of coated vesicle formation (i.e., the capture of cell surface receptors and accompanying membrane into coated vesicles) or any direct evidence for the involvement of ATP (since it was not required that ATP be added directly), these results suggested that ATP might be required for coated vesicle budding. An ATP requirement for coated vesicle formation was more clearly demonstrated for transferrin uptake in semi-intact A431 cells (Smythe et al., 1989). First, sequestration of transferrin into an antibody inaccessible compartment was found to be cytosol dependent and stimulated two to three times in the presence of ATP. As an extension of these studies, we have used this same assay system to measure ^{125}I-BSST internalization into a GSH-resistant compartment. In contrast to the partial ATP dependence observed using Ab inaccessibility, the acquisition of GSH resistance in this same system was found to be absolutely dependent on ATP (Smythe, E., and S. L. Schmid, manuscript in preparation). These results are also consistent with results presented here using intact cells. Secondly, the morphological data of Smythe et al. (1989) strongly suggests that the formation of bona fide sealed coated vesicles, as determined by the analysis of serial sections, was absolutely dependent on the presence of both cytosol and ATP during incubations.

Identification of the enzymes that utilize ATP and the mechanisms by which they mediate events during endocytosis will require further dissection of cell-free assay systems which faithfully reconstitute these events.

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