Intracellular Protein Transduction through a Nucleoside Salvage Pathway

Regulation of gene expression by intranuclear transduction of macromolecules such as transcription factors is an alternative to gene therapy for the treatment of numerous diseases. The identification of an effective intranuclear delivery vehicle and pathway for the transport of therapeutic macromolecules across plasma and nuclear membranes, however, has posed a significant challenge. The anti-DNA antibody fragment 3E10 Fv has received attention as a novel molecular delivery vehicle due to its penetration into living cells with specific nuclear localization, absence of toxicity, and successful delivery of therapeutic cargo proteins in vitro and in vivo. Elucidation of the pathway that allows 3E10 Fv to cross cell membranes is critical to the development of new molecular therapies. Here we show that 3E10 Fv penetrates cells through a nucleoside salvage transporter. 3E10 Fv is unable to penetrate into cells deficient in the equilibrative nucleoside transporter ENT2, and reconstitution of ENT2 into ENT2-deficient cells restores 3E10 Fv transport into cell nuclei. Our results represent the first demonstration of protein transport through a nucleoside salvage pathway. We expect that our finding will facilitate a variety of methods of gene regulation in the treatment of human diseases, open up new avenues of research in nucleoside salvage pathways, and enhance our understanding of the pathophysiology of autoimmune diseases.

The ability to regulate gene expression through intranuclear delivery of macromolecules would significantly impact the treatment of a multitude of human diseases. Effective macromolecular therapy is dependent upon molecular delivery vehicles to circumvent the plasma membrane barrier and facilitate intracellular transport of cargo molecules. The single chain Fv fragment of the 3E10 anti-DNA autoantibody (3E10 Fv) has recently been harnessed as a novel molecular delivery vehicle due to its specific nuclear localization and apparent lack of toxicity (1). 3E10 Fv and Fv fusion proteins readily transduce across cell membranes and penetrate into cell nuclei, and 3E10 Fv has successfully delivered biologically active proteins such as Hsp70 (2) and p53 (3) into living cells in vitro. Moreover, 3E10 Fv mediated full-length p53 protein therapy in vivo (4). The pathway that carries 3E10 Fv across cell membranes and into cell nuclei, however, has not been identified.

Previous studies implicated DNA binding as important in 3E10 Fv transduction into cell nuclei. Specifically, mutations that abrogate DNA binding by the antibody render it incapable of cellular penetration (5). The association between cellular penetration and DNA binding distinguished 3E10 Fv from other protein transduction domains and implied that nucleoside salvage pathways might be involved in 3E10 Fv transport. Both concentrative (CNT) and equilibrative (ENT) nucleoside salvage transporters mediate the uptake of nucleobases and nucleosides by mammalian cells (6). Since any major role of CNTs in 3E10 Fv transport was excluded by previous studies that demonstrated 3E10 Fv penetration into COS-7 cells that lack endogenous CNTs (2, 7), we examined the role of ENTs in 3E10 Fv transport.

EXPERIMENTAL PROCEDURES

Cell Lines—COS-7, K562, and CEM/ENT1 cells were purchased from the American Type Culture Collection (Rockville, MD). PKNTD/ENT1 and PKNTD/ENT2 cells were generated as described previously (8).

Plasmids—A construct for expression of 3E10 Fv in the X-33 strain of Pichia pastoris, pPICZaA-Fv, was generated as described previously (9).

Purification of 3E10 Fv—3E10 Fv was purified from the supernatant of P. pastoris transfected with pPICZaA-Fv as described previously (9).

Cellular Penetration Assays—Purified 3E10 Fv was exchangedialyzed into PBS prior to application to cells. After dialysis, 10% fetal calf serum was added to the buffer. Control buffer was PBS with 10% fetal calf serum. For adherent cell lines (COS-7, PKNTD/ENT1, and PKNTD/ENT2), 50 μl of control buffer or 3E10 Fv was added to cells on 96-well plates for 1 h. After incubation with 3E10 Fv, the antibody fragment was removed, and cells were washed, fixed in chilled 100% ethanol, and stained with the 9E10 α-Myc antibody as described previously (9). For non-adherent cells (K562 and CEM/ENT1), cell pellets composed of ~200,000 cells were resuspended in 100 μl of control buffer or 10 μl 3E10 Fv and allowed to incubate with intermittent shaking at 37 °C for 1 h. Cells were then centrifuged at 100 × g for 2 min and washed three times with PBS. Next, cells were spread on glass slides and allowed to dry overnight. Cells were then fixed in chilled 100% ethanol for 10 min,
washed three times in PBS, and stained with the 9E10 α-Myc antibody.

**Nucleoside Transporter Inhibition Assay**—Nitrobenzylmercaptoapurine riboside (NBMPR) was purchased from Sigma, and a stock solution of 100 mM NBMPR in Me2SO was prepared. To control for the effects of Me2SO in cell culture, Me2SO was added to control buffers not containing NBMPR. The concentration of Me2SO in all control and experimental buffers was 0.1%. COS-7 cells were pretreated for 30 min with control buffer (PBS + 10% fetal calf serum) or buffer containing 10 or 100 μM NBMPR. Buffers were then replaced with control buffer or 10 μM 3E10 Fv in the presence or absence of 10 or 100 μM NBMPR for 1 h. Cells were then washed, fixed, and stained with the 9E10 α-Myc antibody.

**Microscopic Images**—As described previously, images of cells were acquired with an Olympus IX70 inverted microscope with RU reflected light fluorescent attachment and MagnaFire SP Digital Imaging System (Olympus, Melville, NY) (10). The scale bar in cell images in Figs. 1–3 = 5 μm.

**RESULTS**

ENT1 and ENT2 mediate equilibrative nucleoside transport in mammalian cells, and both ENT1 and ENT2 activity is inhibited by high concentrations of NBMPR (8). We therefore tested NBMPR for inhibition of 3E10 Fv transport. Purified 3E10 Fv (constructed with His6 tag for purification and Myc tag for identification) migrated as a single ~30-kDa protein on SDS-PAGE (Fig. 1A). Transduction of 3E10 Fv into COS-7 cells was confirmed by incubating cells with 10 μM 3E10 Fv for 1 h at 37 °C followed by Western blot analysis of cell lysates (Fig. 1B) or immunocytochemical staining of cells (Fig. 1C, top panels). Western blot analysis of cell lysates demonstrated the presence of an ~30-kDa Myc-tagged protein inside cells treated with 3E10 Fv (Fig. 1B, lane 2), which indicated penetration of the full-length antibody fragment. Furthermore, immunocytochemical staining confirmed nuclear localization by 3E10 Fv (Fig. 1C, top right panel), consistent with previous confocal microscopy and immunocytochemical studies on the antibody (2, 10). Next, COS-7 cells were pretreated for 30 min with control buffer or buffer containing 100 μM NBMPR prior to a 1-h incubation with 10 μM 3E10 Fv in the presence or absence of NBMPR. Subsequent immunocytochemical staining of the cells demonstrated that 100 μM NBMPR suppressed nuclear penetration by 3E10 Fv (Fig. 1C, bottom left panel), which suggested that ENT1 or ENT2 is involved in 3E10 Fv transport.

In an attempt to resolve which of the ENTs was linked to 3E10 Fv transport, 3E10 Fv penetration into COS-7 cell nuclei was tested in the presence of a lower dose of NBMPR to take advantage of the different Ki of NBMPR for ENT1 and ENT2 (0.4 nM versus 2.8 μM, respectively) (8). At 10 μM NBMPR, ENT1 activity is completely inhibited, whereas ENT2 retains moderate activity. In contrast to the distinct inhibition of 3E10 Fv transport provided by 100 μM NBMPR (Fig. 1C, bottom left panel), 3E10 Fv successfully penetrated COS-7 cell nuclei in the presence of 10 μM NBMPR (Fig. 1C, bottom right panel). This result suggested that ENT2, not ENT1, mediated transport of 3E10 Fv. The decreased nuclear staining intensity in cells treated with 3E10 Fv + 10 μM NBMPR when compared with cells treated with 3E10 Fv alone likely reflects partial inhibition of 3E10 Fv transport due to the expected >50% suppression of ENT2 activity by 10 μM NBMPR.

As an additional approach to identifying the equilibrative nucleoside transporter(s) involved in 3E10 Fv transduction, we examined 3E10 Fv transduction into the CEM/ENT1 cell line that lacks ENT2 (11). 10 μM 3E10 Fv was applied to the CEM/ENT1 cells for 1 h. As a positive control, 10 μM 3E10 Fv was also

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**FIGURE 1. Inhibition of an equilibrative nucleoside transporter blocks nuclear penetration by 3E10 Fv.** A, SDS-PAGE analysis of purified 3E10 Fv demonstrated a single band of ~30 kDa (Gelcode® Blue stain). B, α-Myc Western blot analysis of lysates of COS-7 cells treated for 1 h with control (lane 1) or 10 μM 3E10 Fv (lane 2) demonstrated penetration of the full-length antibody fragment. Ponseau S staining served as a loading control. C, a high dose of NBMPR inhibits nuclear penetration by 3E10 Fv. COS-7 cells were preincubated with control buffer or buffer containing 100 or 10 μM NBMPR for 30 min. Cells were then treated with control buffer (C, top left panel), 10 μM 3E10 Fv (C, top right panel), 10 μM 3E10 Fv + 100 μM NBMPR (C, bottom left panel), or 10 μM 3E10 Fv + 10 μM NBMPR (C, bottom right panel). After treatment, cells were washed, fixed, and stained with the α-Myc antibody to detect 3E10 Fv penetration. Control cells showed no staining (C, top left panel), and cells treated with 3E10 Fv in the absence of NBMPR exhibited nuclear staining (C, top right panel). In the presence of 100 μM NBMPR, 3E10 Fv failed to penetrate COS-7 cell nuclei as evidenced by a lack of staining (C, bottom left panel). At a low dose (10 μM) of NBMPR, 3E10 Fv penetrated into cell nuclei to a greater extent (C, bottom right panel) than in the presence of high dose NBMPR. Scale bar = 5 μm.
applied to K562 leukemia cells that express both ENT1 and ENT2 (12). As predicted, immunocytochemical staining of the 3E10 Fv-treated K562 cells demonstrated penetration of the antibody fragment into 100% of the cells (Fig. 2A, right panel). CEM/ENT1 cells treated with 3E10 Fv, however, showed no staining (Fig. 2B, right panel), which indicated a limitation or inability of 3E10 Fv to penetrate into ENT2-deficient cells. Data are representative of two independent experiments. Scale bar = 5 μm.

FIGURE 2. 3E10 Fv fails to penetrate ENT2-deficient cells. A and B, K562 cells that express both ENT1 and ENT2 and CEM/ENT1 cells that express ENT1 but not ENT2 were incubated with control buffer or 10 μM 3E10 Fv for 1 h. Cells were then washed, fixed, and stained with the α-Myc antibody. As expected, control K562 (A, left panel) and CEM/ENT1 (B, left panel) cells showed no staining. 3E10 Fv-treated K562 cells exhibited distinct staining (A, right panel), whereas 3E10 Fv-treated CEM/ENT1 cells showed an absence of staining (B, right panel), which indicated a limitation or inability of 3E10 Fv to penetrate into ENT2-deficient cells. Data are representative of two independent experiments. Scale bar = 5 μm.

B

CEM/ENT1 (ENT2-) Cells

PK15NTD/ENT2 Cells

FIGURE 3. Reconstitution of ENT2 restores 3E10 Fv transport into ENT2-deficient cells. A and B, PK15 cells deficient in nucleoside transporters were reconstituted with ENT1 (PKNTD/ENT1) or ENT2 (PKNTD/ENT2) and were treated with control buffer or 10 μM 3E10 Fv for 1 h. Cells were then washed, fixed, and stained with the α-Myc antibody. Control PKNTD/ENT1 (A, left panel) and PKNTD/ENT2 (B, left panel) cells showed an absence of staining. Similarly, 3E10 Fv-treated PKNTD/ENT1 cells exhibited no staining (A, right panel), indicating failure of the antibody fragment to penetrate into the ENT2-deficient cells. By contrast, 3E10 Fv penetrated well into the PKNTD/ENT2 cells as evidenced by nuclear staining (B, right panel). This result indicated that reconstitution of ENT2 restored 3E10 Fv transport into the ENT2-deficient cells. Data are representative of three independent experiments. Scale bar = 5 μm.

ACCELERATED PUBLICATION: ENT2 Mediates Protein Transduction

DISCUSSION

The nucleoside salvage pathways have been studied in detail, but protein transport through or related to nucleoside salvage has not been previously described. The precise interaction between 3E10 Fv and ENT2 remains to be determined, but it is tempting to speculate that 3E10 Fv may be carried into cells by virtue of its binding to nucleosides or nucleobases that are subsequently transported into cells by ENT2. Alternatively, 3E10 Fv may mimic the structure of a nucleoside or nucleobase that is recognized and transported into cells by ENT2. Elucidation of the mechanism by which ENT2 facilitates 3E10 Fv transport should yield further insights into both protein transduction and nucleoside salvage pathways. Furthermore, since ENT2 is located in both plasma and nuclear membranes, it will be important to ascertain whether ENT2 facilitates transport of 3E10 Fv across both cellular and nuclear membranes or whether another pathway is involved in nuclear penetration (13).

With regard to molecular therapy, the linkage between ENT2 and nuclear penetration by 3E10 Fv reported here further
establishes 3E10 Fv as a novel molecular delivery vehicle that is distinct from other protein transduction domains previously described. Endosomal localization by cell-penetrating peptides limits their role in molecular therapy (14, 15), but the identification of 3E10 Fv transport through ENT2 provides a rationale for future studies on the use of 3E10 Fv in delivering molecules such as small interfering RNAs, antisense oligonucleotides, and transcription factors to cell nuclei. It also generates an impetus to determine whether toxic cell-penetrating antibodies utilize a nucleoside salvage pathway in cellular penetration since inhibition of nucleoside transporters might then be investigated as a means of limiting tissue damage by cytotoxic autoantibodies in certain autoimmune diseases. The discovery of intranuclear protein transduction by 3E10 Fv through the ENT2-mediated nucleoside salvage pathway has profound implications for cell biology, pharmacology, and medicine.

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