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Pluripotency transcription factor Sox2 is strongly adsorbed by heparin but requires a protein transduction domain for cell internalization

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ABSTRACT

The binding of protein transduction domain (PTD)-conjugated proteins to heparan sulfate is an important step in cellular internalization of macromolecules. Here, we studied the pluripotency transcription factor Sox2, with or without the nonaarginine (R9) PTD. Unexpectedly, we observed that Sox2 is strongly adsorbed by heparin and by the fibroblasts without the R9 PTD. However, only the R9Sox2 fusion protein is internalized by the cells. These results collectively show that binding to heparan sulfate is not sufficient for cellular uptake, thereby supporting a recent hypothesis that other proteins play a role in cell internalization of PTD-conjugated proteins.

1. Introduction

Nuclear reprogramming of differentiated cells into pluripotent cells, called induced pluripotent stem cells (iPSCs), holds great promise for patient-specific regenerative medicine [1,2]. In the early studies, the genes encoding the transcription factors (TFs) that enabled nuclear reprogramming (Sox2, Oct3/4, c-Myc, Klf4, Nanog, and Lin28) were introduced to the differentiated cells using viral vectors. However, viral delivery of these genes is not appropriate for clinical use, since the delivered genes integrate into the host genome and can cause tumors by mutating and destabilizing the host genome [3,4]. Therefore, our laboratory [5], along with others [6–10], sought to generate iPSCs by delivering these transcription factors directly to the differentiated cells.

Intracellular delivery of these TFs requires transduction across the plasma membrane of the cell. The first barrier to entry that is encountered by the TFs is the heparan sulfate proteoglycans. Cellular uptake of macromolecules, such as these TFs, can be facilitated by fusing them to a protein transduction domain (PTD), also called a cell-penetrating peptide. PTDs are short polypeptides composed of mostly basic amino acids such as arginine and lysine [11,12]. They are thought to be internalized via fluid phase endocytosis (specifically, macropinocytosis), which is triggered by electrostatic interactions between the positively-charged PTD and the negatively-charged heparan sulfate proteoglycans on the cell surface [13–20]. However, more recent studies suggest that other mechanisms may also be involved. For example, Gump and coworkers showed that cellular uptake of a PTD fusion protein still occurs in cells deficient in glycosaminoglycans (GAGs). Internalization of the PTD fusion protein was prevented only when proteins on the cell surface were digested [21]. Naik and coworkers showed that the cellular internalization of both the free polyarginine R_{16} peptide and the R_{16}-DNA complexes was independent of cell surface GAGs [22]. Therefore, hitherto-unidentified proteins or receptors may also mediate cellular uptake of arginine-rich PTDs.

We studied the heparin binding and the internalization of one of the pluripotency transcription factors, SRY (sex determining region Y)-box 2 (Sox2), with or without the nonaarginine (R9) PTD. Using radiolabeled R9Sox2 and Sox2, we showed that both proteins bind equally well to heparin and to fibroblasts. However, only R9Sox2 is able to enter the fibroblasts. Our results thus support the hypothesis that adsorption of the PTD fusion protein to the cell surface is not sufficient for its cellular uptake.

2. Materials and methods

The transcription factors R9Sox2 and Sox2 were synthesized, purified and quantified as described [5]. The *E. coli* S30 cell extract used in the cell-free synthesis of R9Sox2 and Sox2 was prepared as described in reference [23], with modifications reported in reference [24]. The protocols for the heparin and cell binding studies are described below.

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2.1. Heparin binding studies

For the bead saturation experiments, different concentrations of Heparin Sepharose 6 Fast Flow beads (GE Healthcare, Uppsala, Sweden) (0.0016%, 0.008%, 0.04%, 0.2%, 1%, and 5% v/v) in duplicate were shaken with 150 nM of radioactive R9Sox2 or Sox2 in 20% w/v sucrose, 1× PBS and 0.1% v/v Tween 20 (SPTw buffer) at room temperature for 2 h. 0.1% v/v Tween 20 was included in the 100 μL incubation solution to prevent the proteins from adsorbing to the polypropylene microcentrifuge tubes. The microcentrifuge tubes and the Heparin Sepharose beads used in the binding experiments were washed with the SPTw buffer prior to the experiment. After the 2-h incubation, the beads were pelleted at 1700*g* for 10 min. From each tube, 50 μL of the supernatant (which contains the protein not adsorbed to the Heparin Sepharose beads) was spotted onto a Whatman MM filter paper (Whatman, Springfield Mill, United Kingdom). These papers were then dried for 1 h under an incandescent light bulb and immersed in 5 mL of Beckman Ready Safe scintillation cocktail (Beckman Coulter, Brea, CA). The radioactivity from the non-adsorbed R9Sox2 or Sox2 spotted onto the filter papers was quantified using a Beckman LS 3801 liquid scintillation counter (Beckman Coulter). The background radiation, calculated from a filter paper onto which 50 μL of SPTw buffer was spotted, was subtracted from the radioactivity measurements from the non-adsorbed protein. A linear radioactivity calibration curve was constructed by measuring the counts per minute (cpm) of known concentrations of ^{14}C-leucine. There are 20 leucines in R9Sox2 and Sox2, and the ratio of total leucine to ^{14}C-leucine in our CFPS protocol is 321. These values and the calibration curve were used to convert the radioactivity values to protein concentrations. Then, the protein concentrations were first subtracted from, and then divided by the initial protein concentrations to calculate the fraction of protein adsorbed to the Heparin Sepharose beads. Radioactivity from the used polypropylene assay tubes was calculated to ensure that the radiolabeled proteins did not adsorb non-specifically to the tubes; the radioactivity measurements were comparable to the background.

For the protein saturation experiments, different concentrations of R9Sox2 or Sox2 (10, 20, 40, 80, 100, 150, 200, 300, 400, 500 and 600 nM) in triplicate were shaken with 0.01% v/v Heparin Sepharose beads (GE Healthcare) in the SPTw buffer at room temperature for 2 h and 15 min. After the incubation, the beads were pelleted at 1700 g for 10 min. The following supernatant volumes were spotted onto the Whatman MM filter papers (Whatman): 300 μL for 10–80 nM, 150 μL for 100–200 nM, and 50 μL for 300–600 nM of R9Sox2 or Sox2. At lower protein concentrations, higher solution volumes were spotted onto the filter papers to ensure that the radioactivity counts were at least an order of magnitude above the background. The samples were processed as described in the previous paragraph. The simple ligand binding module in SigmaPlot 11 software (Systat Software, San Jose, CA) was used to fit the binding data to the following one-site saturation equation: $y = \frac{B_{max}x}{K_d+x}$, where y is the concentration of the bead-bound protein, x is the concentration of the unbound protein, K_d is the apparent dissociation constant for heparin, and B_{max} is the concentration of available sites for heparin binding.

2.2. Cell binding studies

Human neonatal foreskin (BJ) fibroblasts (passage 6) were cultured in T75 culture flasks (Nunc, Rochester, NY) using fibroblast medium (FM: DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic (Invitrogen, Carlsbad, CA)) in a humidified 5% CO_2 incubator at 37 °C. When the cells were 80% confluent (approximately 1×10^6 cells per T75 flask), they were washed with phosphate-buffered saline solution (PBS) and treated with 60 nM of purified radiolabeled R9Sox2 or Sox2 in serum-free DMEM. The cells were then incubated with the radioactive protein in a humidified 5% CO2 incubator at 37 °C for 4 h. After the incubation, the cells were washed twice with 10 mL PBS and twice with 10 mL of 1 mg/mL heparin (Sigma–Aldrich, St. Louis, MO) to desorb the protein bound to the fibroblasts. The fibroblasts were then treated with either 10 mL of collagenase solution (Accutase, Innovative Cell Technologies, San Diego, CA) for 5 min or with 10 mL of 0.25% Trypsin-EDTA solution (Invitrogen) for 30 min at 37 °C to dislodge the cells. 20 mL of FM was added to dilute the collagenase or trypsin, and the cell suspension was pelleted at 1500 g for 5 min. The cell pellet was washed four times with 1 mL PBS and resuspended in 200 μL PBS. The suspension was added to 5 mL of Beckman Ready Safe (Beckman Coulter) or MP Cytoscint (MP Biomedicals, Burlingame, CA) scintillation cocktail and mixed. The cell-associated radioactivity was measured using a LS3801 liquid scintillation counter (Beckman Coulter). The radioactivity measurements were converted to protein concentrations, as described previously.

Cell viability was analyzed using the trypan blue exclusion assay (Sigma–Aldrich), and the fibroblasts were counted using a hemocytometer. We consistently counted approximately 1×10^6 cells per T75 culture flask.

3. Results and discussion

Our initial aim in this project was to evaluate the functionality of the N-terminal nonaarginine (R9) protein transduction domain (PTD) of the purified R9Sox2 transcription factor by first assessing its affinity to the heparan sulfate proteoglycan layer on the surface of the fibroblasts. Heparin-coated Sepharose beads provided a uniform and well-characterized surrogate for the heparan sulfate proteoglycan layer. First, we evaluated the differential affinities of R9Sox2 and Sox2 as a function of increasing ionic strength or increasing heparin concentrations. These initial experiments did not show a significant difference in heparin affinity (data not shown). To measure the affinities of the R9Sox2 and Sox2 proteins more precisely, radiolabeled R9Sox2 and Sox2 were produced in CFPS using the PANOx-SP system (Fig. S1A). The protein yields were similar for Sox2 with or without the R9 PTD (Fig. S1B). These proteins were then purified by immobilized metal affinity chromatography.

For the heparin affinity studies, different concentrations of beads were incubated with 150 nM R9Sox2 or Sox2 to identify a bead concentration suitable for determining the adsorption affinity (Fig. 1A). A 0.01% v/v bead concentration was chosen because it was high enough to see the pelleted beads after centrifugation, but low enough to avoid complete protein adsorption. At this bead concentration, the difference in concentration between total and unadsorbed protein could be accurately determined by scintillation counting even at low nanomolar total protein concentrations. Different concentrations of R9Sox2 and Sox2 proteins were incubated with 0.01% v/v Heparin Sepharose beads and the amount of protein remaining in the supernatant (i.e., free protein) was measured (Fig. 1B). Data obtained with 10–160 nM free protein solution were fit to a one-site saturation binding model, and the apparent heparin dissociation constants (K_d^{app}) were: 101 ± 28 nM for R9Sox2, and 253 ± 69 nM for Sox2 (Fig. 1C). When our entire data set (obtained from 10 to ~500 nM free protein) was fit to the one-site saturation binding model, the apparent dissociation constants were: 340 ± 93 nM for R9Sox2, and 248 ± 50 nM for Sox2 (Fig. 1D). Taken together, these results suggest that the R9 PTD does not significantly increase the heparin affinity of Sox2.

In the case of R9Sox2, the number of available sites for heparin binding (B_{max}) was also different depending on the data set used.

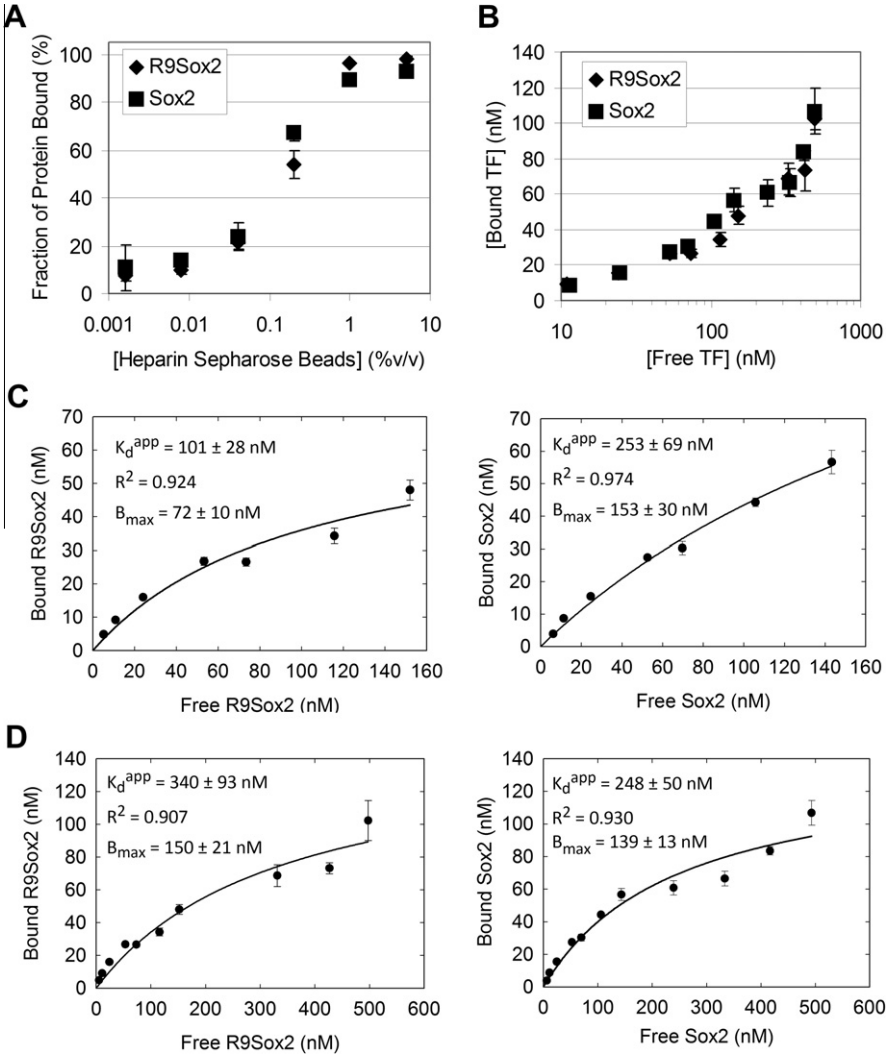

Fig. 1. Adsorption of R9Sox2 and Sox2 to heparin. (A) Binding of R9Sox2 and Sox2 at different bead concentrations. 150 nM of pure R9Sox2 or Sox2 were incubated with different concentrations of Heparin Sepharose beads for 2 h at room temperature. Data points are averages from $n = 2$ tubes. (B) Binding of R9Sox2 and Sox2 to Heparin Sepharose beads. Pure radiolabeled reprogramming factors R9Sox2 and Sox2 were incubated with 0.01% v/v Heparin Sepharose beads. (C) One-site saturation binding curves fit to data obtained after the addition of 10–200 nM R9Sox2 and Sox2. (D) One-site saturation binding curves fit to data obtained after the addition of 10–600 nM R9Sox2 and Sox2. Data points are averages from $n = 3$ tubes.

When the data obtained from 10 to 160 nM free protein were fit to the one-site saturation binding model, the concentration of heparin binding sites (B_{max}) was: 72 ± 10 nM for R9Sox2, and 153 ± 30 nM for Sox2 (Fig. 1C). However, when the entire data set was fit to the model, the concentrations of heparin binding sites for R9Sox2 (150 ± 21 nM) and Sox2 (139 ± 13 nM) were comparable (Fig. 1D). It thus seems that at low concentrations, R9Sox2 preferentially binds to a subset of heparin molecules on the bead surface.

The measured dissociation constants for R9Sox2 and Sox2 are significantly lower than that of the PTD of the HIV-1 TAT protein for heparin and heparan sulfate (~600 nM) [25]. In the case of R9Sox2, the apparent dissociation constant (K_d^{app}) measured with 10–160 nM of unadsorbed protein (101 ± 28 nM; Fig. 1C) agrees well with that reported for the R9 PTD (109 ± 13 nM) [26]. Furthermore, the K_d^{app} 's and the B_{max}'s for R9Sox2 adsorption to heparin differed with the concentration range that was used to obtain the constants (Figs. 1C and D), a difference not observed with Sox2. Finally, the K_d^{app} 's measured from our entire data set for R9Sox2 and Sox2 overlap (Fig. 1D). These observations may be explained by the heterogeneity of the heparin chains on the Heparin Sepharose beads. It is possible that the R9 PTD has a high affinity (~100 nM) to one type of heparin with a relatively low abundance on the beads (~70 nM), while the Sox2 protein may be binding to another more abundant type (~150 nM) with an affinity of 250–350 nM. Since Sox2 lacks the R9 PTD, its heparin binding must be mediated by another region, such as the cationic DNA-binding domain or the nuclear localization sequence. Both heparin and DNA are anionic polymers comprising a sugar backbone.

In order to study the cellular uptake of these proteins, 60 nM of ^{14}C-labeled R9Sox2 and Sox2 in Dulbecco's Modified Eagle Medium (DMEM) were administered to different BJ fibroblast cultures for 4 h at 37 °C. After 4 h, the cells were dislodged from the culture flask using either a gentle 5-min collagenase treatment (Fig. 2A) or a harsher 30-min trypsin treatment (Fig. 2B).

When the fibroblasts were dislodged with collagenase, the amount of cell-associated radiation was similar for the R9Sox2- and Sox2-treated cells. These results were not expected because it was widely believed that PTDs facilitate the internalization of exogenous proteins by heparin-mediated cell surface adsorption [11]. However, transcription factors such as Sox2 have highly cationic DNA-binding domains and nuclear localization sequences.

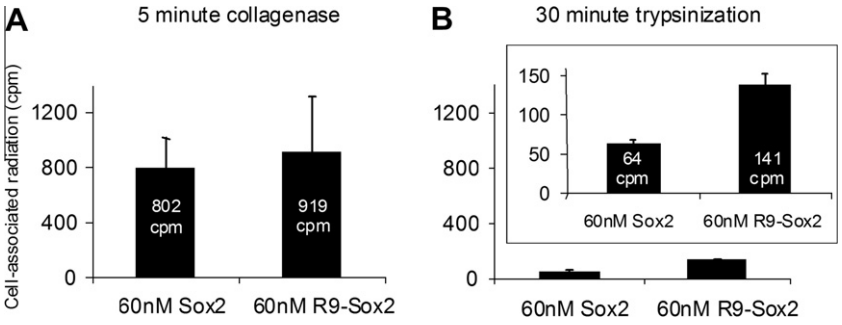

Fig. 2. Trypsinization removes non-internalized R9Sox2 and Sox2 from the surface of BJ fibroblasts. Purified radioactive R9Sox2 and Sox2 were administered to the fibroblasts for 4 h at 37 °C. (A) Similar amounts of Sox2 and R9Sox2 associate with the cells when they are gently dislodged from the plate using collagenase. (B) The 30-min trypsinization step removed the majority of the cell-associated radioactivity for both Sox2 and R9Sox2, suggesting that most of the proteins were bound to the surface of the cells and not internalized.

Since fibroblasts are covered by negatively-charged heparan sulfate, we hypothesized that perhaps the high levels of radiation observed with Sox2-treated cells were due to externally-bound Sox2. This hypothesis is supported by the heparin binding results, which showed that R9Sox2 and Sox2 possess similar affinities to heparin. We then used a trypsinization step [21,27] to both dislodge the cells from the culture plate and remove the surface-bound, but not internalized Sox2. To confirm that the harsh trypsin treatment did not cause cell lysis, we performed a trypan blue exclusion assay on the cells following the trypsinization, and observed retention of membrane integrity (data not shown). However, trypsinization reduced the amount of radiation associated with the Sox2-treated cells to nearly background levels. In contrast, a significant amount of radioactivity was still associated with the R9Sox2-treated cells (Fig. 2B). Cellular internalization of the R9Sox2 was confirmed by the increase in Myb gene expression (a known downstream target of Sox2) in BJ fibroblasts that were trypsinized after the administration of radiolabeled R9Sox2 [5]. These measurements suggest that (i) Sox2 needs a PTD for cell entry, (ii) the R9 PTD indeed has a positive effect on intracellular protein delivery, and (iii) a very small fraction of the administered R9Sox2 is internalized by the fibroblasts.

A closer look at the cell binding studies reveals that the majority (85–90%) of cell-associated R9Sox2 and Sox2, after administration, is bound to the cell surface, instead of being internalized: upon trypsinization, cell-associated radioactivity of R9Sox2 decreased from 919 to 141 cpm, while that for Sox2 decreased from 802 to 64 cpm (essentially a background level). In fact, the R9Sox2 internalization efficiency is very low. In our experiment, 60 nM of R9Sox2 in 10 mL of medium corresponds to approximately 30,000 cpm. Only about 80 cpm of that initial 30,000 cpm is internalized by the fibroblasts (Fig. 2B). Thus, approximately 0.3% of the R9Sox2 available for uptake in the medium actually entered the cells. Despite the low internalization efficiency, the internalized R9Sox2 protein was biologically active; cellular internalization of the R9Sox2 was confirmed by upregulation of myb, an established downstream target of Sox2 [5].

The unexpected result of these experiments was that Sox2 lacking the R9 PTD adsorbed to both the Heparin Sepharose beads and the fibroblasts with high affinity. Several previous reports suggested that cellular entry of proteins mediated by PTDs occurred by non-specific endocytosis of protein adsorbed to the heparan sulfate proteoglycans on the cell surface [28–30]. If heparan sulfate binding was the sole mediator of cellular uptake, then we would have expected both R9Sox2 and Sox2 to be internalized with the same efficiency, since both proteins bind to heparin with comparable affinity. Instead, only R9Sox2 was internalized by the fibroblasts. Our results thus support a more recent hypothesis, that binding of the PTD-containing protein is not sufficient for its cellular uptake [21]. It is possible that the R9 PTD stimulates fluid phase endocytosis by interacting with the cell membrane or with specific membrane proteins [13,19,31]. On the other hand, binding of transcription factors such as Sox2 to the heparan sulfate proteoglycans may be part of an important mechanism for managing cell senescence, apoptosis, and necrosis by preventing the uptake of released transcription factors by neighboring cells, thereby avoiding inappropriate genetic regulation. Protein transduction with other transcription factors such as Oct4, Nanog, and the fate of extracellular Sox2 should be carefully analyzed in order to assess this hypothesis.

In summary, this report provides conclusive evidence for binding of a transcription factor without a protein transduction domain (PTD) to heparin and heparan sulfate-covered fibroblasts with high affinity. This report also contains quantitative uptake data for the Sox2 transcription factor, which is one of the several proteins required for nuclear reprogramming. While both R9Sox2 and Sox2 bind to heparin with similar affinity, only R9Sox2 is able to enter the fibroblasts and upregulate its downstream gene targets. In addition, only 0.3% of the administered R9Sox2 was internalized by the fibroblasts. Much smaller amounts of purified transcription factors would be required for nuclear reprogramming if this internalization efficiency could be improved. Administering smaller amounts of these proteins would be very desirable, since it is difficult to synthesize large quantities of biologically active transcription factors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.016.

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