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Abstract

Transferrin receptor (TfR2), a homologue of classical transferrin receptor 1 (TfR1), is found in two isoforms, α and β . Like TfR1, TfR2 α is a type II membrane protein, but the β form lacks transmembrane portions and therefore is likely to be an intracellular protein. To investigate the functional properties of TfR2 α we expressed the protein with FLAG-tagging in transferrin receptor-deficient Chinese hamster ovary cells. The association constant for binding of diferric transferrin (Tf) to TfR2 α is $5.6 \times 10^6 \text{ M}^{-1}$, which is about 50 times lower than that of TfR1, with correspondingly reduced rates of iron uptake. Evidence for Tf internalization and recycling via TfR2 α without degradation, as in the TfR1 pathway, was also found. The interaction of TfR2 α with Tf was further investigated using atomic force microscopy (AFM), a powerful tool for investigation of the interaction between ligand and receptor at the single molecule level on the living cell surface. Dynamic force microscopy reveals a difference in the interactions of Tf with TfR2 α and TfR1, with Tf-TfR1 unbinding characterized by 2 energy barriers, while only one is present for Tf-TfR2. We speculate that this difference may reflect Tf binding to TfR2 α by a single lobe, whereas two lobes of Tf participate in binding to TfR1. The difference in the binding properties of Tf to TfR1 and TfR2 α may help account for the different physiological roles of the two receptors.

Key words: iron metabolism; transferrin; transferrin receptor 2; atomic force microscopy; functional assay

Abbreviations: Tf, diferric transferrin; TfR1, transferrin receptor 1; HFE, the protein mutated in hereditary hemochromatosis; DMT1, divalent metal transporter 1; TfR2, transferrin receptor 2;

AFM, atomic force microscopy; CHO cells, Chinese Hamster Ovary cells; GFP, green fluorescent protein; FBS, fetal bovine serum; RT-PCR, reverse transcriptase – polymerase chain reaction; PBS, phosphate buffered saline; DFO, deferoxamine mesylate; BSA, bovine serum albumin; TCA, trichloroacetic acid; PTA, phosphotungstic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; K_a , association constant; FACS, fluorescence-activated cell sorting; IP, immunoprecipitation.

Introduction

Iron is essential for all living organisms and required for numerous metabolic processes. In vertebrates, and at least some invertebrates with circulatory systems, almost all circulating iron is carried by transferrin (Tf) to provide iron for cellular needs. The initial event in the cellular uptake of iron is binding of Tf to transferrin receptor 1 (TfR1)¹ on the plasma membrane of cells, followed by endocytosis of the Tf-TfR1 complex. HFE,² the protein which when mutated is responsible for hereditary hemochromatosis, has been shown to combine with TfR1 and reduce its affinity for Tf,³⁻⁶ but the physiological functions of HFE are still not fully understood. In the acidified Tf-bearing endosome, iron is released from Tf and carried into the cytosol by the divalent metal transporter 1 (DMT1).⁷⁻⁹ After release of its iron Tf, still bound to receptor in the acidified endosome, is recycled to the cell membrane, and released from TfR1 at the cell surface where iron-free Tf is not bound by receptor at pH 7.4. Most cells other than hepatocytes have been thought to depend chiefly or exclusively on the TfR1 cycle for securing iron from Tf.¹⁰⁻¹²

A second transferrin receptor, transferrin receptor 2 (TfR2), was cloned and identified as a new member of the transferrin receptor class.¹³ TfR2 has two isoforms; TfR2 α and TfR2 β . TfR2 α is thought to be a type II membrane protein like classical TfR1.¹⁴ TfR2 β is probably an intracellular protein because its amino acid sequence lacks transmembrane portions. Although its affinity for Tf is less than that of TfR1, TfR2 α binds Tf and therefore may participate in cellular iron uptake, while the physiological function of TfR2 β is unknown. Mutations of the *TfR2* gene reduce hepcidin expression, resulting in iron overload and indicating that TfR2 may function primarily as a regulator of hepcidin production. However, the precise mechanisms of TfR2 α involvement in cellular iron metabolism have not been elucidated, largely due to the lack

of information about the properties of the TfR2 α protein. We therefore aimed to characterize the interactions of TfR2 α with Tf by functional assays and atomic force microscopy (AFM), a powerful tool for investigating the interaction between a ligand and its receptor at the single molecule level on a living cell surface.¹⁵

Results

Total protein contents

TfR1-deficient CHO TRVb cells were transfected with TfR2 α expression vector or mock vector, with no detectable change in cell morphology observed in the culture wells by light microscopy. Total protein contents were 113 ± 20 (n = 10) pg/cell for wild-type TRVb cells, 127 ± 19 (n = 10) pg/cell for TRVb-TfR2 α cells, and 120 ± 15 (n = 10) pg/cell for TRVb-mock cells. Thus, transfection of TRVb cells with the TfR2 α expression vector did not cause any remarkable change in cellular protein concentration.

Expression of TfR2 α and its binding to Tf

Transfection of TRVb cells with the TfR2 α expression vector resulted in much higher Tf binding at 4°C compared to wild-type TRVb cells or the mock-transfected clone (Fig. 1). Tf binding to TRVb cells and TRVb-mock cells showed non-saturable, almost linear, behavior characteristic of non-specific binding. In contrast, expressed cell-surface Tf binding sites in TRVb-TfR2 α cells saturated at 2.8×10^4 molecules of Tf/cell, with K_a calculated to be 5.6×10^6 M⁻¹. Since TRVb-TfR2 α cells and TRVb-mock cells were maintained at 30 μ g/ml puromycin but TRVb cells were maintained without puromycin, TRVb-mock cells were used as controls for further studies.

Cell-associated Tf at 37 °C

Total cell-associated Tf at 37 °C increased as a function of Tf concentration in TRVb-TfR2 α cells (supplementary data 1). Cell-associated Tf of TRVb-mock cells, however, also increased as a function of Tf concentration, even though cell-associated Tf was less than that seen in TRVb-TfR2 α cells. To determine whether transfection was responsible for this increase of cell-associated Tf in mock cells, cell-associated Tf was measured in wild-type TRVb cells at 37

°C. There was no remarkable difference between wild-type TRVb and TRVb-mock cells, indicating that transfection itself did not cause the Tf association in mock cells (data not shown). Since TRVb cells lack detectable TfR1, this association with Tf must be receptor-independent. The difference between cell associated Tf in TRVb-TfR2 α and TRVb-mock cells as a function of Tf concentration, presumably due to Tf bound to TfR2 α and Tf internalized via TfR2 α in the former but not the latter, approached a constant value.

Efficiency of the acid-wash method

Our acid-wash method removed approximately 90% of cell surface membrane bound Tf in TRVb-TfR2 α cells compared to normal PBS washing (data not shown). In addition to acid-washing, washing with F-12 medium was necessary to remove bound Tf, suggesting that, like TfR1, TfR2 α binds apotransferrin at acidic pH but not at neutral pH.¹⁴ Addition of cold diferric Tf into F-12 medium at pH 7.4 did not enhance the effectiveness of washing, verifying that washing at pH 7.4 is sufficient for removing cell-surface bound apotransferrin.

Evidence for Tf internalization mediated by TfR2 α

After 1 hour incubation at 37 °C cell-associated Tf in TRVb-TfR2 α cells and TRVb-mock cells represents both internalized Tf and nonspecific cell-surface bound Tf resistant to acid-washing. Tf in TRVb-TfR2 α cells was 26,500 molecules/cell, much higher than the 6,600 molecules/cell seen in TRVb-mock cells (Table 1). Although the effectiveness of the acid-wash method is only about 90%, the difference in cell-associated Tf between transfected and mock cells is too great to be attributed to residual cell-surface bound Tf and therefore represents Tf internalized via TfR2 α . We calculate that about 17,500 molecules of Tf per cell are internalized by TfR2 α (Table 1).

Iron uptake

Iron uptake by TRVb-TfR2 α cells after 1 hour incubation at 37 °C was not clearly different from that of TRVb-mock cells, even though there were small differences at high concentrations of Tf (Fig. 2A). To investigate whether induced TfR2 α protein actually can donate iron to the cells or not, the time course of ⁵⁹Fe uptake was determined. Only after 1 hour did a difference between TRVb-TfR2 α cells and TRVb-mock cells become clear (Fig. 2B). Thus, TfR2 α -associated Tf could donate iron to cells, although its rate of donation is much less than that provided by Tf associated with TfR1. The data also indicate that CHO cells exhibit receptor-independent iron uptake from diferric Tf, as described earlier.¹⁶

Efflux of Tf

To investigate the fate of Tf taken up through TfR2 α , TRVb-TfR2 α cells were incubated with ¹²⁵I-labeled Tf and acid-washed before the time course of efflux was determined (Fig. 3). At time 0, Tf internalization by TRVb-TfR2 α cells was higher than by TRVb-mock cells, in keeping with Tf internalization by TfR2 α as discussed above. Internalized Tf mediated by TfR2 α was exocytosed from the TRVb-TfR2 α cells; approximately 80% of Tf taken up via TfR2 α was released by 30 min. The indication is that an effective efflux route exists for Tf taken up by TfR2 α , suggesting that TfR2 α , like TfR1, is recycled by the cell.

In further investigation of the TfR2 α pathway, the degradation of Tf internalized by TfR2 α was determined. After 60 min, approximately 90% of Tf exocytosed from TRVb-TfR2 α cells was TCA/PTA precipitable, indicating that Tf bound to TfR2 α has recycled without substantial degradation in cells.

Pulse-chase study

To determine the fraction of Tf bound to cell membrane that is internalized, and the recycling time, a pulse-chase experiment was performed (supplementary data 2). Almost all Tf

initially bound to TfR2 α at 4 °C was released into the culture medium after 10 min incubation at 37 °C, and there was no significant internalization of cell surface bound Tf in the single-cycle of this pulse-chase study.

Absence of TfR1–TfR2 heterodimers

The effectiveness of anti-FLAG M2 antibody and anti-TfR1 antibody for immunoprecipitation was confirmed before undertaking these experiments (data not shown). HuH-7 cells, which express detectable TfR1 by Western blotting, were transiently transfected with the TfR2 α expression vector and cell lysates were taken for immunoprecipitation and Western blotting (supplementary data 3). Western blotting by anti-TfR1 antibody for the samples without immunoprecipitation showed that HuH-7 cells and HuH-7 cells transfected with TfR2 α express almost identical amounts of TfR1. Furthermore, Western blotting by anti-TfR2 antibody for the samples not subjected to immunoprecipitation showed that HuH-7 cells transfected with TfR2 α express more TfR2 α protein than non-transfected HuH-7 cells. These data indicated that the transiently transfected cells overexpressed TfR2 α protein and that the antibodies were highly effective for Western blotting. When immunoprecipitation was performed with anti-TfR1 antibody, a band was clearly detected in transfected and non-transfected cells, but no band was found with the anti-TfR2 antibody. If a TfR1-TfR2 heterodimer were present, anti-TfR1 Ab would precipitate that protein, and Western blotting with anti-TfR2 Ab would show the band. When immunoprecipitation was performed with anti-FLAG M2 antibody, a 100 kDa band was detected only in transfected cells when the anti-TfR2 antibody was used as the primary antibody in Western blotting, indicating that transient transfection resulted in expression of immunoprecipitable TfR2 α protein. No band was detected when anti-TfR1 was used as the primary antibody. Prolonged exposure of the

blotted membrane to the developing solution did not make a difference. The possibility that the N-terminal FLAG tag in TfR2 α interferes with dimer formation is unlikely, since recombinant TfR1 lacking the first 120 N-terminal intracellular residues spontaneously dimerizes.¹⁷ These results indicate that no detectable heterodimer of TfR1 and TfR2 α was formed by the TfR2 α -transfected HuH-7 cells. Therefore our experiments detect TfR2, not the heterodimer of TfR1 and TfR2.

Specificity of detection by AFM

HLF cells (human hepatoma) were transiently cotransfected with TfR2 α and green fluorescent protein (GFP) for identification and tested with a diferric Tf coated tip. Retraction force curves were recorded with a Tf coated tip and showed specific unbinding events between diferric Tf on the tip and TfR2 α at the cell surface (in TBS, pH 7.4) (Fig. 4A). The probability of binding between the Tf coated tip and TfR2 at the cell surface reached 26% (n = 7 cells). In contrast, when non-transfected cells were tested with a Tf coated tip, this probability was only 6% (n = 12 cells, p < 0.001, t-test). This indicated that transient transfection of TfR2 α expression vector was adequate for the investigation using AFM (Fig. 4B).

Force histogram for specific interaction between diferric Tf and TfR2 α

We recorded 1500 force curves with a Tf coated tip on TfR2 transfected cells allowing us to collect a total of 573 specific unbinding events in an experiment was performed with a single tip using 2 cells. Events were analyzed and plotted in a force histogram (Fig. 5). A clear peak was visible on the histogram, showing that the mean unbinding force between Tf on the tip and TfR2 at the cell surface was 63 ± 8 pN (at a mean loading rate of 2.8 nN/s). The experiment was repeated several days later with a new tip with cells independently cultured and gave similar results.

Dynamic force spectroscopy

Dynamic force spectroscopy, which consists of measuring the mean unbinding force at different loading rates, was performed with a diferric Tf coated tip on TfR2 transfected cells. As expected, the force was logarithmically dependent on the loading rate (Fig. 6). However, this dependence was small as the force varied from 59 ± 9 pN at a rate of 1.7 nN/s to 62 ± 10 pN at 20 nN/s. In striking contrast the unbinding force between TfR1 and Tf was reported at 39 ± 5 pN at a rate of 1 nN/s and increased up to 94 ± 12 pN at 27 nN/s,¹⁵ indicating that the binding of TfR2 α and diferric Tf differs from those of TfR1 and diferric Tf. Furthermore, the force spectrum of TfR2 transfected cells displayed only one regime (slope) while 2 regimes were clearly visible for TfR1 expressing cells (Fig. 6), which implies different interactions of Tf with TfR1 and TfR2.

Discussion

In their original publications, Kawabata et al. reported that TfR2 α can bind Tf and donate iron to CHO cells,¹³ therefore supporting cell growth.¹⁴ Fleming et al. found that TfR2 expression is not regulated by intracellular iron status and so might be involved in the pathogenesis of hemochromatosis.¹⁸ Although TfR2 is thus shown to function in iron metabolism, its precise physiological role is still unknown.

To avoid confusion by TfR1, our first studies used HuH-7 cells with anti-sense suppressed TfR1 expression.^{19,20} The amount of residual TfR1, however, was still high so that it was not possible to differentiate between Tf binding to TfR1 or to TfR2 α because of the low affinity of TfR2 α for Tf.¹⁴ We therefore turned to TfR1-deficient CHO cells for expressing TfR2 α .^{13,21}

In the present study, the affinity of TfR2 α to Tf was measured in living cells for the first time using ¹²⁵I-Tf to display the binding isotherm at 4 °C. K_a for binding of Tf by TfR2 α was calculated to be $5.6 \times 10^6 \text{ M}^{-1}$, about 35-fold less than that of TfR1 ($2\text{-}3 \times 10^8 \text{ M}^{-1}$ in HuH-7 cells). The lower affinity of TfR2 α in transfected CHO cells accords with a previous report based on a qualitative study by flow cytometry.¹⁴ Quantification by surface plasmon resonance using a recombinant soluble extracellular portion of the receptor gave a binding constant of $37 \times 10^6 \text{ M}^{-1}$, consistent with the present result considering the difference in methodology.²² The deduced amino acid sequence of the extracellular domain of TfR2 α protein is 45% identical and 66% similar to that of TfR1. TfR2 α also possesses the RGD triad, amino acids 678-680,¹³ thought to be critical for binding Tf to TfR1.²³ The lower affinity of TfR2 α for Tf is, therefore, not now explicable.

Although the affinity for Tf of TfR2 α is much less than that of TfR1, the concentration of

iron-bearing Tf in the circulation, about 3×10^{-5} M, is sufficient to saturate TfR2 α . TfR2 α expression has been found in cells with active roles in iron metabolism. Liver, the principal organ of iron storage, expresses a high level of TfR2 α mRNA, as do human hepatoma-derived HepG2 cells¹³ and HuH-7 cells (unpublished observation). K562 cells, of human myelogenous erythroleukemic origin, also express TfR2 α mRNA.¹³ Studies of iron uptake from Tf prior to the discovery of TfR2 did not discriminate between the roles of the two receptors. In future studies, therefore, both receptors require consideration.

Binding of Tf to TRVb-TfR2 α and TRVb–mock cells at 4°C was only 25-50% inhibited by a 100-fold excess unlabeled Tf, possibly because of large, essentially non-saturable, binding. We therefore resorted to use of a binding isotherm with terms for specific (saturable) and non-specific (non-saturable) binding to estimate binding constants for each type of binding.²⁴ Curve fitted parameters for specific binding attributable to transfected TfR2 α were: K_a 5.6×10^6 M⁻¹, and total number of sites, 2.8×10^4 /cell. These contrast with binding constants near 10^8 M⁻¹ and site numbers in the range of $2-5 \times 10^5$ per cell for Tf binding in K562 cells, when TfR1 predominates and curve-fitting to a single class of sites adequately accounts for binding. An apparent binding constant of 6.5×10^9 M⁻¹ is obtained for non-saturable binding of Tf to TRVb-TfR2 α cells, substantially weaker than that derived for specific binding. At Tf concentrations of 10^{-6} M, for example, saturable binding would account for about 24,000 molecules of Tf/cell, while non-saturable binding would account for 6,500 molecules/cell. Nevertheless, both TfR2 α -dependent and non-specific binding contribute to association of Tf with the cells.

In the present study, CHO cells showed a receptor-independent association with Tf that should be considered when investigating the function of TfR2 α expressed in CHO cells. We

find a clear increase of cell-associated Tf after expression of TfR2 α . Since cell-associated Tf represents both cell surface-bound Tf and Tf internalized via TfR2 α , proof of Tf internalization mediated by TfR2 α was required. Cell-associated Tf persisting after acid-washing confirmed the existence of Tf internalization via TfR2 α .

After internalization, efflux of Tf, without substantial degradation, is found. Thus, Tf internalized by TfR2 α , like Tf internalized by TfR1, recycles. An important difference between the two receptors was observed in a pulse-chase study. Less than 12% of Tf bound to the cell surface of TRVb-TfR2 α cells is internalized, most of the Tf being dissociated and released to the medium at 37 °C. In contrast, approximately 30-50% of membrane bound Tf was internalized by human hepatoblastoma-derived HepG2 cells¹² and human hepatoma-derived HLF cells.²⁵ The lower affinity of TfR2 α for Tf may help account for the difference between previous studies and the present work, since HepG2 and HLF cells express TfR1 but TRVb-TfR2 α cells do not. In our pulse-chase study no detectable internalization of membrane-bound Tf in a single cycle was found when the occupancy of TfR2 α by transferrin was about 75%. In the normal circulation, however, the concentration of iron-bearing Tf is close to 3×10^{-5} M so that cell surface receptors are always saturated with Tf and replenished as Tf is internalized. TfR2 α might therefore normally function in iron uptake from Tf, but this has yet to be experimentally confirmed.

Overexpressed TfR2 α protein was also shown to mediate iron uptake although its rate of iron donation was slow. We estimate, from the difference between iron uptake by TRVb-TfR2 α cells and TRVb-mock cells in Fig. 2B, that the rate of iron uptake was approximately 0.2 atoms/receptor/min when cells were incubated with 8.1×10^{-7} M Tf. For comparison, the rate of iron uptake via TfR1 is about 0.5-1 atoms/receptor/min in K562 cells. Thus, TfR2 α may

function in iron-uptake from Tf, albeit with less efficiency than TfR1.

Because of the sequence similarities between TfR1 and TfR2 α , the possibility that the protein monomers combine to form heterodimers was investigated. However, no detectable expression of heterodimers was found in the present studies. The possibility must be considered that our transfection procedure in HuH-7 cells yielded two different cell populations, one expressing TfR2 α and the other failing to do so, so that expression of heterodimer was too low for detection. We cannot exclude the possibility of heterodimer formation that might be detected by more sensitive methods or in other cells.

AFM was used to characterize the interactions between diferric Tf and TfR2 α protein at the single molecule level. We found that the unbinding force needed to detach diferric Tf from TfR2 α (63 ± 8 pN) was different from TfR1, previously reported as 56 ± 7 pN.¹⁵ However, dynamic force measurements revealed striking differences between diferric-Tf-TfR1 and diferric Tf-TfR2 α interactions, which reflected clearly distinct energy landscapes.¹⁵ While Tf-TfR1 unbinding is characterized by 2 energy barriers, only one is present for Tf-TfR2. This obvious difference might arise from different binding points for diferric Tf-TfR1 and diferric Tf-TfR2 α interactions. In addition, this possibility provides a structural interpretation for the energy barriers postulated here. We speculate that the two barriers of the Tf-TfR1 interaction stem from binding of both lobes of diferric Tf (C-lobe and N-lobe), whereas the single barrier of Tf-TfR2 α interaction originates from binding of a single lobe, but we recognize that this speculation requires further investigation.

In conclusion, the present study shows that TfR2 α protein functions in binding Tf and taking up iron and that there must be a difference between TfR1 and TfR2 in their interactions with diferric Tf as revealed by AFM. TfR2 α mRNA lacks an IRE so that expression is not

regulated by intracellular iron status¹⁸ but possibly by the cell cycle.¹⁴ Furthermore, TfR2 cannot compensate for TfR1 in TfR1-knockout mice, which lack a functional Tf cycle, and do not survive gestation.²⁶ A nonsense mutation of the human TfR2 gene causes a hemochromatosis-like disorder.^{27,28} The physiological functions of TfR2 are therefore still unclear, although it likely contributes to the regulation of cellular iron status.

Materials and Methods

Cells and cell culture

TfR1-deficient CHO TRVb cells,²¹ were grown in F-12 Nutrients Mixture (Invitrogen; Grand Island, NY) supplemented with 5 % heat-inactivated FBS (Gemini Bio-Products; Woodland, CA), 100 U/ml penicillin and 100 µg/ml streptomycin. All cultures were maintained at 37 °C in 5% CO₂.

TfR2α expression vector

Total RNA was extracted from K562 cells using the RNeasy Total RNA Isolation System (Qiagen; Madison, WI) following manufacturer's instructions. Complementary DNA was reverse transcribed from 1 µg of RNA using an oligo-dT primer (Qiagen) and MMLV reverse transcriptase (Stratagene; La Jolla, CA), and human TfR2α cDNA was amplified by hot-start PCR. A FLAG sequence was added to the 5'-terminus of the cDNA to enable detection of expressed protein by anti-FLAG antibodies. Oligonucleotides used as PCR primers were:

5'-ACCTTAAGGCCACCATGGATTACAAGGATGACGACGATAAGATGGAGCGGCTTTG-3' and 5'-GGTTCGAAGCAATGAGAGGTGGAC-3'. Conditions for amplification were: 40 cycles of 99 °C for 1 min, 65 °C for 2 min, and 72 °C for 5 min. The amplified DNA fragment was digested with AflIII and BstBI, and then inserted into the bicistronic mammalian expression vector pIRESpuro2 (Clontech; Palo Alto, CA). Orientation and sequence of the inserted TfR2α cDNA fragment were confirmed by sequencing.

Transfection

TRVb cells were grown in 35 mm six-well plates until they reached 70-80% confluence, then transfected with the TfR2α expression vector using Lipofectamine Plus (Invitrogen;

Carlsbad, CA). Selection was performed with 10 $\mu\text{g/ml}$ puromycin (Clontech) for 2 weeks and then the concentration of the antibiotic was raised to 30 $\mu\text{g/ml}$ in order to isolate a clone with high-level expression of TfR2 α . After another two weeks, one viable colony (TRVb-TfR2 α) was obtained, and that was subcultured using a cloning cylinder. As a mock-transfected control, TRVb cells were transfected with the pIRESpuro2 vector without a cDNA insert, and one clone was isolated after 4 weeks' selection (TRVb-mock). These transfectants were maintained at 30 $\mu\text{g/ml}$ puromycin after isolation. Expression of TfR2 α mRNA was confirmed by RT-PCR, and the expression of TfR2 α protein was confirmed by immunoprecipitation-Western blotting using anti-FLAG antibodies (data not shown).

Total protein content

Total protein contents of TRVb, TRVb-TfR2 α , and TRVb-mock cells were determined with the Bio-Rad Protein Assay Kit (Bio-Rad; Hercules, CA) using BSA as a standard. Cells were counted with a hemocytometer.

Iodination of Tf

Diferric human Tf (Boehringer-Mannheim) was labeled with ^{125}I (Amersham Pharmacia Biotech; Piscataway, NJ) by McFarlane's iodine monochloride method.²⁹ To remove unbound ^{125}I , labeled Tf was passed through a 10-DG desalting column (Bio-Rad) two times. Specific activities were in the range of 100 to 200 cpm/ng Tf, and more than 95% of ^{125}I was protein-bound as determined by precipitation with 20% TCA/4% PTA.

^{59}Fe labeling of Tf

Iron was removed from Tf and labeling with ^{59}Fe was carried out by previously described methods.³⁰ Specific activities were in the range of 2000-3500 cpm/ng Fe. In some experiments apotransferrin was labeled with ^{125}I as described above and then loaded with ^{59}Fe .

Binding assay

Tf binding assays were performed as previously reported.³¹ Cell numbers were calculated from protein concentrations determined with the Bio-Rad Protein assay kit. Each experiment was performed in triplicate. The total number of specific binding sites per cell and K_a for binding of Tf to these sites were estimated from non-linear least squares curve fitting to a

saturable binding isotherm:³²
$$\text{occupied sites} = \frac{\text{total sites} \times K_a \times [\text{Tf}]}{1 + K_a \times [\text{Tf}]}$$
,

from which it is also possible to calculate the fraction of sites occupied by Tf at any concentration of free Tf.

Tf and iron uptake

Cells were plated at a density of 1×10^6 cells per well in 35 mm 6-well plates 24 hours before experiments. Cells were preincubated with F-12 three times for 10 min at 37 °C, chilled on ice for 30 min, and then incubated at 37 °C with F-12 containing 2% BSA and labeled Tf at indicated concentrations and times. Cells were then washed with ice-cold PBS five times and solubilized by 0.1 % Triton X-100 for counting.

Acid-wash of cells

To remove cell-surface receptor-bound Tf, cells were washed with ice-cold PBS five times, and then incubated with ice-cold acid wash buffer (0.025 M citric acid, 0.025 M sodium citrate, 200 μ M DFO, 0.15 M sucrose, pH 4.0) for 3 min followed by two 2 min incubations with ice-cold F-12 medium to remove cell surface-bound apotransferrin. Finally, cells were washed once more with ice-cold PBS. This method removed approximately 90 % of surface-bound Tf in TRVb-TfR2 α cells.

Efflux of Tf from the cells

Cells, plated at a density of 1×10^6 cells per well in 35 mm 6-well plates 24 hours before

experiments, were preincubated with F-12 three times for 10 min at 37 °C, chilled on ice for 30 min, and then incubated with F-12 containing 2% BSA and 3.5×10^{-7} M 125 I-labeled Tf for 60 min at 37 °C. After acid-washing, fresh F-12 medium was added and cells were again incubated at 37 °C. At indicated times, F-12 media were collected, cells were washed with ice-cold PBS, and solubilized by 0.1 % Triton X-100. Media and cell lysates were then taken for γ -counting. After counting, media were incubated with 10 % TCA / 2% PTA for 30 min on ice, and then centrifuged at 14,000 rpm in an Eppendorf centrifuge for 20 min. Tf degradations were calculated from radioactivities of supernatants and precipitates.

Pulse-chase study

TRVb-TfR2 α cells were incubated with 5.1×10^{-7} M 125 I-Tf as previously described for 1 hour at 4 °C. Cells were then washed with ice-cold PBS five times. Fresh F-12 medium was added, and cells were incubated at 37 °C. At indicated times cells were chilled on ice and media immediately collected. Cells were again washed with ice-cold PBS and media collected. Cells were then acid washed to quantify surface-bound Tf, and were solubilized for measurement of intracellular Tf.

Immunoprecipitation-Western blotting

Human hepatoma-derived HuH-7 cells were transiently transfected with the TfR2 α expression vector as described above. At 48 hours after transfection, 5×10^6 cells were washed with PBS five times and collected with a cell scraper. Harvested cells were dissolved in 40 μ l of cell extraction buffer of Mammalian Cell Extraction Kit (BioVision Incorporated; Mountain View, CA) following manufacturer's instructions for Western blotting without immunoprecipitation, or in 1.5 ml of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM PMSF containing 1:2,000 Protease Inhibitor Cocktail

(Boehringer-Mannheim; Germany) pH 7.4) for immunoprecipitation-Western blotting. Freezing and thawing were performed 3 times, following which samples were centrifuged at 2,700 x g. Protein G Sepharose (Amersham Biosciences; Uppsala, Sweden) was added and preparations incubated at 4 °C for 8 hours, then centrifuged at 1,500 x g for 5 min. Supernatants were then collected for incubation with anti-FLAG M2 antibody (Sigma), which recognizes FLAG at any location in the target protein, or anti-TfR1 antibody (Zymed Laboratory; South San Francisco, CA) for 8 hours at 4 °C. Protein G Sepharose was added and incubation continued for an additional 8 hours at 4 °C. Samples were centrifuged at 1,500 x g for 5 min and pellets washed with PBS five times. Concentrated dye buffer was added and final concentrations were adjusted to 10mM Tris-HCl, 1mM EDTA, 2.5 % SDS, 5% β-mercaptoethanol and 0.01% bromphenol blue (pH 8.0). Samples were then immersed in boiling water for 5 min and centrifuged at 20,000 x g for 5 min to remove precipitated material. Electrophoresis using a 12 % gradient polyacrylamide gel and transfer to a nitrocellulose membrane was carried out. The transfer membrane was incubated with anti-TfR2 antibody (9F8 1C11) (Santa Cruz Biotechnology; Inc.; CA) diluted 1:200 or anti-TfR1 antibody diluted 1:1000, and then with horse-radish peroxidase-conjugated goat anti-mouse IgG antibody (R&D Systems; Minneapolis, MN) diluted 1:2,000. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific; Rockford, IL) was used as development substrate.

Atomic Force Microscopy (AFM)

The details of the methods for investigation using AFM were previously reported.¹⁵ In brief, the probe of the AFM is a sharp tip placed at the end of a soft cantilever. A piezoelectric scanner allows precisely positioning the tip relative to the sample. A laser beam reflected on the cantilever backside and detected by photodiodes is used to measure the cantilever deflection.

This signal is either used as a feedback to control the scanner (imaging mode) or measured and converted into a force (force spectroscopy mode). Since the cantilever is mounted under the scanner, the optical path is free and the AFM can be coupled to an optical microscope. Diferric Tf was linked to the AFM tip by a three step functionalizing protocol. First, SiN tips are aminosilanized by exposure to APTES vapors. Second, a heterobifunctional PEG linker is anchored to amino-group bearing tips through its NHS end. Third, diferric Tf is attached to the PEG linker free end via a maleimide-cysteine bond.

Measuring the interaction force between Tf and TfR2 α by AFM

HLF cells (human hepatoma) were cotransfected with TfR2 α and GFP for identification as previously described.³³ A diferric Tf coated tip was brought in contact with cell surface, allowing the proteins to bind. The tip is then retracted, resulting first in protein stretching then unbinding. The cantilever deflections during one cycle are recorded in a force curve. A binding- unbinding event between diferric Tf and TfR2 α is represented by a saw-tooth pattern on the curve. It allows calculating the force necessary to unbind the two proteins, using the cantilever spring constant (Hooke's law). The mean unbinding force is obtained by fitting a Gaussian curve to the force histogram. The error on the mean unbinding force is calculated by adding the standard deviation of the sample and the error resulting from the spring constant calibration (10%). Moreover, the unbinding force is related to the pulling rate by:

$$F^* = \frac{k_B T}{x} \ln\left(\frac{x}{k_0 k_B T}\right) + \frac{k_B T}{x} \ln(r_f)$$

(F*: the most probable unbinding force, k₀: dissociation rate constant, r_f: loading rate applied, x: width of the energy barrier along the direction of the applied force, k_B: Boltzmann's constant, T: temperature)

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Figure legends

Figure 1. Tf binding at 4 °C to TRVb (●), TRVb-TfR2 α (▲) and TRVb-mock cells (■). Cells were incubated with ^{125}I -Tf at 4 °C for 1 hour, and washed to remove unbound Tf, then solubilized for counting. TRVb-TfR2 α cells showed a saturable binding curve, with an association constant of $5.6 \times 10^6 \text{ M}^{-1}$ and 2.8×10^4 binding sites per cell. The experiment was performed in triplicate.

Figure 2. Iron uptake at 37 °C as a function of Tf concentration was determined (A). TRVb-TfR2 α cells (▲) and TRVb-mock cells (■) were incubated with ^{59}Fe -Tf at 37 °C for 1 hour, and washed to remove unbound Tf, then solubilized for counting. This experiment was performed in triplicate. Iron uptake at 37 °C with time was also investigated (B). TRVb-TfR2 α cells (▲) and TRVb-mock cells (■) were incubated with $8.1 \times 10^{-7} \text{ M}$ ^{59}Fe -Tf at 37 °C for the indicated time, and washed to remove unbound Tf, then solubilized for counting. This experiment was also performed in triplicate.

Figure 3. Time course of Tf efflux. TRVb-TfR2 α cells and TRVb-mock cells were incubated with $3.5 \times 10^{-7} \text{ M}$ ^{125}I -Tf at 37 °C for 1 hour, washed to remove unbound Tf, then acid-washed to remove Tf bound to cell surface receptors. Cells were then incubated at 37 °C for the indicated times, medium collected and solubilized cells taken for counting. Tf exocytosed into medium from TRVb-TfR2 α cells (●) and TRVb-mock cells (▲), and Tf retained in TRVb-TfR2 α cells (■) and TRVb-mock cells (▼), are shown. Experiments were performed in triplicate.

Figure 4. (A) Retraction force curves on living HLF cells, transfected with TfR2 α . The curves show specific unbinding events between diferric Tf on the probe tip and TfR2 α at the cell surface (in TBS, pH 7.4). (B) Specificity of detection by AFM. HLF cells (human hepatoma) were transiently cotransfected with TfR2 α and GFP and tested with a diferric Tf coated tip. The probability of binding was 26% (n = 7 cells). In contrast, on non-transfected cells, the probability was only 6% (n = 12 cells, *** t-test, p < 0.001).

Figure 5. Force histogram for specific interaction between diferric Tf and TfR2 α on HLF cells. The histogram was obtained from analysis of 573 unbinding events collected over 1500 force curves. The mean unbinding force is 63 ± 8 pN, for a mean loading rate of 2.8 nN/s. The gray line is a Gaussian fit.

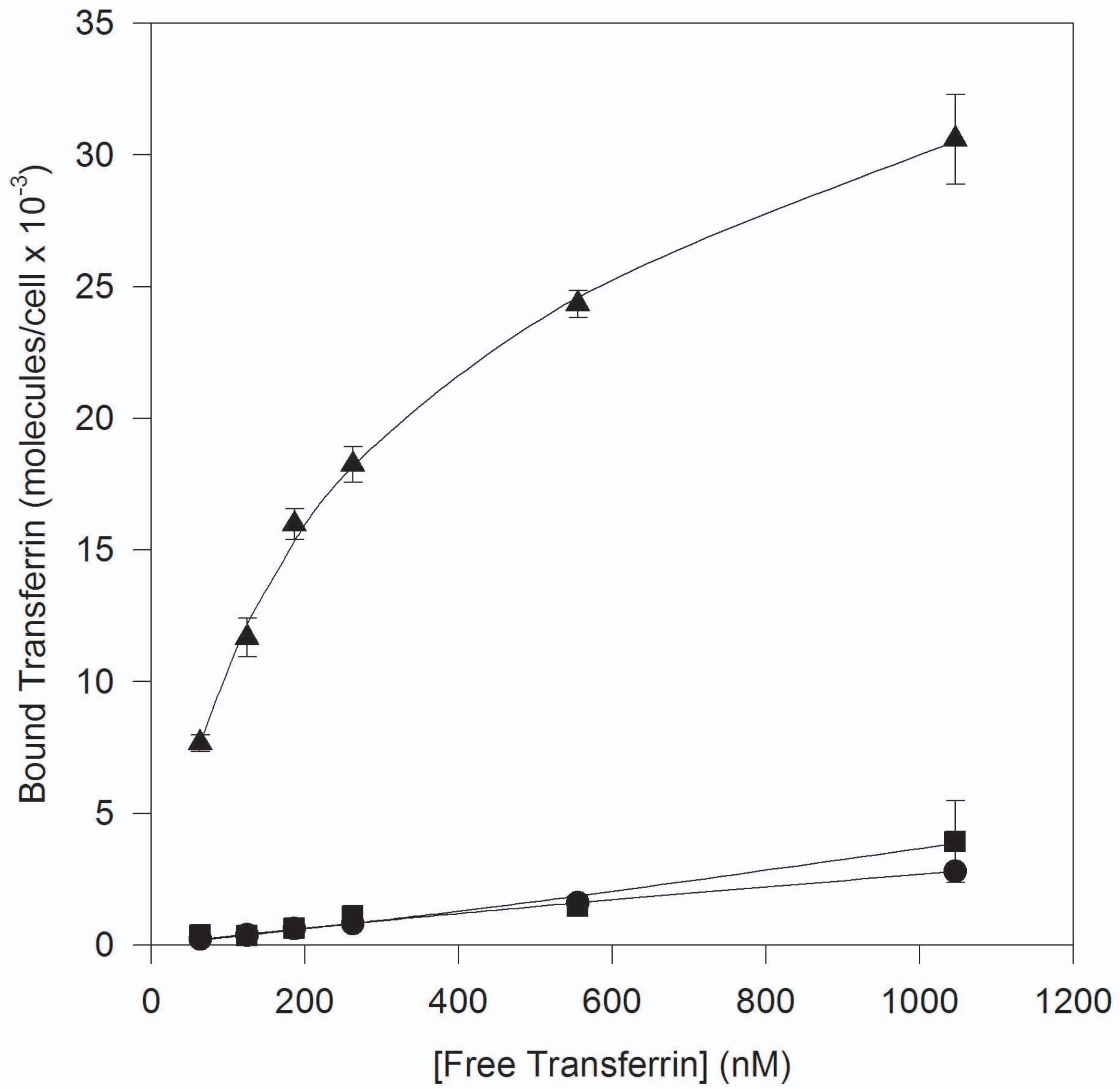
Figure 6. Dynamic force spectroscopy of diferric Tf–TfR2 α (black dots). The unbinding force is plotted as a function of the loading rate logarithm. For comparison, the force spectrum of diferric Tf–TfR1 is also shown. Only one regime is evident for TfR2 but two can be seen for TfR1, clearly revealing different interactions of the two proteins with Tf.

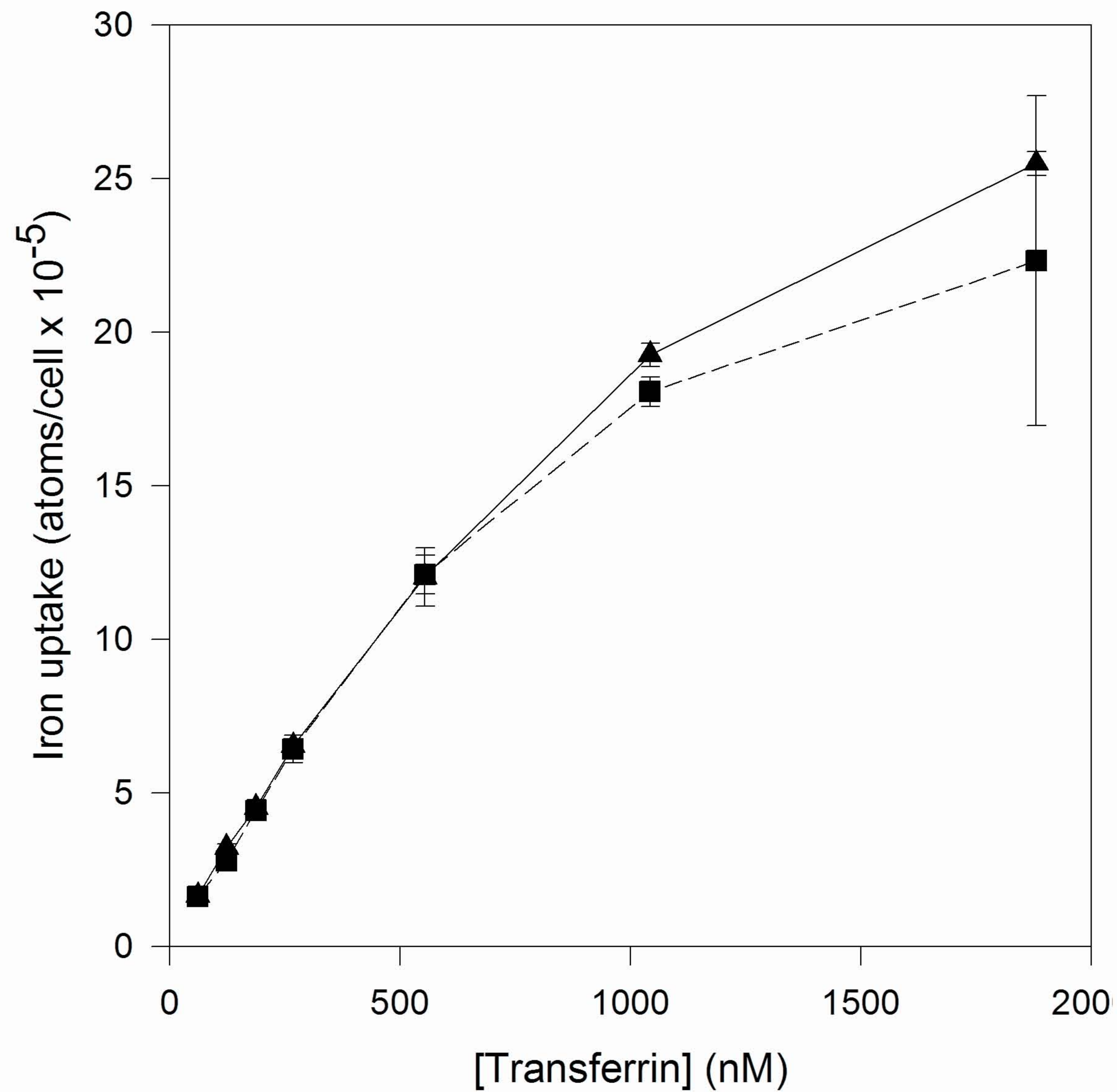
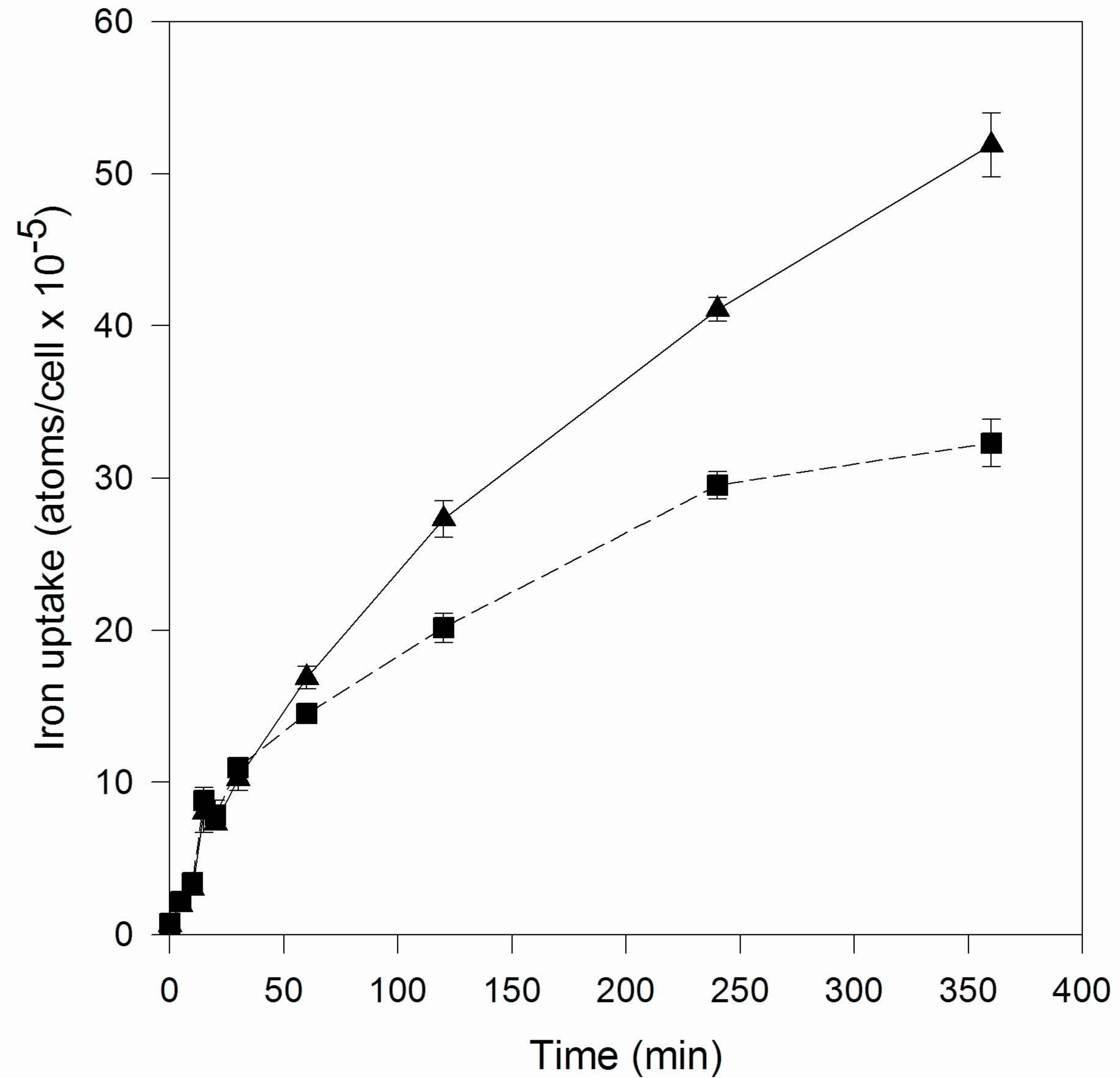
Supplementary data

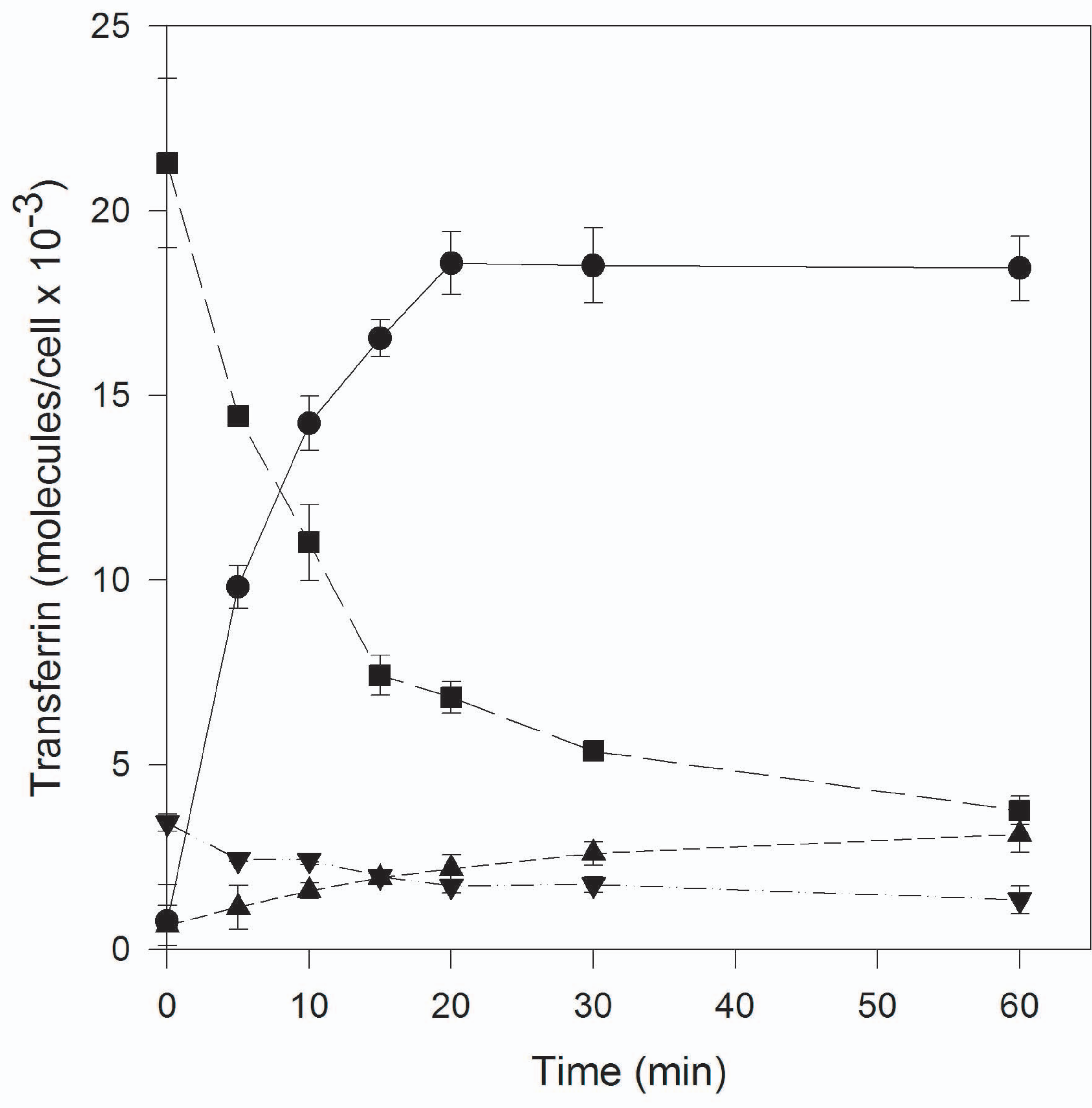
Supplementary data 1. Cell-associated Tf at 37 °C. TRVb-TfR2 α cells (\blacktriangle) and TRVb-mock cells (\blacksquare) were incubated with ^{125}I -Tf at 37 °C for 1 hour, and washed to remove unbound Tf, then solubilized for counting. This experiment was performed in triplicate. The differences between TRVb-TfR2 α and TRVb-mock cells are also shown (\blacktriangledown).

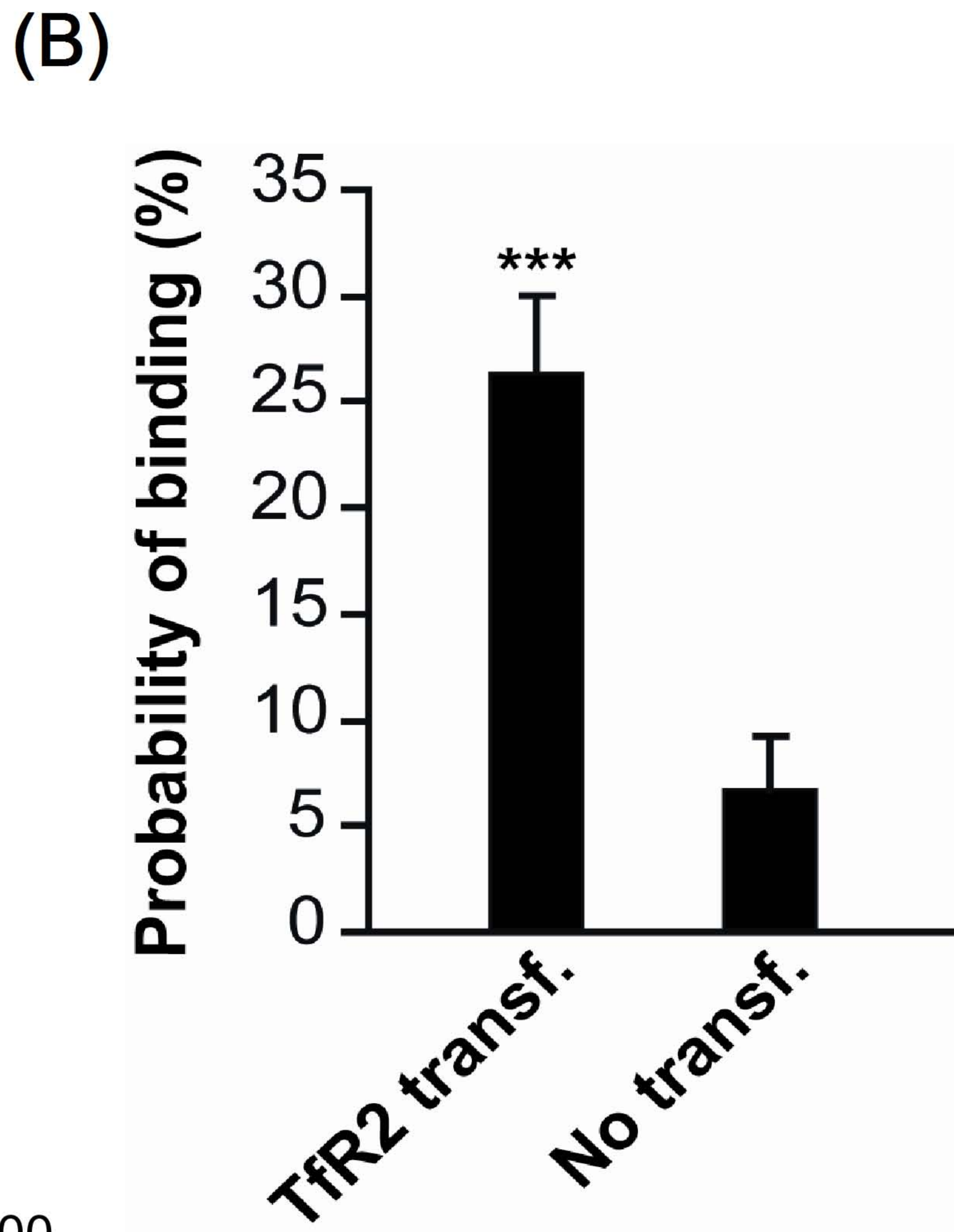
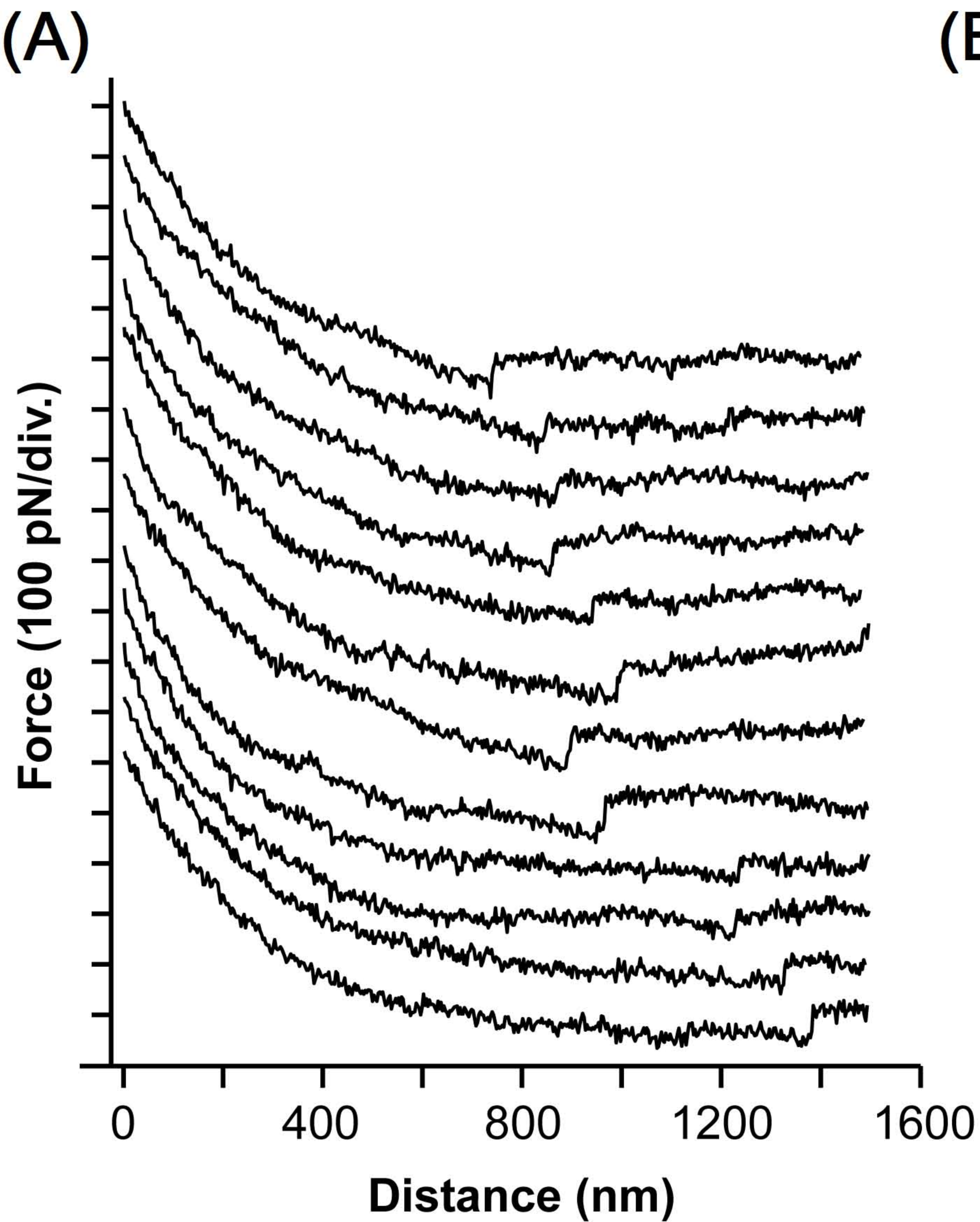
Supplementary data 2. Pulse-chase study of the TfR2 α cycle. TRVb-TfR2 α cells were incubated with 5.1×10^{-7} M ^{125}I -Tf at 4 °C for 1 hour, and washed to remove unbound Tf. Cells were then incubated at 37 °C for indicated time. Medium (\blacktriangle), cell-surface (\bullet), intracellular (\blacksquare) fractions were separately collected and counted for radioactivities. Percentages of each fraction are shown. Experiments were performed in triplicate.

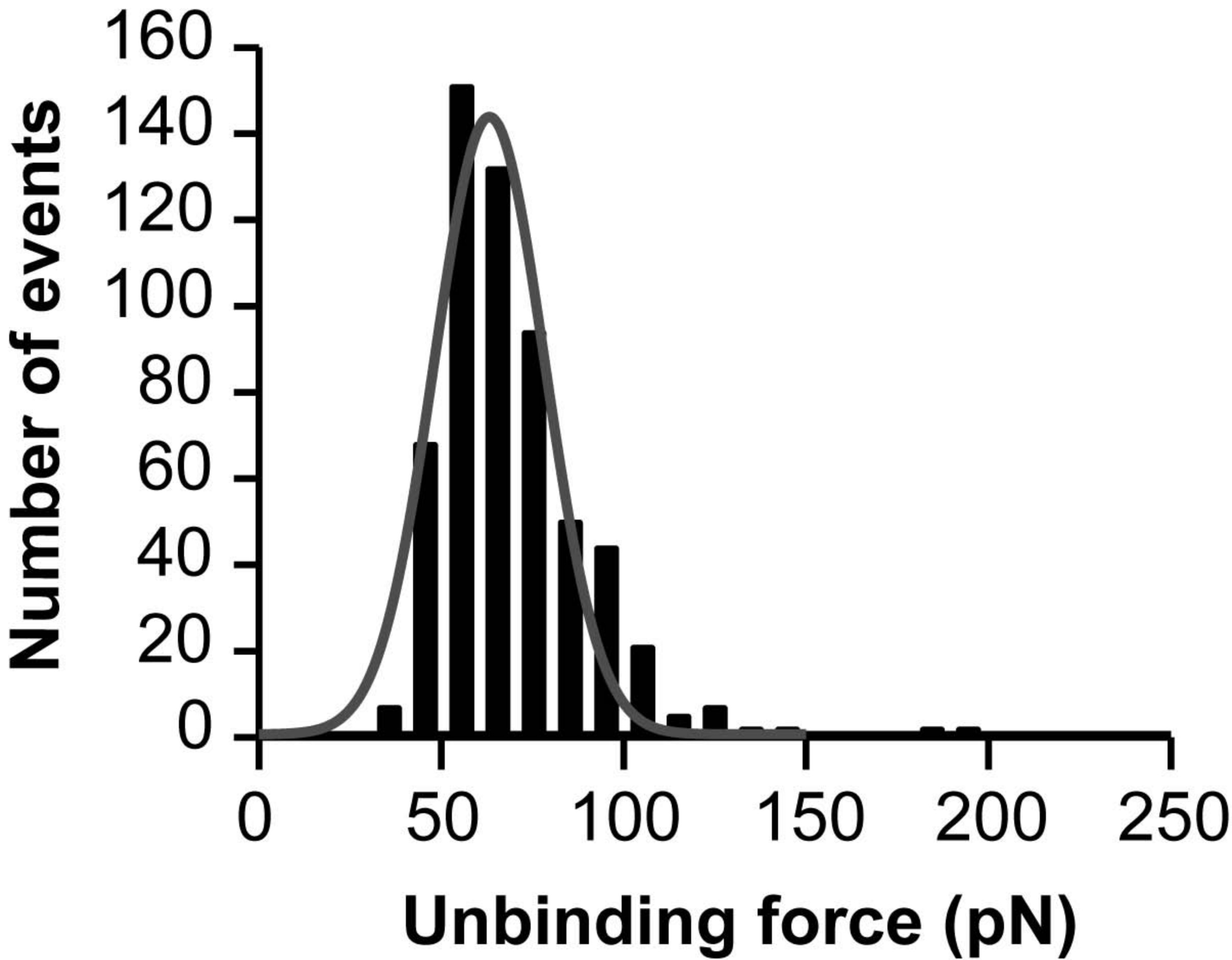
Supplementary data 3. Immunoprecipitation-Western blotting studies. HuH-7 wild-type cells and HuH-7 cells transiently transfected with TfR2 α expression vector were lysed and immunoprecipitated, then applied to Western blotting.

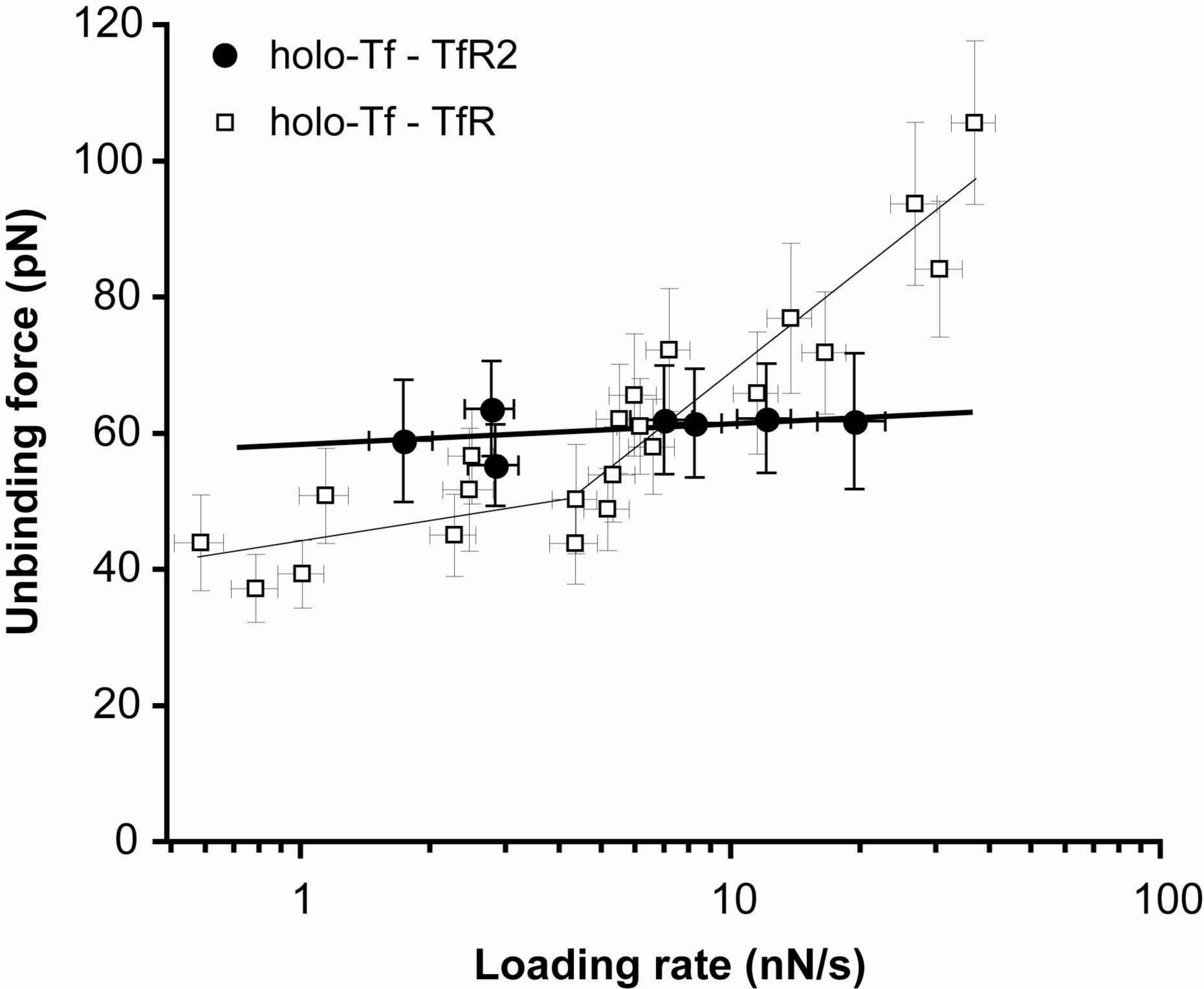


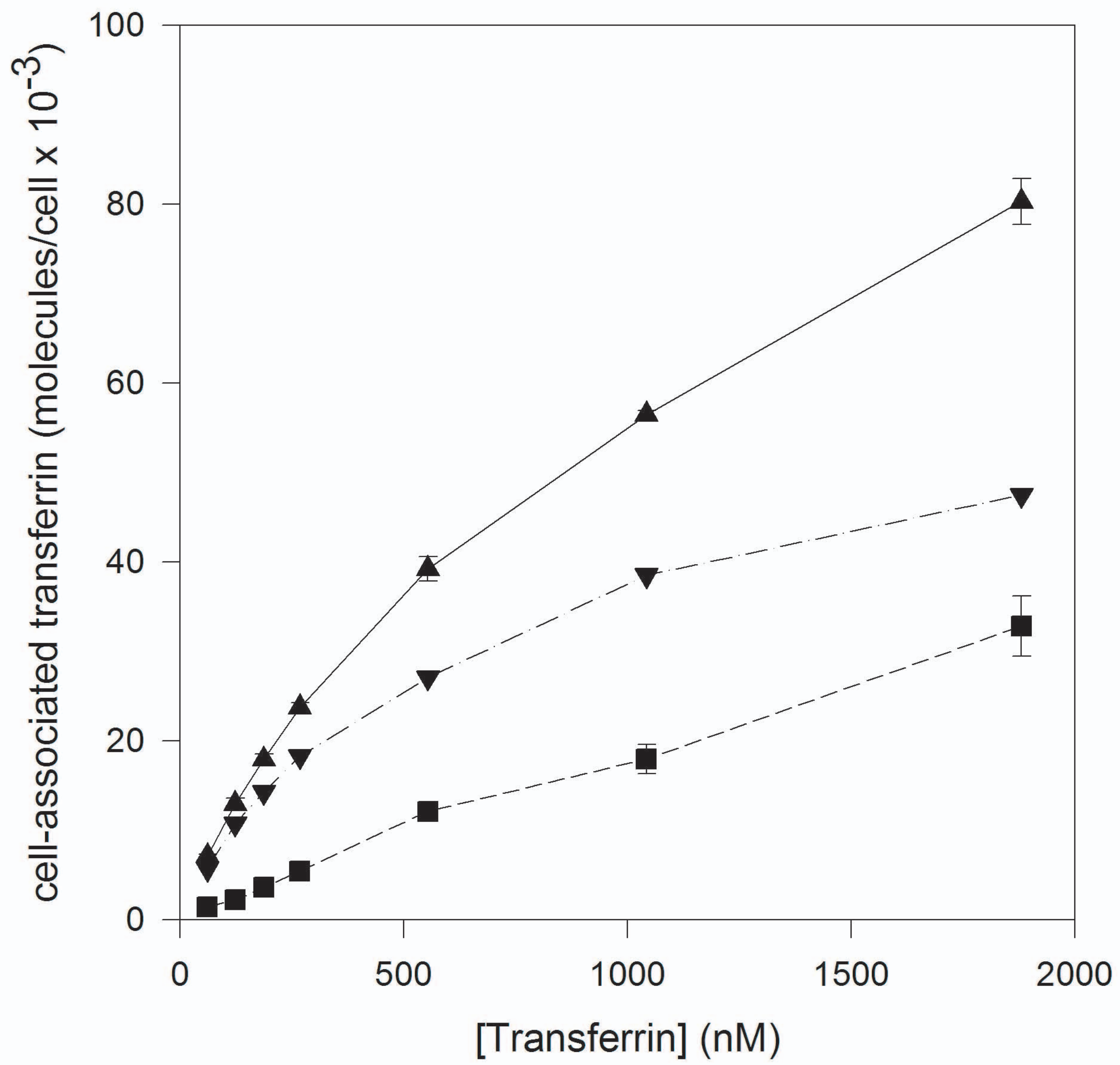
(A)**(B)**

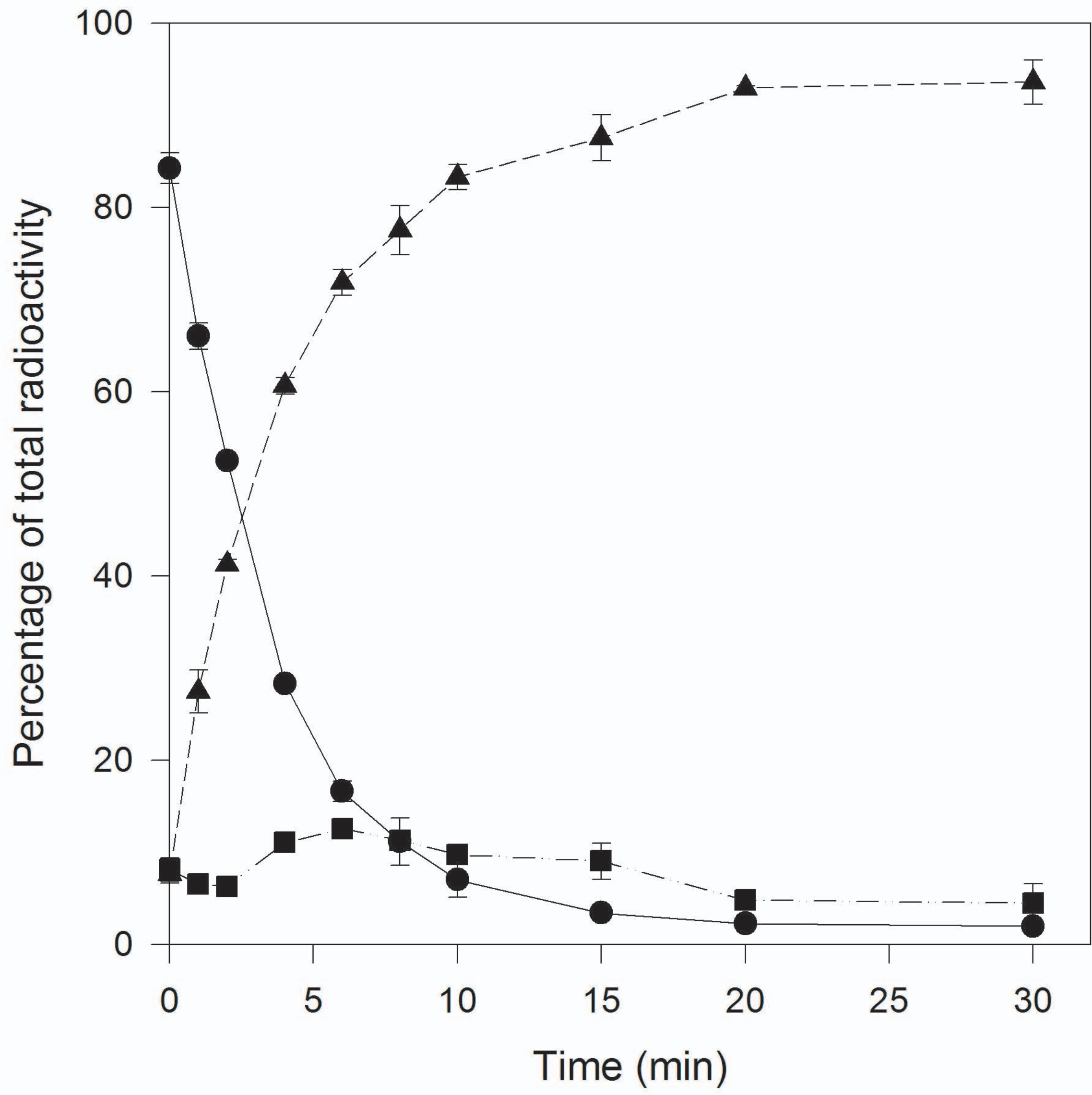






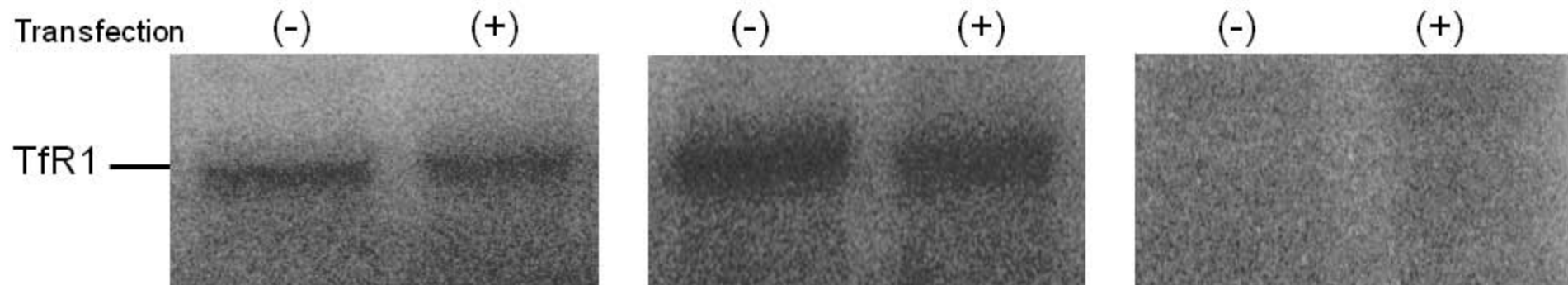






(A)

IP (-) (by anti-TfR1-Ab) (by anti-FLAG-Ab)

**(B)**

IP (-) (by anti-TfR1-Ab) (by anti-FLAG-Ab)

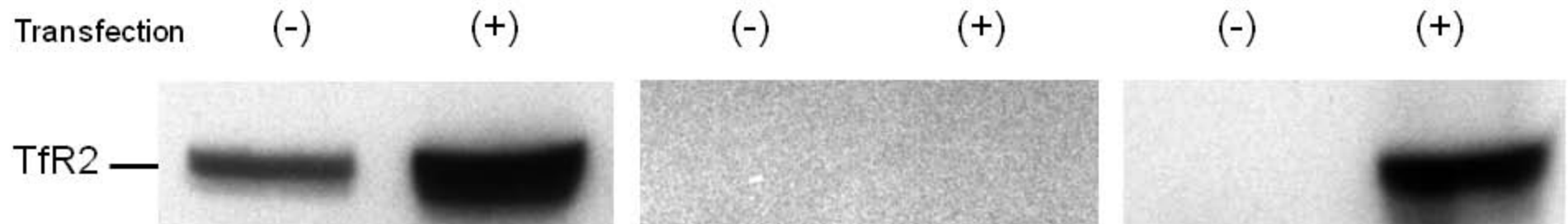


Table 1: Tf Internalized by TRVb-TfR2α Cells via TfR2α

<u>Procedure</u>	<u>Molecules/cell</u>
[1] Total Tf associated with TRVb-TfR2α cells after incubation for 1h at 37 ° C	47,800
[2] Cell-associated Tf after acid washing	26,500
[3] Tf removed by acid washing	21,300
[4] Tf initially bound to TfR2α at cell surface ($10/9 \times [3]$)	23,700
[5] Tf initially bound to TfR2α at cell surface but resistant to acid washing	2,370
[6] Tf internalized by all pathways ([2] – [5]) plus nonspecifically-bound resistant to acid washing	24,100
[7] Tf in mock cells (non-specifically bound and resistant to acid wash)	6,600
[8] Tf internalized via TfR2α in TRVb-TfR2α cells ([6] – [7])	17,500