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Human intrinsic factor expressed in the plant *Arabidopsis thaliana*

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Intrinsic factor (IF) is the gastric protein that promotes the intestinal uptake of vitamin B_{12}. Gastric IF from animal sources is used in diagnostic tests and in vitamin pills. However, administration of animal IF to humans becomes disadvantageous because of possible pathogenic transmission and contamination by other B_{12} binders. We tested the use of recombinant plants for large-scale production of pathogen-free human recombinant IF. Human IF was successfully expressed in the recombinant plant *Arabidopsis thaliana*. Extract from fresh plants possessed high B_{12}-binding capacity corresponding to 70 mg IF per 1 kg wet weight. The dried plants still retained 60% of the IF activity. The purified IF preparation consisted of a 50-kDa glycosylated protein with the N-terminal sequence of mature IF. Approximately one-third of the protein was cleaved at the internal site …PSNP↓GPGP. The key properties of the preparation obtained were identical to those of native IF: the binding curves of vitamin B_{12} to recombinant IF and gastric IF were the same, as were those for a B_{12} analogue cobinamide, which binds to IF with low affinity. The absorbance spectra of the vitamin bound to recombinant IF and gastric IF were alike, as was the interaction of recombinant and native IF with the specific receptor cubilin. The data presented show that recombinant plants have a great potential as a large-scale source of human IF for analytical and therapeutic purposes.

Keywords: *arabidopsis*; cobalamin; intrinsic factor; recombinant.

Vitamin B_{12} (cobalamin, Cbl) is the most complex of the vitamins [1]; it is a complicated system with three transporting proteins and several receptors which together ensure its efficient uptake [2–4]. Intrinsic factor (IF) is responsible for intestinal absorption of vitamin B_{12} facilitating its internalization [2,3,5]. Lack or malfunction of this Cbl binder hampers the uptake of the minute amounts of the vitamin present in food. Only around 1% of the ingested Cbl can be absorbed by passive diffusion [6].

Classical vitamin B_{12} deficiency has been known as pernicious anaemia for a long time [5,7]. The disease is caused by lack of IF and without treatment by injections of 1 mg of the vitamin at regular intervals this condition is lethal [8]. The major disadvantages with such treatment are the time consuming procedure [8] and the relatively high expense [9]. Alternatively, a daily dose of 0.5–2 mg (corresponding to a more than 100-fold excess above the usual requirement) can be given orally [6,9,10], but in this case most of the vitamin is not internalized. High amounts of unabsorbed vitamin B_{12} might present a potential danger for normal growth of intestinal microorganisms and be disadvantageous for the environment. Therefore, the optimal treatment is likely to be ingestion of a normal daily dose of vitamin B_{12} (2–4 μg) complexed to IF, which makes the uptake of Cbl close to natural. However, it is important to mention that on certain occasions oral administration of IF-Cbl will not be beneficial. This concerns those autoimmune cases of pernicious anaemia in which anti-IF antibodies are the reason for Cbl malabsorption [5].

Certain steps are taken to imitate the natural process of Cbl assimilation: porcine IF is added to vitamin supplements by some pharmaceutical companies. However, use of animal proteins in connection with medication becomes more and more problematic. First, the quality of organs obtained from slaughterhouses is quite variable. Second, the products may not be free of pathogens (known at the moment or detected in the future). Third, Muslims may object to treatment with IF of porcine origin for religious reasons.

In recent publications the expression of human IF in recombinant organisms (COS cells, yeast) has been described [11–13], but the amounts obtained and possible price of the protein can by no means fulfil the potential public demand. For instance, in the group of people aged 60 years or more, up to 15% have low levels of serum vitamin B_{12} [14,15]. The syndrome in the elderly population is caused mainly by general gastric malfunction accompanied, beside other symptoms, by low secretion of IF and insufficient

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Abbreviations: Cbl, cobalamin; $CblOH_2$, aquo-cobalamin; Cbi, cobinamide; IF, intrinsic factor; apo-IF, ligand free IF; holo-IF, IF saturated with a ligand; PAS, periodic acid Shiff reagent.

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adsorption of vitamin B_{12} [15]. In addition to anaemia, lack of vitamin B_{12} causes severe neurological symptoms similar to those seen in senile dementia and Alzheimer's disease [14]. As damage to the nervous system caused by vitamin B_{12} deficiency is irreversible, it is of vital importance to discover and treat negative balance at an early stage [14].

Measurement of Cbl in serum, which is widely used for determination of Cbl balance, depends on availability of a suitable IF preparation. The same is true of the Schilling test, which verifies whether vitamin B_{12} deficiency is caused by lack of IF. Alternative techniques for determination of Cbl status in the organism are under debate [16–18], and some of those also incorporate IF as one of the kit reagents.

For the reasons mentioned above it is important to find an effective and pathogen-free source of IF; this would permit the relevant laboratory tests to be performed and eventually optimize the oral treatment of vitamin B_{12} deficiency. We report expression of human IF in the plant *Arabidopsis thaliana* and show that the protein has the key features of native IF. We conclude that recombinant plants may prove to be an excellent source of IF for analytical application and, possibly, for therapeutic development.

Materials and methods

Preparation of the genetic material

A cDNA for human IF was prepared by reverse transcriptase/PCR using human stomach RNA and primers encoding the 5′-region of mature human IF and the 3′-untranslated region. This sequence corresponds to a sequence in GenBank accession no. X76562 and encodes a protein of 399 amino acid residues starting with STQTQSS… and ending with …ANFTQY. Another DNA fragment was synthesized by DNA Technology, Denmark, encoding an extensin-like signal peptide (Ext) with the amino acid sequence MASSSIALFLALNL LFFTTISA and 47 nucleotides from the 5′-untranslated region. This sequence is part of the plant *A. thaliana* cDNA sequence in GenBank accession no. AF104327. These two DNA fragments were fused whereupon the restriction nuclease recognition sequences *Xba*I and *Xma*I were added to enable cloning of the chimeric cDNA into the plant transformation vector CRC-179. CRC-179 was derived from the lbc3-GUS vector [19] by removal of a DNA fragment containing the Gmlbc3 promoter, the gusA gene, and the pAnos termination sequence by digestion with *Hin*dIII; the digestion was followed by self-ligation of the remaining vector to form CRC-179. The Ext/IF DNA fragment and CRC-179 plasmid were mixed and digested with *Xba*I and *Xma*I, purified by phenol/chloroform extraction and ligated with T4-DNA ligase (Roche, Denmark). *E. coli* XL-1 cells were transformed by electroporation with the ligated DNA and selected by growth on low-salt medium containing spectinomycin. Plasmid DNA was produced from one selected colony and used for electroporation of *Agrobacterium tumefaciens*. The Ext/IF insert in *A. tumefaciens* was isolated by PCR and sequenced on both strands by use of specific primers and a DNA Sequencing Kit from Applied Biosystems to check for mutations before transformation of plants.

Culture of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain GV3101(pMP90) carrying the binary plasmid with an insert of human IF cDNA was used for the plant transformation [20]. The bacteria were grown to stationary phase in 200 mL liquid culture at 28–30 °C, 250 r.p.m. in sterilized Luria–Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per L H_2O) carrying added rifampicin (100 $\mu g \cdot mL^{-1}$), gentamycin (50 $\mu g \cdot mL^{-1}$), and streptomycin (100 $\mu g \cdot mL^{-1}$) for the pPZP vector. Cultures were started from a 1 : 200 dilution of a 5-mL overnight culture and grown for 16–18 h. Bacteria were harvested by centrifugation for 10 min at 5500 ***g*** at room temperature and then resuspended in 400 mL inoculation medium [10 mM $MgCl_2$, 5% w/v sucrose and 0.05% v/v Silwet L-77 (Lehle Seeds, Round Rock, TX, USA)].

Plant growth

A. thaliana plants (ecotype Col-0) were grown to flowering stage in growth chamber, 22 °C day/18 °C night with metal halide lighting (175 $\mu Einsteins \cdot m^{-2} \cdot s^{-1}$) for 16 h per day, humidity 70%. Between 20 and 25 plants were planted per 64 cm^2 pot in moistened soil mixture: 40 kg soil orange and 40 kg soil green (Stenrøgel Mosebrug A/S Kjellerup, Denmark), 25 L 4–8 mm Fibroklinker (Optiroc, Randers, DK), 12 L Vermiculite (Skamol, DK), and 300 g Osmocote plus NPK 15-5-11 (Scott's, UK).

To obtain more floral buds per plant, inflorescences were removed after most plants had formed primary bolts, relieving apical dominance and encouraging synchronized emergence of multiple secondary bolts. Plants were transformed by *Agrobacterium tumefaciens* when most secondary inflorescences were ≈ 7–13 cm tall.

Transformation of plants

A. thaliana plants were transformed by the floral dip method [21]. The suspension of recombinant *Agrobacterium tumefaciens* was added to a 400-mL beaker and plants were dipped into the suspension such that all above-ground tissues minus the rosette were submerged. After 10–15 s of gentle agitation in the suspension the plants were moved to a sealed plastic bag and incubated in a horizontal position for 24 h at room temperature and normal daylight. The plants were then moved to the growth camber and the plastic bag was removed. Here the plants were grown for 3–4 weeks until siliques were brown and dry. Seeds were harvested and allowed to dry at room temperature for 7 days.

Selection of transformants

Seeds were surface sterilized by a treatment with 0.5% sodium hypochlorite containing 0.05% v/v Tween-20 for 7 min followed by submergence in 70% ethanol for 2 min, and then three rinses with sterile water.

To select for transformed plants the sterilized seeds were plated on kanamycin selection plates at a density of ≈ 2000

seeds per 144 cm^2 and grown for 8–10 days at 21 °C under light for 16 h per day. Selection plates contained 1 × MS medium (Duchefa, Haarlem, NL #M 0222), 1% (w/v) sucrose, 0.9% (w/v) agar noble (Difco), 50 $\mu g \cdot mL^{-1}$ kanamycin, 50 $\mu g \cdot mL^{-1}$ ampicillin, pH 5.7. After selection the transformed plants were transferred to soil mixture and grown in climate chambers (see Plant growth). Seeds were selected through five generations of growth on selective medium. Seeds from the last generation were used for production of IF.

Preparation of the affinity matrix for IF purification

$CblOH_2$ was coupled to an insoluble matrix containing amino-groups using a modified version of the method described first by Nexø [22]. AEH Sepharose 4B was equilibrated with 2 mM $CblOH_2$ in 0.2 M NaH_2PO_4, pH 7.5 and incubated at 65 °C for 1 h with periodical shaking. Then, the suspension was placed on ice for 1 h, which stabilizes the thermo-labile bond between the cobalt atom of Cbl and the amino group. At that point the matrix can be either used for application or stored in a refrigerator. Before adsorption of Cbl-binding proteins on Sepharose–Cbl, the matrix was extensively washed from excess of free Cbl with cold 0.2 M NaH_2PO_4 pH 7.5. The approximate concentration of Cbl in packed Sepharose was 0.5 mM as judged by visual comparison with the standard solutions. Application of the adsorbent is described below.

Purification of IF from plants

The recombinant plants were harvested after 4 weeks and either used immediately or stored frozen at −80 °C. The raw material (500 g) was milled on ice by a blender to a fine powder. Cold phosphate buffer (1 L 0.2 M NaH_2PO_4, pH 7.5) was added and the mixture homogenized. The suspension was left for 1 h at 5 °C, then filtered through two layers of fabric and centrifuged (3000 ***g***, 20 min, 5 °C). The supernatant was filtered through Watman paper (3 mm Chr) on a Buhner funnel and kept frozen at −20 °C until use. The thawed extract from plants was centrifuged (15 000 ***g***, 10 min, 5 °C) and filtered through Watman paper. The solution obtained (1.2 L) was applied to the affinity column (5 mL) with immobilized Cbl, and adsorption of IF was carried out at 5 °C and a flow rate of 5 $mL \cdot min^{-1}$. The matrix was washed with 100 mL cold buffer with high ionic strength (0.1 M Tris, 1 M NaCl pH 7.5). The material was then equilibrated with the elution buffer (0.2 M NaH_2PO_4 pH 7.5) and left at 37 °C overnight. Increased temperature caused detachment of IF–$CblOH_2$ (as well as of some amount of free $CblOH_2$) from the matrix. The IF–$CblOH_2$ complex was separated from the free ligand by dialysis against the elution buffer at 5 °C overnight. The protein sample obtained (15 mL) was subjected to gel filtration on a Sephacryl S-200 column (290 mL) equilibrated with 0.1 M Tris, 1 M NaCl pH 7.5. The gel filtration was conducted at room temperature and the flow of 10 $mL \cdot h^{-1}$. The fractions with red protein were pooled and concentrated to 8 mL by ultrafiltration on an Amicon membrane (pores with the cut off molecular mass of 10 000). The protein was stored frozen at −20 °C.

Small-scale extraction of IF

One or two leaves were ground with a pestle in 1 mL of a cold phosphate buffer (0.2 M, pH 7.5) in a mortar. The sample was centrifuged (10 000 ***g***, 5 min) to remove debris and stored at −20 °C until measurement of Cbl binding capacity.

Purification of IF from gastric juice and recombinant yeast

Purification of the natural human IF, porcine IF and the recombinant human IF from yeast was performed as described elsewhere [22,13]. Both proteins were obtained as holo-forms, i.e., in complex with $CblOH_2$.

Preparation of apo-IF

The isolated holo-IF was subjected to exhaustive dialysis against 5 M guanidinium chloride (30 °C, for 3 days with three changes of the solution). Removal of Cbl from the sample was monitored visually by disappearance of red colour. The Cbl-binding capacity of the protein was restored by an overnight dialysis at 5 °C against the renaturing buffer (0.1 M Tris, 2 M NaCl pH 7.5) followed by 0.2 M NaH_2PO_4 pH 7.5.

Measurement of Cbl binding capacity and relative affinity of Cbl and Cbi to IF

The binding capacity was measured by using ^{57}Co-cobalamin (Cbl*) [23]. Binding of Cbl and its analogue Cbi to apo-IF was carried out as described elsewhere [23]. In short, the radioactive ligand Cbl*, mixed with increasing concentrations of 'cold' Cbl or Cbi, was added to IF and excess of the ligand was removed by charcoal precipitation. The amount of IF-associated radioactivity is expected to be reversely proportional to the concentration of the unlabeled ligand, if it is capable of IF binding.

Electrophoretic assay

SDS/PAGE, gel staining by Coomassie Brilliant Blue, staining of carbohydrates by periodic acid Shiff (PAS) reagent, Western blotting and reactions with antibodies were performed according to the standard procedures. The polyclonal antibodies used for Western blotting were raised in rabbits against native human IF.

Binding of IF to cubilin

Specific binding of IF–Cbl complex to the immobilized receptor cubilin was conducted on a BIAcore 2000 equipment as described earlier [24]. In short, recombinant cubilin was coupled to the surface of a sensor chip activated by carbodiimide. Binding of IF–Cbl to cubilin was registered by plasmon resonance signals from the chip surface when the reaction cell was washed with a flow of IF–Cbl over the concentration range 10–50 nM. Dissociation from the receptor was induced by exclusion of IF–Cbl from the buffer.

Results and discussion

Comparison of the extraction methods

Extraction of IF from the homogenized fresh plants yielded the best results when a neutral buffer with ionic strength of 0.2–0.5 M was used. Thus, the amount of binding capacity extracted by 0.2 M NaH_2PO_4 pH 7.5 corresponded to 70 mg of active IF per 1 kg plant wet weight. An analogous procedure with water or citrate buffer pH 4.5 ensured liberation of approximately 50 mg IF per 1 kg wet plant material. Freezing and storage of the plant material at −80 °C prior to extraction did not influence the results. When plants were dried at 37 °C overnight and stored at room temperature from 1 day to 1 year the amount of extracted active IF decreased to 40 mg and 30 mg, respectively (calculated per 1 kg of wet weight or 150 g of dry weight).

Purification of recombinant IF from plants

The purification procedure included the following major steps: homogenization, removal of debris, adsorption on affinity matrix and gel filtration (see Materials and methods). The IF elution peak (Fig. 1) practically coincided with that of BSA (67 kDa). The fractions with red protein obtained after gel filtration were pooled and analyzed by SDS/PAGE (Fig. 1 inset). The major band of 50 kDa stained by Coomassie (lane 2) had the N-terminal sequence of mature human IF (STQTQSS…). Two bands of smaller size (30 and 20 kDa) corresponded to the fragments: (1)STQTQSS… and (285)GPGPTSA… Staining with PAS reagent (lane 4) revealed presence of carbohydrates both on the full IF molecule (50 kDa) and on the smaller C-terminal fragment (20 kDa), the size of which would have been only 12.8 kDa if only the peptide core had been counted. Lane 5 shows PAS staining of recombinant human IF from yeast, which revealed only one band on electrophoresis. The analysis conducted demonstrates that IF isolated from the recombinant plants contains two kinds of the protein molecules: IF_{50} (two-thirds) and IF_{30+20} (one-third). Both of them can bind Cbl as follows from the spectral analysis at 280 nm and 356 nm (see Absorbance spectroscopy, below).

Comparison between recombinant plants and yeast shows similar levels of IF production: 70 mg and 40 mg per 1 kg of wet weight, respectively. The production expenses calculated per 1 kg of biomass were significantly lower for the plant source. In addition, the purification technique for IF from plants was simpler due to expression of the protein in the unsaturated apo-form in contrast with the B_{12} saturated holo-IF from yeast [13].

Absorbance spectroscopy

The absorbance spectrum (Fig. 2) recorded for recombinant human IF from plants (saturated with $CblOH_2$ at pH 7.5) was quite typical for a Cbl binder [25]. All IF molecules appear to be saturated with $CblOH_2$. Thus, the theoretically calculated extinction coefficient of IF–$CblOH_2$ in the UV-part of the spectrum was $\varepsilon_{280} = 59\,400 \cdot M^{-1} \cdot cm^{-1}$ according to $\varepsilon^{IF}{}_{280} = 40\,300 \cdot M^{-1} \cdot cm^{-1}$ of the protein moiety [26] plus overlapping absorbance of $CblOH_2$ $\varepsilon^{Cbl}{}_{280} = 19\,100 \cdot M^{-1} \cdot cm^{-1}$ at pH 7.5 (IF:Cbl = 1 : 1). If we conjecture that, for example, 30% of IF in the preparation is incapable of Cbl binding, then the apparent extinction will be equal to $1.3\varepsilon^{IF}{}_{280} + \varepsilon^{Cbl}{}_{280} = 71\,500 \cdot M^{-1} \cdot cm^{-1}$ when calculated per mole of Cbl. At the same time, relation of A_{280} to the molar concentration of Cbl in the sample [25] gave the value of $\varepsilon_{280} = 61\,900 \cdot M^{-1} \cdot cm^{-1}$, which was quite close to the theoretically predicted coefficient. In other words, all molecules of the purified protein contained bound Cbl. Other extinction coefficients of recombinant IF from plants (Fig. 2) were practically identical to those of recombinant IF from yeast [13] and gastric human IF [25].

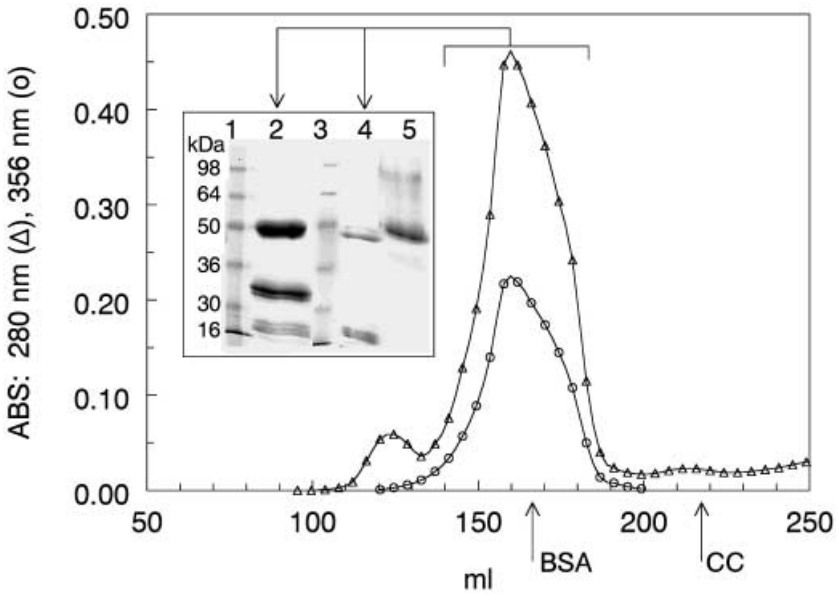

Fig. 1. Gel filtration of recombinant human IF on Sephacryl S-200. A preparation of IF (10 mg, 15 mL) was subjected to gel filtration on a Sephacryl S-200 column (290 mL) run with a flow of 12 $mL \cdot h^{-1}$. Fractions of 4.2 mL were collected. Elution volumes of (67 kDa) and cytochrome *c* (CC, 12 kDa) are marked with arrows. Inset: SDS/PAGE of the isolated preparation. Coomassie stained lanes: 1, standards; 2, recombinant IF from plants. PAS stained lanes: 3, standards; 4, recombinant IF from plants; 5, recombinant IF from yeast.

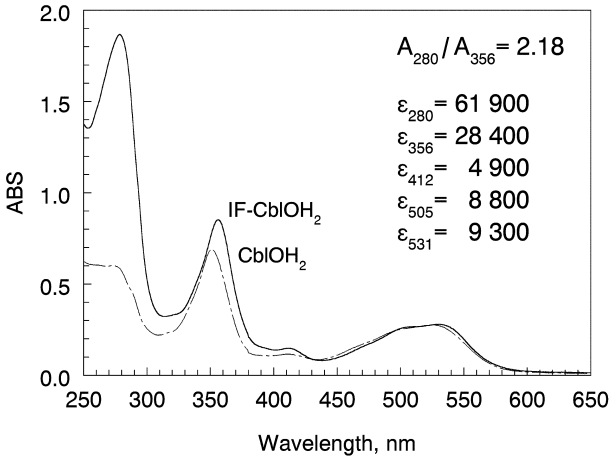

Fig. 2. Absorbance spectrum of recombinant holo-IF from plants. The spectrum of IF–$CblOH_2$ complex (solid line) was recorded with 0.5 nm steps in 0.1 M Tris, 1 M NaCl pH 7.5. The extinction coefficients of IF–$CblOH_2$ were determined as described elsewhere [25]. The depicted spectrum corresponds to 30 μM of the protein–ligand complex. The spectrum of the free ligand 30 μM (dash-dotted line) is given for a comparison.

Binding of Cbl and Cbi to plant IF

When the radioactive ligand Cbl* was subjected to competition with the 'cold' ligands (Cbl or Cbi) added at increasing concentrations, only Cbl efficiently substituted for Cbl* (Fig. 3). The incomplete corrinoid Cbi appeared to be a poor substrate with point of half-saturation shifted to a 10^5-fold higher concentrations in comparison with Cbl. This result does not differ from the data obtained for gastric human IF, see Fig. 3, dashed lines, and [23].

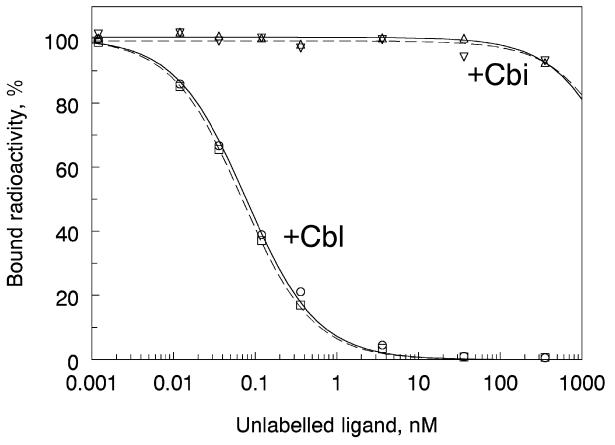

Fig. 3. Binding of Cbl and Cbi to recombinant and gastric IFs. Radioactive ligand Cbl* was prevented from binding to IF by increasing concentrations of nonradioactive substrates (either Cbl or Cbi) added to Cbl* prior to mixing with the binder. Solid and dashed lines correspond to recombinant IF from plants and human gastric IF, respectively. Points of half saturation correspond to $S^{Cbl}{}_{0.5} = Cbl^*{}_{free0.5} + 0.5 \cdot IF_{total} \approx IF_{total}$ for Cbl; and $S^{Cbi}{}_{0.5} = (K_{Cbi}/K_{Cbl}^*) \cdot Cbl^*{}_{free0.5} + 0.5 \cdot IF_{total} \approx 0.5 \cdot IF_{total} \cdot K_{Cbi}/K_{Cbl}^*$) for Cbi assuming $K_{Cbl} \ll IF_{total}$ and $IF_{total} \approx Cbl^*{}_{total}$. The ratio K_{Cbi}/K_{Cbl}^* can be estimated as 10^6 from $S^{Cbi}{}_{0.5}/S^{Cbl}{}_{0.5} \approx 5 \cdot 10^5$.

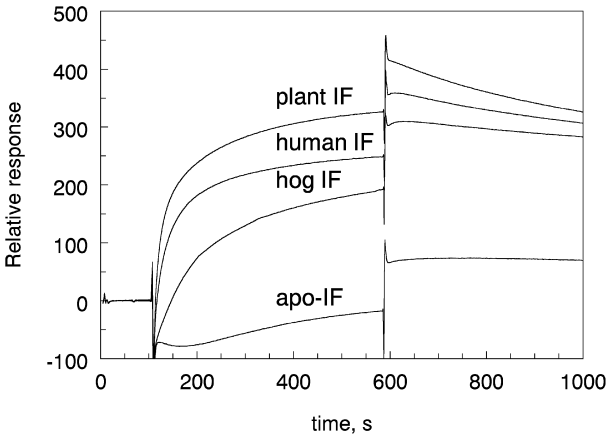

Fig. 4. Association and dissociation of IF and cubilin. IFs from different sources at concentration 50 nM were exposed to the specific receptor cubilin immobilized on the surface of a registration chip. The relative response was measured on BIAcore equipment. The lower curve was recorded for recombinant human apo-IF from plants and it represents nonspecific adsorption. The curves for human and porcine apo-IFs were of similar shape and are not shown. The records for apo-IF were subtracted from holo-IF curves before the fit to an exponent: $y = a1 + a2*exp(-a3*x)$. The value of $a3$ is equal to $k_+[IF] + k_-$ (increasing curves) or k_- (decreasing curves), see Table 1.

Table 1. Rate constants of interaction between IF and cubilin.

Source of IF	k_+ ($nM^{-1} \cdot s^{-1}$)	k_- (s^{-1})	K_d (nM)
Plant	6.0×10^{-4}	7.5×10^{-4}	1.2
Human	7.1×10^{-4}	5.3×10^{-4}	0.75
Porcine	2.5×10^{-4}	3.3×10^{-4}	1.3

Binding of IF to the specific receptor cubilin

When IF–Cbl complexes from different sources were exposed to the IF-specific receptor cubilin immobilized on a detector chip [4], all proteins showed rapid binding to the surface of the chip (Fig. 4). The apo-form of IF is known to be almost incapable of this binding, which was also demonstrated on the example of apo-IF from plants (Fig. 4, lower curve). The calculated kinetic parameters of the interaction between cubilin and IFs from different sources are presented in Table 1. Both the natural proteins and the recombinant product from plants had comparable dissociation constants of $K_d \approx 1$ nM.

Conclusions

Human IF was successfully expressed in *A. thaliana* plants at high yield: 70 mg of the active protein (capable of Cbl-binding) per 1 kg wet weight (40 mg per 150 g dried plant material). The protein was quite stable during storage both as frozen wet substance and as a dried powder. The properties of isolated recombinant IF from plants were

comparable to those obtained for human gastric IF in terms of the IF–$CblOH_2$ spectrum, the relative affinity to Cbl or the analogue Cbi, and the binding to the IF receptor cubilin. Comparison between recombinant plants and yeast in terms of yield, expenses and technological complexity during IF expression undoubtedly favours the plant source. The data presented show that plants may be an excellent source for a large scale production of IF for diagnostic and therapeutical purposes.

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