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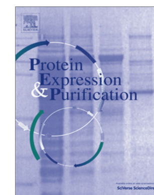

# A versatile ionic strength sensitive tag from a human GM-CSF-derived linear epitope

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ABSTRACT

A 7-mer hGM-CSF-derived linear epitope (APARSPS) is herein described as a novel and small tag taking into account its particular binding affinity in native conditions that could be easily modified by increasing or lowering the ionic strength. Thus, a 3.4 or 3.8-fold binding increment was observed in high NaCl concentration when the tag was fused to IFN-α2b or when the peptide was in its native environment, respectively. The high salt concentration increased the affinity of the binding interaction and improved the APARSPS epitope binding to the paratope allowing one to design an immunoaffinity chromatography purification step in which the high ionic strength was useful to adsorb the fusion protein to the immunoaffinity matrix and the low ionic strength at pH 9 was valuable to desorb it (a 470-fold purification with a 94%-purity was attained in only one step). Also, this short tag did not affect the functionality of the fusion protein and it was able to be detected both in the natural molecule (hGM-CSF) as in the tagged one with the same high detection limit: 273 pg of each protein.

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## Introduction

Epitope tagging constitutes a well-known technique for detecting, purifying, quantifying and tracking recombinant proteins. Considering the vast possibilities and diversity of universal tags [1,2], the epitope-derived ones constitute a subset of them, for which the specificity of their interactions is mainly exploited by generating canonical pairs of epitopes–paratopes that are optimal for immunochemical purposes. In this regard, it is possible to find short epitopes such as Softag 1 (13 amino acids, [3]), heavy chains of protein C (HCP)-tag (12 amino acids, [4]) T7-tag (11 amino acids, [5]), c-myc-tag (11 amino acids, [6]), hemagglutinin (HA)-tag (9 amino acids, [7]) FLAG-tag (8 amino acids, [8]), among others, that are commercially available and many that are used in their respective labs as in-house reagents [9–11]. The use of short polypeptides avoids protein insolubility and functional impairment, features that are common when proteins or large polypeptides are employed as fusion partners.

Many of the above-mentioned epitopes are appropriate as tags for protein purification by immunoaffinity chromatography. In this regard, several authors have described mild desorption conditions to carry out the elution step. In particular, Burgess et al. [3,12–14] have identified a singular and outstanding specificity for some mAbs that confers the property of being sensitive to polyol compounds. These antibodies, named polyol-responsive mAbs, were intensively described by those authors and successfully used to purify distinct antigens by immunoaffinity chromatography.

In a similar direction of research, our work shows the finding of an ionic strength-sensitive epitope, providing a condition to manage the epitope–paratope affinity in order to perform different immunochemical approaches.

Preceding research in our lab described the immunodominant regions of the human granulocyte and macrophage-colony stimulating factor (hGM-CSF) by means of a monoclonal antibody (mAb)[1] panel [15]. The binding sites on hGM-CSF were accurately delineated using cytokine-derived overlapping peptide scans and combinatorial hexapeptide libraries prepared by SPOT synthesis on cellulose membranes [15,16]. Two mAbs (CC1H7 and CC3C12) recognized the same epitope APARSPS that corresponds to the *N*-terminal region of the protein and has a linear conformation. Also, this peptide was useful to purify the corresponding mAb when the sequence APAR was immobilized as ligand for affinity

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[1] *Abbreviations used:* ANOVA, analysis of variance; BSA, bovine serum albumin; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; GM-CSF, granulocyte macrophage-colony stimulating factor; HRP, horseradish peroxidase; i.p., intraperitoneally; IFN, interferon; mAb, monoclonal antibody; MDBK, Madin–Darby Bovine Kidney; MEM, Minimum Essential Medium; n, sample size; O.D., optical density; ORF, open reading frame; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% (v/v) Tween 20; PVDF, polyvinylidene difluoride; rh, recombinant human; S.D., standard deviation; SBA, specific biological activity; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline containing 0.05% (v/v) Tween 20; VSV, vesicular stomatitits virus; WB, Western blot; wt, wild type.

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chromatography [17]. In connection with these findings, current investigation showed that the binding strength between the epitope in non-denaturing conditions and the paratope could be regulated by adding or subtracting NaCl to the environment solution. The epitope in the context of GM-CSF or used as a tag fused to IFN increased its affinity constant to the corresponding mAb when high ionic strength was used and decreased it as ionic strength was reduced. These properties helped us to define a new epitope tag as a single and versatile reagent to perform a variety of immunochemical approaches (ELISA, immunoprecipitation, immunoaffinity chromatography) in which the binding between the 7-mer peptide and the related CC1H7 or CC3C12 mAb paratopes could be finely regulated by the ionic strength. Therefore, a rational study leading to the description of a novel ionic strength sensitive tag was developed.

## Materials and methods

### Cytokines

*Escherichia coli*-derived cytokines [hGM-CSF and human Interferon-α2b (hIFN-α2b)] were kindly supplied by Zelltek S. A., Argentina.

### Antibodies

The antibodies used in this work were obtained and characterized in our lab. Taking into account that mAb CC1H7 and CC3C12 recognize the same hGM-CSF epitope [15], the former was selected to carry out this investigation.

The mAb producing clones (mouse $IgG_1/\kappa$) were established from BALB/c splenocytes following immunization with *E. coli*-derived rhGM-CSF (mAb CC1H7) and *E. coli*-derived rhIFN-α2b (mAb CA5E6), using standard fusion protocols [15,18,19]. Hybridoma cell lines were gradually expanded and cloned by the limiting dilution method. Thus, clones CC1H7 and CA5E6 were injected intraperitoneally (i.p.) in BALB/c mice ($2 \times 10^6$ hybridomas in 0.5 ml phosphate-buffered saline, pH 7: PBS) primed with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane; Sigma, USA). 7–10 days post-implantation, ascitic fluid was daily drained and centrifuged at 2000 rpm. mAbs were purified from ascites using protein A-Sepharose 4B Fast Flow (GE Healthcare, USA) according to the manufacturer's protocol.

Polyclonal antibodies (pAbs) anti-rhIFN-α2b were obtained by immunization of rabbits with *E. coli*-derived cytokine using standard protocols [20].

### Cell culture

Chinese hamster ovary (CHO-K1) cells were grown in the basal culture medium previously described [21] supplemented with 5% (v/v) foetal calf serum (FCS; Bioser, Argentina; growth medium).

Madin–Darby bovine kidney (MDBK) cells were grown in Minimum Essential Medium, MEM (Gibco, USA) supplemented with 10% (v/v) FCS (growth medium). For bioassays, MEM supplemented with 2% (v/v) FCS (assay medium) was employed.

### Assembly of peptide-tagged IFN molecules

The peptide genes were fused to the coding sequence of the hIFN-α2b using overlapping mutagenesis PCR for site-directed insertion [22]. Using this technique, the peptide genes were inserted between the signal peptide for eukaryotic expression and the coding sequence of hIFN. The plasmid pCI-neo-IFNα2b wt was used as template for mutagenesis reaction. This plasmid, expressing wild type hIFNα2b, was obtained as it was previously described by Ceaglio et al., 2008 [23].

Two sets of oligonucleotides were used for the chimeric construction: external and internal primers. The former, shared in all mutagenesis reactions, were the vector-specific 30-mer primers: IFNalfa Fw and IFNalfa Rv (Table 1). The gene sequences for insertion were present in 39–47-mer internal primers (Table 1) which hybridized within the peptide signal region. For each mutant, two separated first-round PCR reactions, including an external primer and a gene-specific one (internal primer) to insert the corresponding peptide, were performed using *Pfu* polymerase. Then, the two PCR-products were gel-purified and 50 ng of each first-round PCR products were combined in a second-round of PCR reaction which used the two flanking (external) primers to amplify the *pep-ifn* cDNA as one fragment.

After each mutation was added to the ORF of the *ifn*, each ~950 bp fragment was subcloned into pCI-neo plasmid, using *Eco*RI and *Xba*I restriction sites that had been added to the *pep-ifn* by means of the PCR primers at the 5′ and 3′ ends, respectively. All clones were sequenced prior to use.

### Gene expression in mammalian cells

Peptide-fused IFN-α2b analogs were transiently expressed in CHO-K1 cells using 6-well plates. pCi-neo vectors were introduced into the cells by liposome-mediated gene transfer, using 10 μg of LipofectAMINE 2000 (Invitrogen, USA) and 10 μg of plasmid DNA.

In order to obtain stable cell lines expressing the APAR-tagged proteins, the supernatant of a 72 h-post-transient transfection was replaced by selective medium, i.e., fresh growth medium containing 400 μg/ml Geneticin (G418) (Gibco, USA). Selective medium was changed every 3–4 days until death of control cells.

Fusion protein-producing cell lines, assayed by sandwich ELISA – Type 2 (see below), were cloned by limit dilution method. The clones of each IFN variant with the highest expression level were cultivated for large-scale production. Cells were grown until confluence in 500-cm$^2$ triple flasks using growth medium. The medium was then changed to basal medium supplemented with 2% (v/v) FCS (production medium). Every 96 h, conditioned medium was harvested and replaced with fresh production medium. Harvests were clarified by centrifugation and stored at −20 °C. The growth in triple flasks was continued for up to 40 days.

### Quantification of IFN by sandwich ELISA – Type 1

Fusion proteins were quantified by a sandwich ELISA using the mAb CA5E6 as capture antibody in order to bind to it by the IFN counterpart. Briefly, 96-well plates were coated overnight at 4 °C with 100 ng per well of mAb in 50 mM carbonate-bicarbonate buffer (coating buffer, pH 9.6). After blocking 1 h at 37 °C with 1% (w/v) bovine serum albumin (BSA) in PBS, plates were incubated with serial 2-fold dilutions of *E. coli*-derived rhIFN-α2b standard (Zelltek S. A., Argentina) from 10 ng/ml to 0.16 ng/ml or serial 2-fold dilutions of test samples for 1 h at 37 °C. Then, plates were incubated with an appropriately diluted rabbit anti-rhIFN-α2b polyclonal antibody for 1 h at 37 °C. Finally, horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG antibodies (DAKO, Denmark) diluted 1:1000 were added to the wells. After 1 h, plates were incubated with substrate solution (0.5 mg/ml *o*-phenylenediamine, 0.015% (v/v) $H_2O_2$ in 50 mM phosphate-citrate buffer). Reaction was stopped by the addition of 2 N $H_2SO_4$ and the absorbance was measured at 492 nm with a microtiter plate reader (Labsystems Multiskan MCC/340, Finland). Between steps, plates were washed with PBS containing 0.05% (v/v) Tween 20 (PBST). Dilutions were prepared in PBST containing 0.1% (w/v) BSA. The assay was performed in triplicate.

**Table 1**
Oligonucleotides designed to build the different APAR-tagged hIFN-α2b molecules.

| Oligonucleotides | | Sequences |
|---|---|---|
| Internal | APARGGG Fw | **GCACCCGCACGAGGAGGTGGT**TGTGATCTGCCTCAAACCC |
| | APARGGG Rv | **ACCACCTCCTCGTGCGGGTGC**GCCCACAGAGCAGCTGACT |
| | GGGAPAR Fw | **GGTGGTGGTGCTCCCGCCCGC**TGAAAACTGGTTCAACATGG |
| | GGGAPAR Rv | **GCGGGCGGGAGCACCACCACC**TTCCTTACTTCTTAAACTTT |
| | APARDDDDK Fw | **GCACCCGCACGAGATGATGATGATAAA**TGTGATCTGCCTCAAACCCAC |
| | APARDDDDK Rv | **TTTATCATCATCATCTCGTGCGGGTGC**GCCCACAGAGCAGCTTGACT |
| | APARSPS Fw | **GCACCCGCCCGCTCGCCCAGC**TGTGATCTGCCTCAAACC |
| | APARSPS Rv | **GCTGGGCGAGCGGGCGGGTGC**GCCCACAGAGCAGCTTGA |
| External | IFNalfa Fw | TAACGAATTCACATCTACAATGGCCTTGAC |
| | IFNalfa Rv | ATAGTCTAGAGTCTTTGAAATGGCAGATCA |

All primers are depicted in 5′–3′ orientation.
The tag sequences are indicated in bold letters.

*Identification of peptide-tagged IFN by sandwich ELISA – Type 2*

Peptide-tagged IFN was also identified by a sandwich ELISA where the unique difference with the above immunoassay lay in the use of mAb CC1H7 as capture antibody that binds to the fusion protein by the hGM-CSF epitope counterpart. Briefly, 96-well plates were coated overnight at 4 °C with 200 ng per well of mAb in 50 mM carbonate-bicarbonate buffer (pH 9.6). After blocking, the ELISA continued as it was described by sandwich ELISA – Type 1.

*ELISA – Type 2 variant A*

To analyse the elution performance of several reagents for rAPARSPS/hIFN purification by affinity chromatography, a modified sandwich ELISA – Type 2 (referred to as ELISA-elution assay) was performed to mimic the affinity chromatography procedure. After the capture step of the tagged IFN by the immobilized mAb CC1H7, a 1-h incubation step with one of the following elution reagents was carried out: 100 mM glycine–HCl buffers (pH 2 and pH 3); 100 mM sodium acetate buffers (pH 4 and pH 5); PBS; PBS-1 M NaCl; 100 mM Tris–HCl buffer (pH 8); 100 mM glycine–NaOH buffers (pH 9 and pH 10); 100 mM sodium phosphate buffers (pH 6, pH 11 and pH 11.7). The tagged IFN that remained bound to the mAb was identified by adding pAbs anti-rhIFN-α2b and further goat anti-rabbit IgG antibodies conjugated with HRP. A simultaneous test to control the mAb CC1H7 susceptibility to the elution conditions was also performed. Thus, a 15-min incubation step with the above-mentioned solutions was carried out before the rAPARSPS/hIFN capture. Then, the ELISA proceeded as above.

*ELISA – Type 2 variant B*

To explore the differential binding properties of the pair APARSPS epitope – CC1H7 paratope, in the presence of high or low ionic strength, a modified sandwich ELISA – Type 2 was also performed. In this sense, we compared the behavior of the peptide APARSPS as the naturally occurring *N*-terminal end of the rhGM-CSF molecule or as the tag added to the IFN-α2b. Thus, after immobilizing the mAb CC1H7 onto polystyrene plates and carrying out the subsequent blocking step, the rhGM-CSF or the fusion protein were appropriately diluted in PBS or PBS-1 M NaCl solutions and they were added to different wells. The assay was performed in quadruplicate, i.e., 4 wells of each condition. Besides, each protein was evaluated as 2 sets of 4 wells of incubation in the presence of PBS and 3 sets of 4 wells of incubation in the presence of PBS-1 M NaCl as binding conditions. Then, the following reagents (post-binding conditions) were tested: PBS, PBS-1 M NaCl and 100 mM glycine–NaOH buffer (pH 9).

Simultaneously a control of no added solution during the elution reagent step was also analysed, i.e. a set of 4 wells were incubated in a humid atmosphere without adding any putative desorption reactive in order to analyse the maximum fusion protein quantity that mAb CC1H7 was able to capture. Then the assay proceeded as mentioned above.

*Statistics of ELISA – Type 2 variants*

After the colorimetric reaction, the read out of the assays (O.D.) were evaluated by ANOVA. When the ANOVA produced significant results ($p < 0.05$), a post hoc Tukey's multiple comparison test was applied.

*Ionic strength effect on the mAb CC1H7dissociation constant*

A competitive ELISA described by Friguet *et al.* 1985 [24] was used to determine the dissociation constants of the complex produced between the *E. coli*-derived rhGM-CSF and the mAb CC1H7, either adding or not adding NaCl to the solution used during incubations. The bivalency effect of the antibodies was considered [25]. For the competition, five successive twofold dilutions of rhGM-CSF (from 280 nM to 17.5 nM) were preincubated overnight at room temperature with a constant amount of mAb (0.37 nM or 0.78 nM, depending if NaCl was added or not to the solution). Then, an aliquot of each mixture was incubated in microtiter plates firstly coated with 160 ng ml$^{-1}$ of rhGM-CSF diluted in coating buffer and then blocked with 5% (v/v) FCS in PBS. The plates were incubated for 2 h at 37 °C. After washing with PBST, the plates were incubated for 1 h at 37 °C with HRP-labelled rabbit anti-mouse IgG antibodies (DAKO, Denmark) diluted 1:2000 with PBST containing FCS 0.5% (v/v). The plates were washed with PBST and the colorimetric reaction proceeded as it was described by sandwich ELISA – Type 1. The assay was performed in triplicate.

*Purification of rAPARSPS/hIFN-α2b variant*

The preparation of the immunoaffinity column was performed using CNBr-activated Sepharose-4B (GE Healthcare) according to the manufactureŕs standard protocol. Briefly, 375 mg lyophilized powder was weighed out, swelled and washed with 1 mM HCl. The highly purified mAb CC1H7 (about 0.9 mg) was dissolved in 0.1 M NaHCO3 (pH 8.3) – 0.5 M NaCl (coupling buffer) and it was coupled to 1 ml of the swelled gel. The excess of ligand was washed with coupling buffer and the remaining sites were blocked with 0.1 M Tris–HCl buffer (pH 8). After that, the gel was washed three times with 0.1 M sodium acetate buffer (pH 4) – 0.5 M NaCl and 0.1 M Tris–HCl buffer (pH 8) – 0.5 M NaCl, alternatively. Then, it was washed with the selected elution buffer to be used during the immunoaffinity chromatography procedure. Finally, the gel was ready to use and it was packed in a XK16/20 column (200 × 16 mm i.d., GE Healthcare). The bed height (0.5 cm) was adjusted using two flow adapters.

CHO cell-derived rAPARSPS/hIFN-α2b-containing supernatants were adjusted to pH 7.5, 2 M NaCl and Triton X-100 was added to a final concentration of 0.3% (v/v).The sample was filtered through a 0.45 μm cellulose acetate membrane filter (Nalgene, USA) and applied to the immunoadsorbent previously equilibrated with 0.3% (v/v) Triton X-100 in 100 mM Tris–HCl buffer (pH 7.5), 2 M NaCl at a flow rate of 0.5 cm/min. After sample loading at a flow rate of 0.25 cm/min, the column was washed at a flow rate of 0.5 cm/min for 15 min with each of the following solutions: (A) equilibration buffer, (B) 0.5 M NaSCN in 100 mM Tris–HCl buffer (pH 7.5) – 0.1% Triton X-100 and (C) 100 mM Tris–HCl buffer (pH 7.5). Elution of bound rAPARSPS/hIFN-α2b was accomplished using 0.1 M glycine–NaOH buffer (pH 9) at a flow rate of 0.25 cm/min. Column fractions were analyzed by sandwich ELISA – Type 1 and – Type 2 and those containing the higher concentrations of the fusion protein were pooled. Protein content was measured by the Bradford method [26]. Purified rAPARSPS/hIFN-α2b variant was concentrated and diafiltered against ultra-pure water using Amicon Ultra-4 centrifugal filter units with 10-kDa cut-off membrane (Millipore, France).

### *Protein purity analysis*

The purity of the fusion protein after chromatography procedure was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE analysis was performed according to the standard method [27]. Gels were run under reducing conditions at 200 V at room temperature in a Mini Protean III system (BioRad, USA). Protein bands were visualized by silver nitrate staining (Silver Stain Plus kit, Bio-Rad, USA) and the purity parameter was determined by densitometry of the stained gel in which the amount of rAPARSPS/hIFN was determined as a percentage of the total protein in the corresponding fraction using the ImageMaster™ TotalLab Version 1.0 (GE Healtcare) according the manufactureŕs instructions.

### *Western blot*

For Western blot analysis, proteins were submitted to electrophoresis in the conditions mentioned above and then electrotransferred onto a polyvinylidene difluoride PVDF membranes (BioRad, USA). Blots were then blocked for 1 h with 5% (w/v) non fat milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) at room temperature followed by washing with TBST (3 times, for 5 min each). Either rabbit anti-rhIFN-α2b polyclonal antibody or CC1H7 mAb as the first antibodies were diluted (1:1000) in the blocking buffer and incubated for 1 h. After washing three times with TBST, the second antibodies, HRP-conjugated goat anti-rabbit IgG (DAKO, Denmark) or goat anti-mouse IgG (DAKO, Denmark) were diluted (1:2000) in the blocking buffer and incubated for 1 h. After washing with TBST, immunoreactive bands were visualized using an ECL™ Chemiluminescent Western Blotting Analysis System (GE Healthcare).

### *Interferon functional integrity assay*

To evaluate the potentiality of tag APARSPS to impair the biological activity of IFN, the in vitro specific antiviral action of the tagged cytokine was quantified. The antiviral activity of rhIFN-α2b was determined by its ability to inhibit the cytopathic effect caused by vesicular stomatitits virus (VSV) on MDBK cells [28,29]). For this, MDBK cells were seeded into culture microtiter plates in growth medium ($2.5 \times 10^4$ cells per well) and incubated at 37 °C overnight. After removing culture supernatants, serial 2-fold dilutions of rhIFN-α2b WHO international standard (NIBSC 95/566) from 20 U/ml to 0.16 U/ml or serial 2-fold dilutions of tagged IFN test samples in assay medium were added. Plates were incubated for 6 h at 37 °C and, after removal of supernatants, an appropriate dilution of VSV virus was added (the maximum dilution from a stock that produce 100% of cytopathic effect in a control without IFN). Virus replication was allowed to proceed until the cytopathic effect was clearly observable in control wells (no IFN). The medium was discarded and cells were fixed and stained simultaneously with a solution of 0.75% (w/v) crystal violet in 40% (v/v) methanol. After 10 min, plates were washed with water and the remaining dye was solubilized in 20% (v/v) acetic acid. The plates were read at 540 nm with a microtiter plate reader and the signal intensity of each dilution was reported as the mean of the absorbance measured in five wells.

Specific biological activity (SBA) was determined as the ratio between the IFN biological activity and the peptide-tagged IFN concentration measured by sandwich ELISA – type 1. Statistical analysis was performed using Student's *t*-test for single comparison between means. Probabilities lower than 0.05 were considered significant.

## Results and discussion

### *hGM-CSF epitope-tagged proteins*

Previous works from our group [15] demonstrated than only four amino acids, sequentially located in the *N*-terminus of the hGM-CSF ($A_1PAR_4$), comprised the non-conformational epitope recognized by mAb CC1H7. This particularly short linear epitope prompted us to evaluate it as affinity tag for different immunochemical purposes with the aim of not distressing the functional integrity of the tagged protein target.

Taking into account that a spacer arm between the target protein and the tag could improve the antibody recognition, we tested the following spacer arms linked to the *C*-terminal end of the APAR sequence: GGG (with minimal probabilities of structural interferences), DDDDKY (useful for adding a cleavage site to split the tag from the protein) and SPS (three natural occurrence amino acids from hGM-CSF that are present in the cytokine sequence at positions 5–7). Also, the tag was fused at the *C*-terminal of the target protein using the linker GGG at the *N*-terminal end of the APAR sequence. Therefore, the following sequences were tested as tags: APARGGG, APARDDDDK, APARSPS and GGGAPAR.

We selected the hIFN-α2b as model protein to evaluate the APAR sequence functionality as tag, making comparisons with the taǵs properties in the context of the natural counterpart: hGM-CSF.

The epitope-tagged IFN was expressed in mammalian CHO cells considering that naturally produced-hIFN-α2b is *O*-glycoslylated. This eukaryotic host was selected in order to simultaneously analyze if natural glycosylation of rhIFN could be affected by the tag incorporation. Protein expression was evaluated by Western blot (WB). Controls were assessed using pAb anti-rhIFN-α2b that has the ability to recognize the *O*-glycosylated and non-glycosylated IFN-derived isoforms.

In WB experiments, the epitope APAR was faintly recognized by the mAb CC1H7 in the context of GGG or DDDDK at the *C*-terminal end of the peptide or strongly recognized by the same antibody in the context of SPS spacer-arm (Fig. 1A). Considering the recognition pattern given by the mAb CC1H7, a main immunodetected band corresponding to the *O-glycosylated* tagged protein was observed. This band matches with that visualized by Ceaglio et al. [23] who reported the same IFNα2 isoform after *N*-deglycosylation of novel *N*-glycosylated-IFNα2b muteins. The resulting molecules exhibited two bands (21 kDa and 19 kDa) corresponding to the isoform *O*-glycosylated at Thr106 and the non-glycosylated one, respectively. Hence, the incorporation of the 7-mer tag did not

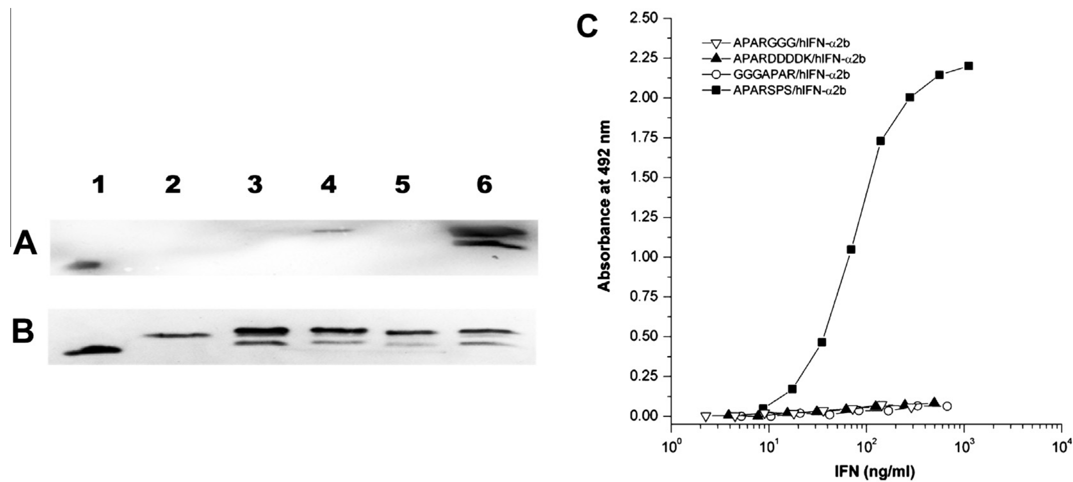


**Fig. 1.** APAR-tagged hIFN-α2b expression in CHO-K1 cells using different spacer arms: SPS, GGG and DDDDK. Conditioned media from transfected-CHO-K1 cells were assayed to detect the fusion proteins by Western blot using the mAb CC1H7 (A) or the pAb anti-hIFN-α2b. (B) Additionally, the binding of fusion proteins in native conditions was assessed by ELISA sandwich – Type 2 where mAb CC1H7 was immobilized in solid phase. (C) Samples: *E. coli*-derived rhGM-CSF (A1), *E. coli*-derived hIFN-α2b (B1), CHO-derived hIFN-α2b (2), CHO-derived APARGGG hIFN-α2b (3), CHO-derived APARDDDDK hIFN-α2b (4), CHO-derived GGGAPAR hIFN-α2b (5) and CHO-derived APARSPS hIFN-α2b (6).

affect glycosylation because the same Thr at position 106 of the IFN or 113 of the tagged-IFN was also *O*-glycosylated.

Moreover, the expression of the different tagged proteins was confirmed by the rabbit anti-rhIFN-α2b polyclonal antibodies (Fig. 1B) and the specific binding to the tagged protein was also confirmed employing a mAb of irrelevant specificity (anti-rh erythropoietin), that did not show cross reaction (data not shown).

To evaluate the property of the mAb CC1H7 to recognize the tagged protein in native conditions, the sandwich ELISA – Type 2 (that use the mAb CC1H7 as capture antibody) was carried out. Fig. 1C shows the ability of the antibody to bind to soluble rAPARSPS/hIFN only. None of the other variants were captured by the solid phase-immobilized mAb. Therefore, both folded and unfolded conditions showed that the APAR epitope must be expressed in the context of SPS (as in the natural protein) to elicit its binding capability. Besides, the expression of GGG at the *N*-terminus of the APAR sequence completely prevented the recognition of APAR-tagged IFN in native or denatured condition.

The preceding research by Oggero et al. [15] described the linear epitope APAR by spot synthesis technique [16] using the mode of replacement scan where substitutional analyses of the minimal epitope peptide were performed to evaluate the role of individual amino acid residues in the antibody-peptide interaction. When using this method, which immobilizes the peptide on cellulose membranes, the contributions of the amino acids Ser5 and Pro6 were considered irrelevant. However, the addition a Ser5, Pro6 and Ser7 to the fusion protein, instead of three glycine residues or three aspartic acid and one lysine residues, improved the binding of mAb CC1H7 to APAR when the peptide was fused to the *N*-terminus of the IFN. It is likely that the occurrence of SPS emulates with more fidelity the natural epitope in the hGM-CSF molecule that composed the CC1H7 paratope during the *in vivo* B-clonal selection. Consequently, we decided to continue working with APARSPS as affinity tag for further evaluations.

### *Immunochemical analyses and functional integrity of rAPARSPS/hIFN*

The immunochemical recognition of rAPARSPS/hIFN and rhGM-CSF was compared by WB experiments performing 8 serial 2-fold dilutions from 4375 pg of each protein. The detection limit (determined as the last dilution where it was possible to detect protein) was 273 pg for both proteins indicating that peptide APARSPS is a valuable tool for protein tagging because it was identically recognized by mAb CC1H7 in the context of the naturally occurring molecule (hGM-CSF) or in the context of a new one as hIFN-α2b (Fig. 2).

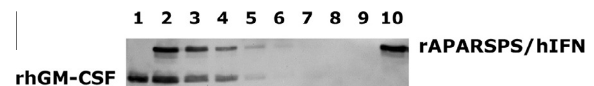


**Fig. 2.** The detection limit of APARSPS tag-containing proteins (rhGM-CSF and rAPARSPS/hIFN) assayed by Western blot was evaluated using mAb CC1H7. A mixture of equivalent masses of rhGM-CSF and rAPARSPS/hIFN was immunochemically analyzed by exploring successive 2-fold dilutions of each protein from 4375 pg to 34 pg (lanes 2–9). Also, both proteins were separately tested (lane 1: rhGM-CSF, lane 10: rAPARSPS/hIFN).

On the other hand, the biological activity of the tagged-IFN was evaluated by exploring the antiviral potency on MDBK cells challenged with VSV virus. No differences were observed as SBA of rAPARSPS/hIFN (188 ± 14 IU/ng) was comparable ($p < 0.05$) with that of the wild type protein (197 ± 17 IU/ng). Thus, tagging the *N*-terminal tail of the cytokine with APARSPS sequence did not impair the IFN-receptor binding.

The perturbation of protein function by tagging will depend on the physicochemical features of both counterparts and the extent of changes that tag attachment will produce on the protein site that is active for displaying its biological action. Nevertheless, the results presented herein suggest that tagging a protein with the 7-mer peptide could be valuable considering that the shorter the tag, the smaller the possibility to alter the protein structure and function.

### *Characterization of APARSPS as tag for immunoaffinity purification*

#### *A-Selection of elution conditions*

In order to select the most appropriate condition to purify APARSPS-tagged proteins using mAb CC1H7, the elution performances of several reagents were initially evaluated in a polystyrene support (microplates). A modified ELISA, termed ELISA-elution assay, was used [14,30,31]. This procedure was based on a standard sandwich ELISA with the exception that the antigen–antibody complex was treated with a particular reagent which was proposed as a candidate to be used in the elution condition. The method

constituted an efficient alternative to avoid the complete affinity chromatography process for each elution reagent to be screened. Thus, a modified sandwich ELISA – Type 2 was performed following the procedure described above. Each reagent was tested in two forms: on the one hand, the immobilized mAb CC1H7 was incubated with rAPARSPS/hIFN and then the elution reagent (post-binding solution) was added to analyze its ability to dissociate the mAb-antigen complex (procedure A). On the other hand, the mAb susceptibility to the elution reagent was analyzed incubating the polyestyrene-adsorbed antibody with the elution reagent as pre-binding solution, i.e., before the incubation with the rAPARSPS/hIFN (procedure B). This last evaluation was valuable to predict if a successive re-binding of the antigen to the antibody might be carried out as it is common during the reuse of an immunoaffinity matrix. Fig. 3 shows the elution profile of different reagents. In each procedure. the incubation with PBS as pre-binding solution was used as a control of the maximum binding that could be attained between the immobilized mAb and the rAPARSPS/hIFN protein during the experiment. Therefore, the bound antigen percentage for each procedure was calculated as follow:

$$\text{Bound antigen}(\%) = 100 \times \text{P/PBcontrol}$$

Where P represents the absorbance value that corresponds to the bound antigen after assaying with the corresponding procedure and the absorbance value $PB_{control}$ corresponds to the bound antigen after assaying with procedure B and using PBS as a pre-binding solution.

From the data we concluded that increasing the pH to the alkaline range: 9–11.7 showed better stripping conditions to recover the highest percentage of the bound rAPARSPS/hIFN and preserved the binding integrity of the antibody. Nevertheless, pH 2-being also the condition with the highest elution capability- altered the binding integrity of the antibody as it was observed when mAb was incubated at pH 2 previous to the addition of the tagged protein. Therefore, making an extrapolation to a CC1H7-based immunoaffinity matrix for a chromatographic purpose, the utilization of an acidic elution buffer (pH 2) would be detrimental for further antibody–antigen binding, i.e. a fast decrease in the capacity might be observed after re-cycling the matrix in order to be used in a further antigen-binding step.

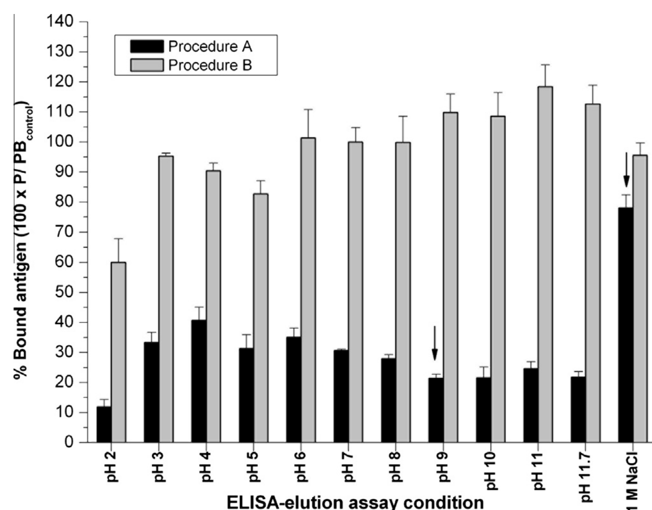


**Fig. 3.** Characterization of APARSPS as tag for immunoaffinity chromatography purification. The selection of the elution conditions was carried out by ELISA sandwich – Type 2 (variant A, see M and M). Different pHs or ionic strengths conditions were used as post-binding solutions (Procedure A, ■) or as pre-binding solutions (Procedure B, ■) to evaluate their effect on the rAPARSPS/hIFN capturing step. *P*: absorbance value representing the bound antigen after assaying with each procedure. $PB_{control}$: absorbance value representing the bound antigen after assaying with procedure B and using PBS as a pre-binding solution. The arrows show the best condition (pH 9.0) for fusion protein desorption without affecting the ability of the antibody to further bind and the condition (NaCl) that, contrarily, improves its binding.

Interestingly, when PBS was used as diluent of rAPARSPS/hIFN and simultaneously as solution for post-binding, a 70% desorption was observed indicating that a low ionic strength or a longer incubation period in the same solution of PBS could be responsible for this behavior (Fig. 3). Otherwise, when a high ionic strength buffer (PBS-1 M NaCl) was evaluated as an elution condition, a higher absorbance signal and the consequently higher binding was observed in correlation with that obtained using PBS. The comparison between the post-binding effect of PBS with and without NaCl showed a very highly significant difference ($p < 0{,}001$) using ANOVA followed by Tuckey post hoc test. The NaCl content increased the recognition of the fusion protein, suggesting that it might stabilize or enhance the antigen–antibody affinity.

*B-Effect of ionic strength on mAb CC1H7 binding to APARSPS*

In order to evaluate the ionic strength effect on the native binding conditions, affinity constants were determined by the method of Friguet et al. [24] using the original protein (rhGM-CSF, with the naturally occurred-APARSPS sequence) with or without NaCl addition. Fig. 4A shows a 3,8-fold increase of the affinity constant of rhGM-CSF protein when NaCl was included in the solution. To analyze if ionic strength affects the binding of APARSPS tag in the context of another protein, e.g., hIFN-α2b, the sandwich ELISA-Type 2 was used (Fig. 4B). The binding augmentation was calculated as the ratio between the rAPARSPS/hIFN concentration needed to produce an absorbance value of 0.8 with and without NaCl. A 3.4-fold increase of binding was observed in the presence of NaCl. Therefore, it was confirmed that high salt concentration increased the affinity of the interaction and improved the APARSPS epitope binding to the paratope.

This untypical ionic strength dependence of the interaction between mAb CC1H7 and the APARSPS epitope in comparison with other antigen–antibody interactions was also described by Dimitrov et al. [32]. These authors discovered that a gradual increase in ionic strength resulted in a considerable increase in the association rate of binding between the human antibody BO2C11 and the antigen Factor VIII. They concluded that the combination of charged and hydrophobic residues found at the binding interfaces of BO2C11 and FVIII may create a unique molecular microenvironment vulnerable to the ionic strength effects. They also mentioned that the increase of the ionic strength of environment might decrease the solvatation energy and thus benefit the association. This conclusion agrees with that of Kumagai and Tsumoto [33] who considered that, in general, the driving force in antigen–antibody binding is originated from an increase in the entropy of solvent molecules displaced from the interface upon complexation. Therefore, taking into account that the APARSPS epitope comprises a combination of non-polar residues (Ala *x* 2, Pro *x* 2), polar uncharged residues (Ser *x* 2) and a charged residue (Arg), the ionic strength might dehydrate the binding interfaces of both molecules and thus it might strengthen the binding energy of different intermolecular interactions, i.e., those interactions that develop when epitope and paratope approach more closely each other. Consequently, it is probable that the phenomenon that Van Oss et al. [34] described during the interaction of an epitope and its paratope took place in greater intensity when high ionic strength was used. Particularly, they described that after the close encounter of the epitope and the paratope, the water of hydration is expelled and a direct interfacial bonding takes over, bringing more closely the chains in the vicinity. At this distance the short-range forces prevail and the overall strength of the binding depends on the goodness of fit the two surfaces [35].

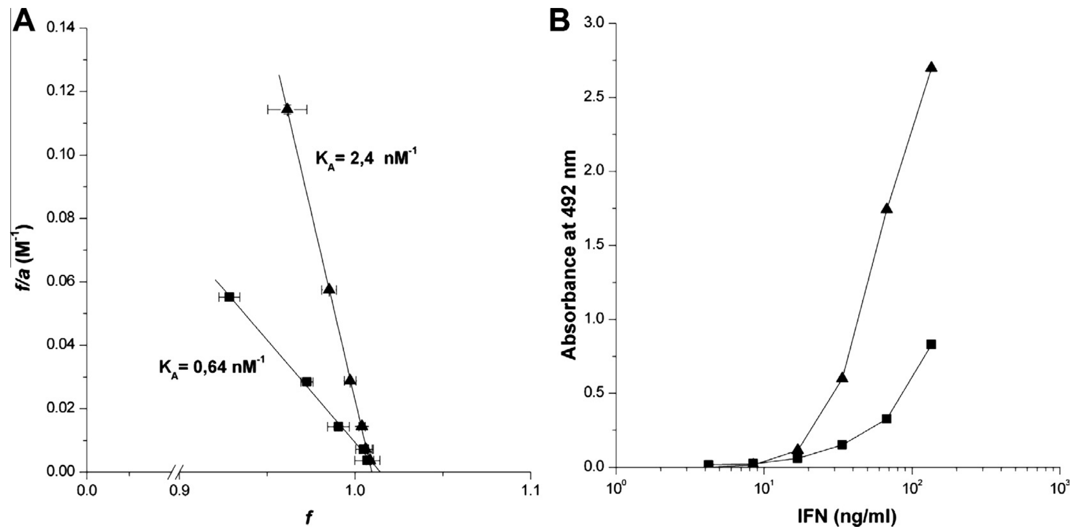


**Fig. 4.** The ionic strength effect on mAb CC1H7 binding to APARSPS was analysed by quantifying the affinity constant ($K_A$) by ELISA using the rhGM-CSF (A) or the rAPARSPS-tagged hIFN-α2b. (B) Samples were assayed in PBS (■) or PBS-1 M NaCl (▲).

In conclusion, despite the fact that amino acid composition of the paratope is unknown, its higher binding with the epitope in the presence of high ionic strength clearly shows that hydrophobic effect is responsible of this behavior.

*C-Binding/post-binding assay to optimize the immunoaffinity chromatographic procedure*

To optimize the binding and desorption conditions of an APARSPS-carrying protein during an affinity chromatography, a comparison test between the rAPARSPS/hIFN and the hGM-CSF was carried out. Hence, another sandwich ELISA – Type 2 (variant B) was performed introducing some modifications (Fig. 5). In this assay, the proportion of antigen that remained bound to the immobilized mAb CC1H7 after each treatment was estimated as follows:

$$\text{Bound antigen after treatments}(\%) = 100 \times T/T_M$$

Where T represents the absorbance value that corresponds to the antigens that remained bound after the incubation with the different combinations of binding and post-binding solutions and $T_M$, the absorbance value that corresponds to the maximum antigen binding capacity that could be attained when the assay was carried out in the presence of PBS-1 M NaCl as binding and post-binding solutions.

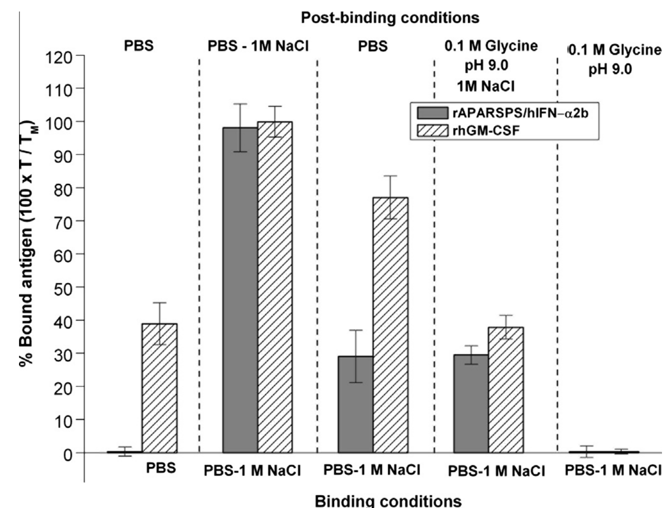


**Fig. 5.** Binding/post-binding assay to optimize the immunoaffinity chromatographic procedure. A comparison test between the rAPARSPS/hIFN and the rhGM-CSF was carried out by ELISA sandwich in order to optimize the binding and desorption conditions during the immunoaffinity chromatography. Two conditions for binding were assayed: PBS or PBS-1 M NaCl. After specific protein binding, the desorption conditions were analyzed using the same solutions and including 0.1 M glycine–NaOH buffer (pH 9). *T*: absorbance value corresponding to the antigens that remained bound after the incubation with the different combinations of binding and post-binding solutions. $T_M$: absorbance value corresponding to the maximum antigen binding capacity that could be attained when the assay was carried out in the presence of PBS-1 M NaCl as binding and post-binding solution.

In Fig. 5 the binding profile of both molecules (fusion protein and rhGM-CSF) is shown. Thus, the binding was improved after incubating the antigens in the presence of high ionic strength both in the binding and post-binding condition ($p < 0.05$; highly significant). When binding and post-binding states were accomplished in PBS, no binding was measured in the case of the fusion protein and only less than 40% was detected for rhGM-CSF. Probably a higher affinity constant of mAb CC1H7 for rhGM-CSF (the protein which was used as immunogen for antibody generation) could explain the higher binding to the cytokine than to the fusion protein. Moreover, when the binding of both molecules was previously carried out in the presence of 1 M NaCl, a partial desorption was observed after using a post-binding solution with lower ionic strength (PBS). The elution potency of PBS was higher with APARSPS-tagged protein than rhGM-CSF, most likely caused by differences in the affinity constant between mAb/rhGM-CSF and mAb/rAPARSPS-tagged protein.

Therefore, an ionic strength-dependent binding between APARSPS and the CC1H7 paratope was confirmed and became worthy of interest to be used to give the proper environment during an immunoaffinity chromatographic procedure where the ionic strength governs the tagged-protein adsorption and induces its gentle release from its paratope by increasing or decreasing the salt concentration, respectively.

Furthermore, using the same ELISA sandwich procedure described above, the APARSPS epitope (as the natural aminoacid sequence of rhGM-CSF and as the IFN-tagged aminoacid sequence) was completely desorbed from the CC1H7 paratope by means of a solution of glycine (pH 9) (Fig. 5), representing the best assayed condition to desorbed the proteins. In order to analyze whether pH 9 by itself or the combinantion with lower ionic strength were sufficient to produce antigen desorption, both proteins were

**Table 2**
Determination of chromatographic parameters for the purification of rAPARSPS/hIFN-α2b using the novel ionic strength sensitive tag.

| Step | rAPARSPS/hIFN[a] (μg) | Proteins[a] (μg) | Purity (%) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Supernatant | 36.7 | $17.9 \times 10^3$ | 0.20 | 1 | 100 |
| Flowthrough | 13.3 | $17.9 \times 10^3$ | 0.074 | 0.4 | 36 |
| Wash (A) | 0.46 | n/d | n/d | n/d | 1.3 |
| Wash (B) | 0.08 | n/d | n/d | n/d | 0.22 |
| Wash (C) | 0.18 | n/d | n/d | n/d | 0.49 |
| Elution | 22.1 | 23.6 | 94 | 470 | 60 |
| Retained[b] | 22.7 | | | | 100 |
| Eluted | 22.1 | | | | 97[c] |

n/d: non determined. The protein concentration was too low for the detection limit of Bradford method.
A, B and C refer to the washing solutions mentioned in M&M.
[a] The fusion protein concentration was determined by the sandwich ELISA – Type 1 and total proteins were determined by Bradford method.
[b] Retained refers to the quantity of the fusion protein that was retained by the immobilized CC1H7 mAb. It was calculated as the difference between the amount of fusion protein loaded to the affinity matrix minus the amount of fusion protein that passed through the matrix without being retained and minus the amount of it that was washed during the procedure.
[c] The real yield of eluted fusion protein was calculated from the retained fraction.

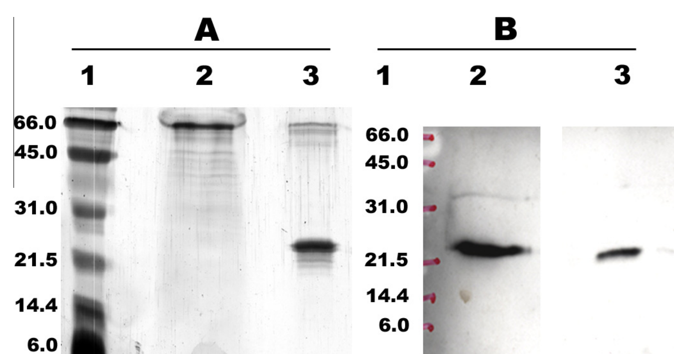


**Fig. 6.** rAPARSPS/hIFN immunoaffinity purification analyzed by SDS–PAGE followed by silver staining (A) or Western blot. (B) Lane 1: molecular mass standards, lane 2: CHO-derived rAPARSPS/hIFN containing culture supernatant (14 ng in each procedure), lane 3: immunoaffinity-purified rAPARSPS/hIFN (2850 ng for procedure A and13 ng for procedure B).

incubated in the presence of 100 mM glycine–NaOH pH 9 – 1 M NaCl. Thus, a partial desorption was observed during the last condition indicating that pH 9 or ionic strength by themselves are not appropriate to reach the complete elution of the assayed antigens.

Considering the results previously discussed, the mildest condition to carry out a CC1H7 mAb-based immunoaffinity chromatography to purify APARSPS-tagged proteins.was the combination of low ionic strength and pH 9.

*Affinity purification of APARSPS-tagged interferon: determination of chromatographic parameters*

Fifteen stable CHO clones expressing rAPARSPS/hIFN were isolated showing a productivity that varied from 2.1 to 55.2 ng/$10^6$ cell/day in the presence of 2% (v/v) of FCS. The clone with the higher productivity was used for large scale production as it was summarized in M&M.

The chromatographic procedure described below was performed in order to establish the chromatographic parameters. The best conditions for binding and desorption were selected taking into account the above-mentioned analysis. Also an increment in the concentration of NaCl was used (from 1 M to 2 M) to guarantee a better binding condition (data not shown). Thus, 98.5 ml of supernatant was pre-conditioned by adding solid NaCl up to 2 M, 0.1% (v/v) Triton X-100 and by adjusting the pH to 7.5 with a solution of 1 M HCl. Finally, it was applied to the CC1H7-affinity matrix previously conditioned in 2 M NaCl, 0.1% (v/v) Triton X-100, 25 mM Tris–HCl buffer (pH 7.5). The fusion protein-containing supernatant was loaded onto the immunoaffinity gel. The APARSPS-tagged IFN was detected in the flowthrough fractions before the theoretical capacity was achieved (252 μg of fusion protein/ml of immunoaffinity gel). Then, a washing step was carried out to remove components that bound non-specifically to the matrix. Two high ionic strength solutions and one of low ionic strength were used. The last wash was made with the low ionic strength solution aiming to prepare the matrix for the further quantitative elution procedure. The mentioned preparation consisted in removing traces of the detergent Triton X-100 and avoiding the presence of high ionic strength in the eluates. Thus, 2% of the applied amount of rAPARSPS/hIFN was desorbed during the complete washing step but only 0.49% was eluted during the 15-min washing with the low ionic strength solution. This ionic strength decrease was useful to remove Triton X-100 and to reduce the ionic content.of eluates but did not produce the elution of the tagged-IFN. Contrarily, the binding/post-binding assays carried out before (Fig. 5) showed that a decrease of the ionic strength during 1 h was responsible for eluting almost the 70% of the fusion protein.

Previous investigations demonstrated that after the first encounter between an epitope and paratope, much of the interstitial water is expelled [36–38]. For this reason, the presence of high ionic strength during the antigen binding might increase the binding energy to an extent that it is difficult to lessen by reducing the ionic strength only for 15 min. Thus, the proper elution condition for the chromatography procedure coincided with those shown in Fig. 5: decreasing the ionic strength and increasing the pH up to 9. In fact, after washing the gel with the above-mentioned solutions, the fusion protein eluted sharply at low ionic strength solution, pH 9 (data not shown).

Taking into account the amount of fusion protein that was retained by the immunoaffinity gel (Table 2), 97% was recovered during the elution step. Only a slightly leakage of the cytokine was observed during washing steps (that were useful to remove a high content of contaminants). A purity of 94% was achieved considering the ratio between specific protein quantified by ELISA sandwich – Type 1 and the total protein quantified by Bradford method. The eletrophoreticprofile of purified rAPARSPS/hIFN-a2b revealed a good purity level for a single step procedure with BSA being the main contaminant, as judged by SDS–PAGE followed by silver staining (Fig. 6A).

Furthermore, the identity of the purified rAPARSPS/hIFN analyzed by Western blot analysis (Fig. 6B) and the preservation of its biological activity (182 ± 20 IU/ml) were confirmed.

The static binding capacity of the CC1H7-immobilized-affinity matrix was at least 22.7 μg of fusion protein/ml of gel, calculated

as the amount of APARSPS-tagged IFN loaded to the affinity matrix, subtracting the amount of it that passed through the matrix without being retained and the amount of it that was washed during the procedure. The immunoaffinity chromatography protocols from Subramanian et al. [39] establish that current immobilization techniques render antigen-binding efficiencies in the range of 10–30%. Therefore, our method was in the lower limit of this range indicating that it functions well.

Finally, despite the fact that the static binding capacity was moderated in this sort of matrixes, about a 470-fold purification and 94%-purity were achieved with by this single step. Thus, mAb CC1H7 was able to recognize specifically the tag APARSPS in an environment that showed a large quantity of bovine serum albumin from culture medium.

## Conclusions

Previously, we characterized a mAb anti-rhGM-CSF as the cognate partner that binds to the cytokine-derived linear epitope, APARSPS, *N*-terminally located in the protein [15]. In the present work we have described this small amino acid sequence as tag to carry out several immunochemical assays (Western blot, competitive and sandwich ELISAs and immunoaffinity chromatography). Interestingly, changes of the ionic strength were found to increase or diminish the affinity between the amino acid sequence APARSPS and the mAb anti-rhGM-CSF in native conditions. Therefore, a 3.4 or 3.8-fold binding increment was observed in high NaCl concentration when the tag was fused to IFN-α2b or when the peptide was present in the natural protein hGM-CSF (Fig. 4). Taking advantage of this property, an immunoaffinity chromatography was developed by immobilizing the mAb CC1H7 and by purifying the fusion protein rAPARSPS/hIFN-α2b using a low ionic strength solution and pH 9. A purity of 94% and a purification factor of 470 were attained (Table 2 and Fig. 6).

In consequence, the 7-mer hGM-CSF-derived linear epitope constitutes an interesting tool to carry out biotechnology processes as tagging with this short label, that is sensitive to variations of the ionic strength and represents a great advantage to manage its binding properties. Also, this short tag showed to preserve the protein functionality.

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