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Targeting antigens to an invariant epitope of the MHC Class II DR molecule potentiates the immune response to subunit vaccines

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ABSTRACT

Recombinant subunit and peptidic vaccines in general present a reduced immunogenicity in vaccinated individuals with respect to the whole pathogen from which they derived. The generation of strong immune responses to these vaccines requires the use of potent adjuvants, high antigen doses and repetitive vaccinations. In this report, we document the enhanced antibody response obtained against two recombinant subunit vaccines by means of targeting to antigen-presenting cells by a recombinant single chain antibody. This antibody, named APCH1, recognizes an epitope of MHC Class II DR molecule preserved in different animal species, including humans. We showed that vaccinal antigens translationally fused to APCH1 antibody and produced by recombinant baculoviruses in insect larvae (*Trichoplusia ni*), elicited an increased antibody response in comparison with the same antigens alone or fused to a carrier molecule. These results suggest that targeting of antigens to this invariant MHC Class II epitope has immunopotentiating effects that could circumvent the reduced potency of peptidic or subunit vaccines, opening the possibility of widespread application of APCH1 as a new adjuvant antibody of general use.

1. Introduction

The generation of strong serological responses to protein or peptide antigens in humans or animals usually requires the use of potent adjuvants, most of which cannot be used in commercial vaccines because of deleterious side effects they may cause. The antibody response to most protein antigens requires specific cooperation between B and T cells. In order to deliver the helper signal, $CD4^+$ T cells must recognize processed antigens in the context of the major histocompatibility complex Class II molecules (MHC II) found on the membrane of antigen-presenting cells (APCs).

Antigens may be targeted to APCs through their conjugation to monoclonal antibodies (mAbs) directed against surface molecules on these cells (Barber, 1997; Fossum et al., 1992). Following this idea, different membrane proteins were studied as targets with different effects observed among them (Skea and Barber, 1993). These results were basically related to the chance of each of these surface molecules to be endocyted and the particular pathways they may take upon cellular endocytosis (Snider and Segal, 1989).

In particular, MHC II-targeted antigens were described to enhance antigen presentation and induction of antibody responses against peptide and protein antigens (Carayanniotis and Barber, 1987, 1990; Carayanniotis et al., 1988). This approach was reported to generate potent recombinant subunit vaccines that could be produced in adjuvant-free formulations. Targeting to MHC II molecules also produced specific effects on the Ig isotype profiles induced and even in the generation of long-term memory responses (Skea and Barber, 1993; Snider et al., 1997).

In the present work, we present the development of the recombinant single chain antibody (scFv) APCH1, directed to an invariant epitope of the porcine MHC II DR molecule also preserved in other species. The APCH1 molecule had the ability to drive vaccine antigens to APCs, increasing the specific antibody responses against peptide and subunit vaccine antigens in two different animal models. Our results show that humoral responses were dramatically augmented for peptide antigens fused to APCH1 and that the induced humoral responses can provide protection to the natural host after challenge with virulent rabbit hemorrhagic disease virus (RHDV) when using sub-protective doses of a subunit vaccinal antigen.

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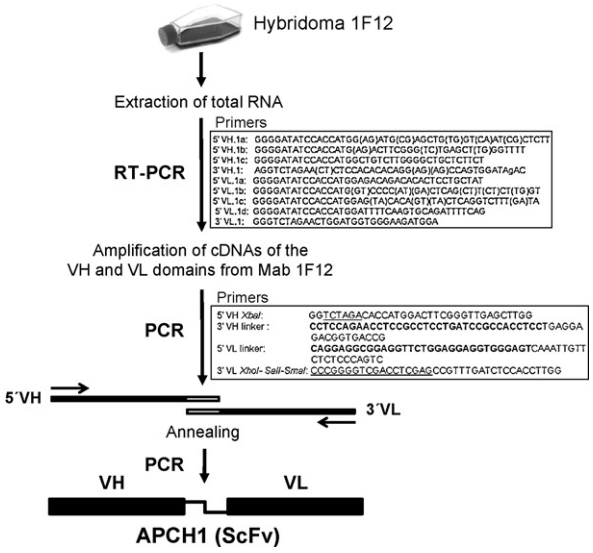

Fig. 1. Cloning strategy of APCH1 single chain antibody from hybridoma 1F12.

2. Materials and methods

2.1. Cloning of VH and VL domain sequences of IF12 and construction of scFv APCH1

The hybridoma producing the monoclonal antibody (mAb) 1F12 (Brodersen et al., 1998; Bullido et al., 1997) with specificity for a porcine MHC II DR molecule epitope was kindly provided by Dr. Javier Domínguez (Biotechnology Department, INIA, Spain). Hybridoma cells were cultured in RPMI 1640 medium supplemented with 0.01 mM piruvic acid (SIGMA), 2 mM L-glutamine and antibiotics. The recombinant single chain (scFv) version of mAb 1F12 was obtained as previously described (Froyen et al., 1993; Chaudhary et al., 1990) (Fig. 1). Briefly, total RNA was extracted from disrupted cells according to the Qiagen RNeasy extraction kit (Qiagen) protocol. All samples were subjected to on-column DNase digestion using the Qiagen RNase free DNase treatment (Qiagen) during RNA isolation. Isolated RNA was quantified and 1 μg was used as a template in a reverse transcriptase reaction using AMV-RT polymerase (Promega) to produce the DNA copies (cDNA) from total mRNA obtained from the hybridoma cells.

Copy DNA fragments corresponding to the mAb 1F12 VH and VL domains were obtained by PCR using as template the cDNA described above. For this purpose, a set of primers comprising constant murine VH and VL sequences was used in different PCR DNA amplifications (Fig. 1).

After sequence analysis of some colonies transformed with pGEM VH and pGEM VL, a new set of primers was designed (Fig. 1). These primers allowed us to fuse the VH and VL domain of MAb IF12 into scFv antibody fragments via a flexible and synthetic hinge of Ser-Gly repetition in different PCR steps. The resulting APCH1 construct (scFv from mAb 1F12) was assembled with the VH-(Gly_4-Ser_3)-VL arrangement and cloned into pGEM-T easy vector to obtain pGEM APCH1, which was used to transform DH5α cells. This vector was used to fuse different vaccine polypeptides in frame with the scFv APCH1 antibody.

2.2. Recombinant baculovirus

Production of recombinant baculovirus expressing the RHDV AST/89 VP60 (Barcena et al., 2000) was previously described (Perez-Filgueira et al., 2007). The DNA sequence encoding for the canine parvovirus (CPV) VP2-derived peptide 2L21 (Langeveld et al., 1995; Langeveld et al., 1994) fused to the β-glucuronidase (βGUS) gene was obtained from plasmid pBI2L21-GUS (Gil et al., 2001). The 2L21-GUS chimeric gene was cloned into the baculovirus transfer plasmid *pBacPak* 8 (Clontech) under control of the polyhedrin promoter, using the restriction enzyme sites *Xba*I and *EcoR*I. DNA sequences for the full length VP60 protein from RHDV AST/89 strain or for the 2L21 epitope flanked by *Xba*I and *Sma*I restriction sites were produced by PCR and translationally fused to 3′ end of the APCH1 gene through a pGem intermediate plasmid (Clontech). Both APCH1-fusion genes were finally transferred to the *pBacPak 8* transfer vector and corresponding recombinant baculoviruses were obtained by homologous recombination as previously described (Oviedo et al., 1997). A *pBacPak 8* vector without insert was used to produce a control baculovirus (BacWT) following the same protocol. Recombinant baculoviruses were propagated and amplified in sf21 insect cells to reach infective titers of $\sim 10^8$ pfu/ml (O'Reilly et al., 1992) and stocks were kept at 4 °C for daily use and at −80 °C for long-term storage.

2.3. Insect growth conditions and inoculation

Trichoplusia ni (*T. ni*; Cabbage looper) larvae were reared following previously described methodology (Gomez-Sebastian et al., 2008; Perez-Filgueira et al., 2006). For all experiments, fifth-instar larvae (last instar larvae before pupation) of about 300 mg weight, were injected near the proleg (forward the body cavity) with 10 μl of recombinant baculovirus diluted to reach the number of pfu per dose selected. Infected larvae were kept in growth chambers at 28 °C and collected at the indicated times. Larvae were immediately frozen and kept at −20 °C until processed.

For time–dose assays, groups of 20 larvae were infected with 10^5, 10^4 or 10^3 pfu of the different baculoviruses, separated into 4 groups of 5 larvae each and finally processed at 24, 48, 72 and 96 hpi. Total protein extracts were analyzed for production of APCH1-fusion proteins by Western blot assay.

2.4. Analysis of protein extracts

Total soluble protein (TSP) fractions were obtained from *T. ni* larvae as described previously (Perez-Filgueira et al., 2006). Total concentration of extracted protein was quantified by Bradford assay (Bradford, 1976).

Quantification of recombinant proteins production in larva was performed by capillary electrophoresis using the Experion system (Bio-Rad, USA) following the manufacturer's instructions. Briefly, crude protein samples (1 μl) were mixed with sample buffer provided, heated and diluted to 1/90 in deionized water, and 6 μl of each diluted sample was loaded in Pro260 chips (Bio-Rad, USA) for analysis.

For Western blot assays, 20 μg of TSP per lane were resolved by SDS-PAGE and transferred into nitrocellulose membranes (Bio-Rad, USA). Membranes were blocked overnight at 4 °C with PBS–0.05% Tween 20 (PBST) and 4% skim milk (blocking buffer, BF) and then incubated at room temperature (RT) for 1 h using monoclonal antibodies (mAbs) 2E11 for VP60 and 3C9 for peptide 2L21 at 1:200 (Ingenasa, Spain). Blots were then washed 3 times with PBST and anti-mouse IgG-horseradish peroxidase (HRP)-labeled conjugate diluted 1:4000 in BF (KPL, UK) was finally added for 1 h as secondary antibody. After extensive washing with PBST, protein bands were detected using ECL Western-blotting detection system on Hyperfilm ECL films (Amersham, USA).

2.5. Analysis of recombinant APCH1-2L21 molecule binding to antigen-presenting cells

To determine whether recombinant APCH1 scFv retained the MHC II-binding capacity; we carried out fluorescence and immuno-

histochemical analyses using a baculovirus-derived APCH1-2L21 fusion protein. Glutaraldehyde fixed alveolar macrophages primary cultures were incubated overnight at 4 °C with 100 μg of TSP from infected larvae expressing the recombinant APCH1-2L21 protein (~6 μg of recombinant protein). The next day, cells were washed with PBST and incubated with the 2L21-specific mAb 3C9 (Ingenasa, Spain). Positive and negative controls were included by incubating samples with the original mAb 1F12 at a concentration of 10 μg/ml or using TSP extracts from larvae infected with BacWT baculovirus, respectively. Detection of immunocomplexes was carried out after an additional washing step. For fluorescence assays, fixed cells were incubated with a fluorescein-conjugated (Alexa FluorTM488) goat anti-mouse Ig serum (Molecular Probes) diluted 1:1000 in PBS. For immunohistochemical assays, samples were incubated with HRP-labelled goat anti-mouse Ig conjugate (Amersham Pharmacia) at the same dilution and immunocomplexes were detected using 3,3′-diaminobenzidine (DAB) as substrate. Microscopy was carried out in a Leica photomicroscope with a digital camera and digitized images were obtained with Qwin program (Leica Microsystems, Germany).

2.6. Analysis of immunogenicity of the APCH1-fusion proteins

Adult female Swiss mice ($n = 4$ per group) were immunized intraperitoneally on days 0, 15 and 30 with crude larvae extract containing 2 μg of APCH1-2L21 protein. Two other groups of mice were equally immunized with 2 μg of synthetic 2L21 peptide or recombinant protein 2L21 fused to β-glucuronidase (β-GUS) antigen as a carrier. A third control mice group was immunized with the same amount of larvae extracts but infected with the BacWT baculovirus. Vaccines for primo-vaccinations were formulated using Freund's complete adjuvant and booster formulations were produced with incomplete Freund adjuvant. Two weeks after the last immunization, mice were bled and the sera analyzed for the anti-2L21 antibodies using a previously described indirect ELISA carrying the 2L21 synthetic peptide as detecting antigen absorbed to the plates (Gil et al., 2001).

Similar experiments were done in mice ($n = 4$ per group) but using 2 μg of VP60 or APCH1-VP60 as vaccinal antigens and following the same immunization protocol as above. Anti-VP60 antibodies induced were determined by a commercial ELISA test (Ingezim RHDV DAS, Ingenasa, Spain).

In all cases, antibody titers were expressed as the log 10 of reciprocal of the maximum serum dilution that yield at least 2.5 times the OD values of preimmune sera.

Four two-month-old New Zealand White rabbits ($n = 2$ per group), free from anti-RHDV antibodies, were used to compare the immune response and protection induced by the APCH1-VP60 antigen vs. the VP60 alone. A single dose of 10 μg of VP60 (Perez-Filgueira et al., 2007) or APCH1-VP60 proteins were used to immunize the rabbits diluted in PBS and in absence of any adjuvant. A single dose of antigen was administered by intramuscular route and all rabbits were challenged intramuscularly with 100 LD50 of homologous RHDV (Spanish isolate AST/89) at 30 days post vaccination (dpv). Blood samples were collected from the marginal ear vein of each animal at 15 and 30 dpv and 15 days after challenge. Anti-VP60 antibody analysis was carried out by the same commercial ELISA test used for mice (Ingezim RHDV DAS, Ingenasa) and antibody titers were also expressed as described above.

3. Results

3.1. Production of recombinant APCH1 fusions by baculovirus in T. ni larvae

As it is shown in Fig. 1, the scFv APCH1 was produced as a single construct fusing the light (V_L) and heavy (V_H) chain variable domains from mAb 1F12 (Brodersen et al., 1998; Bullido et al., 1997), separated by a flexible linker peptide. This construct, corresponding to a ~26 kDa polypeptide, was translationally fused to the coding sequences of the canine parvovirus-derived 2L21 peptide and RHDV VP60 capsid protein. Recombinant baculoviruses, BacAPCH1-2L21 and BacAPCH1-VP60, carrying the corresponding chimeras, were produced and further amplified in sf21 cells to reach titers higher than 10^8 pfu/ml.

Experiments were carried out in order to determine optimal conditions for production of these recombinant fusion proteins in insect larvae. Fifth-instar larvae were inoculated with 3 different doses of recombinant baculoviruses (10^3, 10^4, and 10^5 pfus/dose), processed at different times post-inoculation and analyzed by SDS-PAGE and Western blot. Infected larvae extracts analysis showed that APCH1-2L21 and APCH1-VP60 recombinant proteins were easily identified as major reacting bands of 28 and 72 kDa respectively (Fig. 2). Recovery yields were dependant on the concentration of baculovirus inoculated, being 10^5 pfus per dose the best condition for both chimeras (data not shown).

Both baculoviruses showed maximum expression levels at 72 hpi (Fig. 2). Recombinant protein expression levels were of about 2.8 and 3.2 mg/g of insect fresh biomass for APCH1-2L21 and APCH1-VP60 respectively.

3.2. Analysis of the recombinant APCH1 capacity to bind MHC Class II molecules on APCs

Alveolar macrophages express abundant MHC II DR molecules on their surface. To demonstrate the functionality of recombinant APCH1 scFv fused to vaccine antigens, we incubated fixed non-permeabilized alveolar swine macrophages with recombinant protein APCH1-2L21, and the staining pattern was compared to that obtained with mAb 1F12. The binding of APCH1-2L21 to MHC II DR molecules was detected using a secondary antibody (3C9) specific for an epitope of the peptide 2L21.

Recombinant fusion APCH1-2L21 protein bound to the macrophages showed in all cases a characteristic surface staining pattern. This peripheral labeling was observed by both immunohistochemistry and immunofluorescence (Fig. 3A3 and B3). This staining was also very similar to that found with mAb 1F12 (Fig. 3A2 and B2), suggesting that recombinant APCH1 fused to epitope 2L21

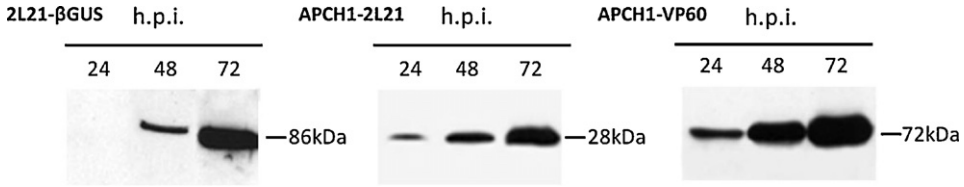

Fig. 2. Production of recombinant fusion proteins 2L21-βGUS, APCH1-2L21 and APCH1-VP60 in *Trichoplusia ni* larvae. Fifth-instar larvae were inoculated with 10^5 infectious doses of recombinant Bac2L21-βGUS, BacAPCH1-2L21 or BacAPCH1-VP60 baculoviruses. Infections progressed for 24, 48 and 72 h post-infection and total protein extracts were collected at the times indicated. Crude preparations were resolved by SDS-PAGE (20 μg of TSP/lane) and analyzed by Western blot using mAb 3C9 for peptide 2L21 and 2E11 for VP60.

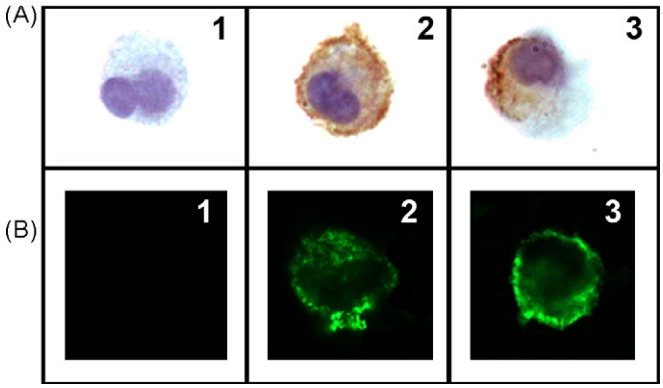

Fig. 3. Binding of fusion protein APCH1-2L21 to alveolar pig macrophages in comparison to mAb 1F12 from which derived the single chain antibody APCH1. Surface decorated macrophages were visualized by immunohistochemistry (A) and immunofluorescence (B). Figure shows binding to MHC Class II molecules on surface of macrophages of mAb 1F12 (2) or APCH1-2L21 (3) in comparison to control cells incubated with an irrelevant antibody (1). The binding of APCH1-2L21 was detected using a secondary antibody directed to an epitope of the peptide 2L21 (mAb 3C9).

was fully functional, and recognized MHC II molecules on the surface of swine alveolar macrophages APCs.

3.3. *APCH1 molecule potentiates the antibody immune response to the fused subunit vaccine antigens*

Two model antigens were assayed in mice and rabbits to study whether APCH1 molecule may facilitate antigen presentation to the immune system and concomitantly enhance specific humoral responses. In a first set of experiments, groups of mice were immunized with vaccines containing the synthetic peptide 2L21 alone or fused to β-GUS, following the immunization protocol described in Section 2. ELISA results from sera obtained from these animals 15 days after the last immunization, showed an increased antibody response elicited by the APCH1-2L21 antigen compared to those of the 2L21 alone or fused to β-GUS (Fig. 4). Antibody titers induced by the APCH1-2L21 chimera were ~600 times higher than those elicited by the synthetic 2L21 peptide alone and ~25 times higher than for the fusion protein β-GUS-2L21. In a second set of experiments, groups of mice were immunized with vaccines containing the VP60 antigen alone or fused to APCH1. An enhanced antibody response was also obtained in mice immunized with antigen APCH1-VP60 with respect to those immunized VP60 protein alone. In this case, APCH1 molecule increased two times the antibody titers against VP60 (Fig. 4).

To test if APCH1 molecule could act as an adjuvant in other animal species, rabbits were immunized with a single dose of recombinant VP60 protein (10 μg) in the absence of adjuvants. This dose was previously determined as non-protective against RHDV challenge (Perez-Filgueira et al., 2007). A second group of animals were immunized with an identical dose of APCH1-VP60 fusion protein. Rabbits immunized with APCH1-VP60 showed higher antibody responses than those observed in animals immunized with non-fused VP60 protein (Fig. 4). Interestingly, rabbits immunized with APCH1-VP60 survived up to 54 dpi to the infective RHDV challenge (rabbits #1 and #2), while rabbits (#3 and #4), vaccinated with the sub-protective dose of recombinant VP60, died at 48 and 60 h after virus challenge showing characteristic clinical signs of RHDV. The non-protected rabbits showed clinical signs typical of RHD (apathy, dullness, ocular haemorrhaging and cyanoses of mucous membranes, ears and eyelids, anorexia, increased respiratory rate, convulsions, ataxia, posterior paralysis). The deaths from rabbit haemorrhagic disease (RHD) usually occurred 2–3 min after the onset of neurological signs. Necropsies were performed on death rabbits and gross pathological observations were typical of RHD (pale yellow or greyish liver with marked lobular pattern, petechial and echymotic, multifocal haemorrhages of the lung, lung oedema, lung congestion, splenomegaly, poor blood coagulation, swollen, dull, pale to patchy reddish discolouration of the kidney).

4. Discussion

Antigens may be targeted to APCs by conjugating them to antibodies directed against surface molecules on APCs (Fossum et al., 1992). This methodology was originally developed to enhance immune responses with low doses of antigen and without the use of adjuvants (Carayanniotis and Barber, 1987). It was also described to bias immune responses towards specific directions such as antibody production (Carayanniotis and Barber, 1990), $CD4^+$ T cell stimulation (Schjetne et al., 2003) or even cytotoxicity-mediated immunity (Sloots et al., 2008). Albeit immunotargeting has been assayed against a range of surface molecules, efficiency in inducing the expected response will vary with the targeted molecule (Snider and Segal, 1989). In particular, targeting to MHC II molecules has been shown to strongly enhance primary humoral responses, both systemic and mucosal (Carayanniotis et al., 1988; Snider et al., 1997).

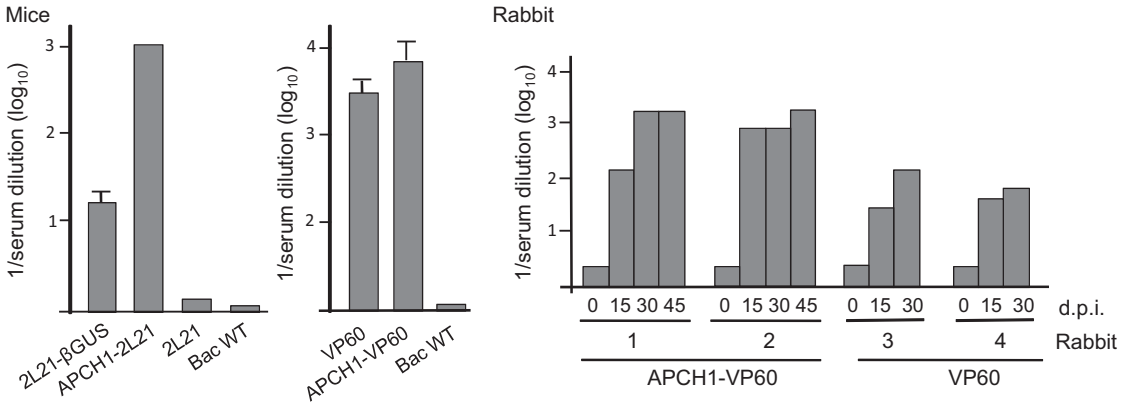

Fig. 4. Antibody response to peptide 2L21, recombinant 2L21-βGUS, APCH1-2L21, VP60 and APCH1-VP60 in mice intraperitoneally immunized with an oil formulation with Freund's complete adjuvant in the first immunization and incomplete in the following. This figure also shows the antibody immune responses obtained in rabbits (2 animals per group) immunized intramuscularly with a subimmunogenic dose of 10 μg of VP60 protein or with an identical dose of recombinant APCH1-VP60 fusion protein, in both cases in absence of adjuvant. Animals received a single dose and were challenged with infective RHDV at 30 dpi. Serum samples were taken at 15, 30 and 45 dpi and analyzed by ELISA. Rabbits immunized with VP60 died after RHDV infection, while rabbits immunized with the same dose of APCH1-VP60 survived to the virus infection and presented none or low increase in antibody titers measured 15 days after challenge exposure.

Mechanisms of intracellular trafficking previously described for B lymphocytes could be used to explain how MHC II targeted proteins and peptides may induce an enhanced antigen presentation (Weber et al., 1990). According to this, newly synthesized class II molecules may be targeted to endosomal compartments following transport to the cell surface and rapid internalization. Support for this hypothesis was obtained in studies showing that the N-terminal endosomal targeting signal in the cytoplasmic domain of class II-associated invariant chains (Ii) also functions as an efficient internalization signal, resulting in rapid endocytosis of MHC II-Ii complexes from the cell surface into endosomal compartments in which Ii dissociates from the complex (Roche et al., 1993). Once there, they get in contact with processed antigens and eventually they may bind peptides that will be later exposed on the cell membrane. In this way, MHC II-targeted antigens may follow this endocytic route to reach intracellular compartments where they can be processed and associated to other MHC II molecules for antigen presentation (Snider and Segal, 1989).

In this work, we generated a scFv (APCH1) from a mAb (1F12) with specificity against an invariant epitope located in the β-chain of the DR molecule of the MCH II antigen of swine (Bullido et al., 1997). To demonstrate the adjuvant properties of APCH1 we selected two well characterized veterinary vaccine antigens. The first one consisted of a linear antigenic peptide derived from the VP2 capsid protein of canine parvovirus. This 21-mer peptide has been shown to be effective in protection of dogs and minks against parvovirus infection (Langeveld et al., 1995; Langeveld et al., 1994). Our laboratory has followed different approaches to increase the immunogenicity of this peptide, including its fusion to the carrier molecule β-glucuronidase (Gil et al., 2001) or to a tetramerization domain derived from P53 cellular protein (Gil et al., 2007). In both cases, this vaccine immunogenicity was increased. In the present work, we demonstrated that APCH1 molecule may increase the immune response against 2L21 peptide with respect not only to the peptide alone but also fused to the carrier molecule β-glucuronidase.

The second vaccine antigen utilized in this study was the VP60 capsid protein from RHDV. This antigen constitutes the only RHDV protein containing epitopes involved in the induction of neutralizing antibodies during infection. Consequently, a number of recombinant subunit vaccines were developed based on this protein produced in different expression systems (Laurent et al., 1994; Boga et al., 1997; Castanon et al., 1999; Fernandez-Fernandez et al., 2001; Martin-Alonso et al., 2003; Gil et al., 2006). In a previous work, we have efficiently expressed this protein in insect larva as living biofactories and we determined the minimum protective dose in presence or absence of Freund's adjuvant (Perez-Filgueira et al., 2007). Interestingly, in this study we did not only obtained higher antibody titers in immunized rabbits with the APCH1-VP60 fusion protein with respect to VP60 alone, but also able to protect the rabbits with a subimmunogenic dose. It is of particular interest considering that subunit vaccines developed for rabbits have to be produced at very low cost. By reducing the vaccine dose needed to protect animals we are decreasing the vaccine production costs. Also it is relevant that in the case of the RHDV vaccine we have not used any other adjuvant than APCH1 and rabbits were protected without increase of specific RHDV antibodies after virus challenge. It suggests that the virus replication in infected animals was efficiently controlled by antibodies generated by the vaccine, indicating a very solid protection.

In conclusion, our results suggest that targeting antigens to APCs through the APCH1 molecule could enhance humoral immune responses against peptide and subunit antigens in two different animal models. These results may provide a proof of principle for other vaccine antigens. Moreover, the fact that the original mAb 1F12 is specific to an invariant epitope preserved in other mammals (Brodersen et al., 1998), opens the possibility of widespread use of APCH1 in humans and other animal species, as a new adjuvant molecule of general use. Current studies are in progress to analyze the adjuvant properties of APCH1 for subunit vaccines in cattle and swine.

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