Accelerated Publications

Coxsackievirus and Adenovirus Receptor (CAR) Binds Immunoglobulins

Steven D. Carson* and Nora M. Chapman

Department of Pathology and Microbiology, University of Nebraska Medical Center, 986495 Nebraska Medical Center, Omaha, Nebraska 68198-6495

Received July 9, 2001; Revised Manuscript Received August 28, 2001

ABSTRACT: The coxsackievirus and adenovirus receptor protein (CAR) serves as the cell surface receptor for group B coxsackieviruses and most adenoviruses, but the physiological function and ligand for this protein remain to be described. An affinity column was constructed with the recombinant extracellular domain of the CAR (rECAR) to isolate potential ligands by affinity chromatography. Immunoglobulins G and M were consistently isolated from human sera passed through the column, suggesting that the CAR may be an immunoglobulin-binding protein. Further investigation revealed that the affinity-purified immunoglobulins bound to rECAR-coated immunoassay plates, and the peroxidase-labeled rECAR bound the immunoglobulins on ligand-overlay blots. The peroxidase-labeled rECAR was incorporated into immunoprecipitates formed between the affinity-purified immunoglobulins and rabbit antibodies against human immunoglobulins, but not into immunoprecipitates formed between mouse IgG and rabbit antibodies against mouse IgG. The CAR present in HeLa cell lysates also bound to the affinity-purified immunoglobulins on Immobilon membranes, showing that the association is not limited to the recombinant protein. These results demonstrate that the CAR binds IgG and IgM present in serum, and reveal a direct interaction between the coxsackievirus and adenovirus receptor and the immune system.

Group B coxsackieviruses (CVB)[1] and adenoviruses (Ad) cause a variety of human diseases, including inflammatory heart disease, in which immune cell infiltrates and antibodies against heart antigens have been found (*1*, *2*). CVB have recently been developed for delivery of cytokines and as potential vehicles for vaccination (*3*, *4*), and Ad have received considerable attention as vectors for delivery of therapeutic genes and vaccines. The cell surface protein that binds CVB and Ad [coxsackievirus and adenovirus receptor (CAR)] is an immunoglobulin superfamily protein (*5*−*7*), for which the physiological function has not yet been established. Elucidation of CAR function and identification of its natural ligand should be of pressing interest in view of the widespread efforts toward using Ad-based vectors for gene therapy as well as inadequate knowledge of the pathologies associated with CVB and adenovirus infections.

EXPERIMENTAL PROCEDURES

cDNA encoding the extracellular domain of the CAR, beginning with the amino-terminal Leu (*7*), was modified by polymerase chain reaction (PCR) amplification from an IMAGE consortium clone (GenBank accession number N31467 from a human cDNA library constructed by B. Soares and M. Fatima Bonaldo from normal foreskin melanocytes). Primers (RCPTRPR4, 5′GGCTCGAGCTGAGTATCACTACACCTGAAGA; and RCPTRPR5, 5′GCATACGCTCAGCTTTATTTGAAGGAGGGACAAC) were used to add an *Xho*I site at the 5′ end (N119, numbering as in GenBank accession number Y07593) and a *Bpu*1102I site at the 3′ end of the open reading frame (N768). The PCR product was ligated in *Xho*I, and *Bpu*1102I digested, pET-15b+ (Novagen). This construct, upon transformation into *Escherichia coli* strain BL21 (DE3) and induction of T7 RNA polymerase expression with isopropyl β-D-thiogalactopyranoside (IPTG), produces a protein with a 23-amino acid extension at the amino-terminal end. The CAR sequence ends at Ala(217) (GenBank accession number Y07593, signal sequence residues numbered −1 to −19) with an additional GluGln pair before the stop codon. The MGSSHHHHHH-SSGLVPRGSHMLE-(CAR 1−217)-EQ protein was isolated from inclusion bodies using nickel-chelate affinity chromatography, and is called the rECAR.

The rECAR, protein A, hemocyanin (*L. polyphemus*, Sigma), and monoclonal antibody RIg9 (*8*) were coupled to horseradish peroxidase (Sigma) using the two-step glutaraldehyde procedure as described by Engvall (*9*). Peroxidase-labeled rabbit anti-mouse immunoglobulins (adsorbed to improve specificity) were from DAKO.

Affinity columns were prepared by coupling the rECAR, in 0.1 or 0.2 M $NaHCO_3$, to AffiGel-10 (Bio-Rad). The rECAR (1−2 mg) was added for each milliliter of AffiGel and allowed to couple overnight at 4 °C. Concentrated Tris buffer [0.5 M Tris, 1 M NaCl, 0.2% NaN_3 (pH 7.6); 10×

* To whom correspondence should be addressed. Phone: (402) 559-4710. Fax: (402) 559-4077. E-mail: scarson@unmc.edu.

[1] Abbreviations: CVB, coxsackievirus B; CAR, coxsackievirus and adenovirus receptor; Ad, adenovirus; rECAR, recombinant extracellular domain of CAR; TBS, Tris-buffered saline; PMSF, phenylmethanesulfonyl fluoride; HRP, horseradish peroxidase; DTT, dithiothreitol.

10.1021/bi015571y CCC: $20.00
Published on Web 11/07/2001

TBS] was added to block reactive sites on the AffiGel. The affinity gel was poured into glass columns and washed with each of the buffers to be used in subsequent affinity chromatography.

Serum was prepared from citrated plasma obtained from the blood bank by adding $CaCl_2$ to a final concentration of 15 mM from a 1 M stock. The plasma was allowed to stand in a glass beaker at 37 °C until it was clotted, and then placed at 4 °C overnight. The clot was disrupted in a blender (low speed), and phenylmethanesulfonyl fluoride (PMSF) was added to a final concentration of 2 mM from an 80 mM stock solution in ethanol. Benzamidine was added to a final concentration of 10 mM. The serum and residual clot were centrifuged in a Beckman JA10 rotor for 1 h at 8000 rpm. The serum was filtered through Whatman No. 1 paper and then through a 0.45 μm filter (Nalgene) before application to the affinity column.

For affinity chromatography, the rECAR−AffiGel complex was equilibrated in TBS with 5 mM $CaCl_2$ and 5 mM benzamidine. Serum was allowed to flow through the column by gravity. The column was washed with 150 mL of the equilibration buffer (>15 column volumes), followed by 100 mL of TBS with 10 mM disodium ethylenediaminetetraacetic acid (EDTA). Proteins bound to the column were then eluted with TBS and 6 M urea. Fractions were assayed for protein using Coomassie G250 (*10*), and those containing protein were pooled and concentrated on YM100 ultrafiltration membranes (Amicon; initial experiments used YM10 membranes).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was adapted from Laemmli (*11*) and used a mini-gel apparatus (Bio-Rad). Proteins were prepared for electrophoresis either without reduction (reducing agent omitted) or with reduction (sample solvent contained 60 mM dithiothreitol). Protein blots were prepared by electrophoretic transfer of samples from polyacrylamide gels to PVDF membranes (Immobilon) (*12*). Blots were blocked with 6% Carnation milk in TBS and 0.1% Tween 20 before incubation with probes. Peroxidase activity was detected on Immobilon membranes using ECL+Plus (Amersham) and BioMax film (Kodak). Developed films and stained gels were documented by computer scanning using a flatbed scanner and Micrografx Picture Publisher software.

ELISA experiments used Immulon 2 96-well plates (Dynatech). Plates were coated with proteins in $NaHCO_3$ (0.1 or 0.2 M) and blocked with 10 mg/mL bovine serum albumin in TBS and 0.1% (v/v) Tween 20. Subsequent washes and protein dilutions were carried out with TBS and 0.1% Tween 20. Peroxidase activity was developed with *o*-phenylenediamine (*9*). Dual peroxidase and protein staining of immunoprecipitates in agarose were as previously reported (*13*), using 4-chloro-1-naphthol as the chromogen.

Native CAR binding to affinity-purified immunoglobulins was demonstrated by ligand-overlay blotting and elution. Affinity-purified immunoglobulins were separated by SDS−PAGE and transferred to PVDF membranes. The blots were cut into sections and incubated with octyl glucoside lysates of HeLa (constitutively express CAR) or RDt3 cells (CAR-negative cells transfected to express a truncated version of the CAR, CARt3) (*14*) to which DMEM was added to a final concentration of 10%, Tween 20 to 0.1%, and Carnation milk to 10%. The IgG and IgM were localized using either the rECAR−HRP or the protein A−HRP conjugate on a separate section of the blot, and the corresponding regions of the experimental sections were excised. Control sections were taken from HRP-negative regions of the blot. The excised membrane pieces were soaked in 50 μL of 3× SDS sample solvent with DTT and heated, and the eluates were collected. Eluted proteins were analyzed for the CAR and CARt3 by probing Western blots with MoAb.E1 (*15*) followed by HRP-labeled rabbit anti-mouse immunoglobulins.

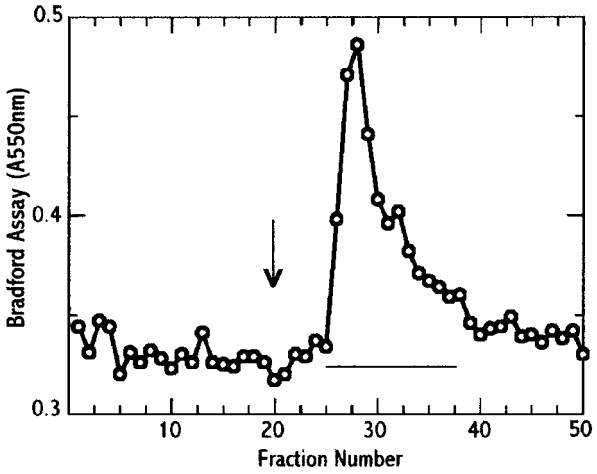

FIGURE 1: Affinity chromatography of serum on the rECAR−AffiGel complex. Serum was passed through the affinity column and the flow-through collected. The column was washed with equilibration buffer. TBS and 10 mM EDTA were applied to the column, and the eluate was collected beginning with fraction 1. TBS and 6 M urea were applied (beginning at fraction 20; arrow). Fractions 25−38 were pooled for analysis of bound proteins eluted with urea. An absorbance peak was observed in fractions 2−8 in some experiments.

Protein sequence analysis was carried out at the University of Nebraska Medical Center Protein Structure Core Facility using automated Edman chemistry.

RESULTS

Affinity chromatography is a highly selective method that can be used to isolate proteins on the basis of known interactions, or to identify unknown proteins on the basis of their interaction with the affinity ligand. We employed this approach to identify ligands for the CAR. Human serum, a potential source of a CAR ligand, was tested for the presence of proteins that bind to the recombinant extracellular domain of the CAR (rECAR) immobilized on AffiGel-10. Protein was consistently eluted from the washed affinity column with 6 M urea (Figure 1). The protein-containing fractions were pooled and analyzed on polyacrylamide gels in the presence of SDS. Three predominant protein bands, corresponding to about M_r values of 25 000, 52 000, and 78 000, were detected with Coomassie blue stain (Figure 2A). Proteins from a similar gel were transferred to PVDF and subjected to amino-terminal sequence analysis. The 78 kDa band returned a sequence of EVQLVESGGG, and the sequence for the 52 kDa band was determined to be EVQLVESGGA. Search of protein sequence databases showed that the sequences correspond to the V region of human immunoglobulin heavy chains. This result suggested that the 78 and 52 kDa bands were the heavy chains of IgM and IgG, and that the third band (25 kDa) was probably the immunoglobulin light chain. To confirm the identities, proteins were transferred from

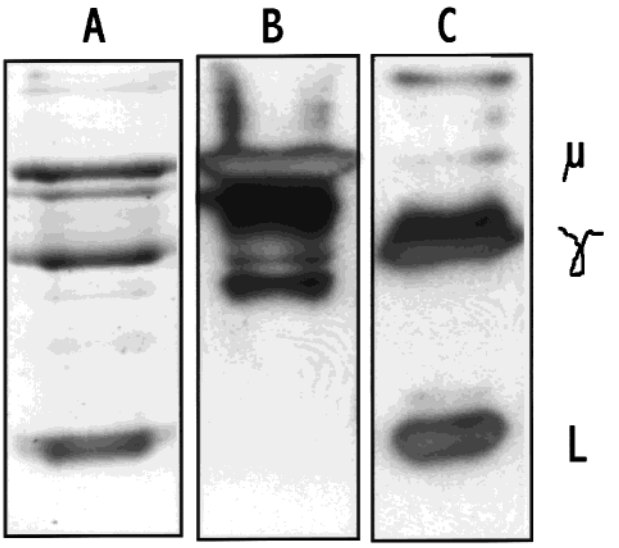

FIGURE 2: SDS−PAGE analysis (10 to 15% polyacrylamide gradient) of proteins eluted from the rECAR-affinity column. Coomassie staining (A) detected proteins near 78, 52, and 25 kDa. Western blots of a similar gel were probed with antibodies against the human immunoglobulin mu chain (B) and anti-human IgG [gamma and light chain reactivity (C)].

polyacrylamide gels to PVDF and probed with antibodies specific for the human mu chain or antibodies against human IgG (gamma and light chains). These blots (Figure 2B,C) confirmed that the proteins that eluted from the rECAR affinity column were indeed IgM and IgG.

The initial series of 17 serum samples from 59 donors (median of three donors per serum preparation) returned essentially pure IgG and IgM from the affinity column in every experiment. On the basis of the quantitative analysis of five of these preparations, recoveries averaged 2 ± 0.4 μg of immunoglobulin protein per milliliter of serum passed over the affinity column. To determine if the affinity chromatography was removing all of the rECAR-binding immunoglobulins from the serum, three sera were passed through the column, and the unbound fractions were rechromatographed. The second chromatography recovered $51 \pm 15\%$ as much immunoglobulin as had been isolated in the initial affinity chromatography. This result showed that the first affinity chromatography had isolated most, but not all, of the rECAR-binding immunoglobulins in the sera, and that the rECAR-binding immunoglobulins were present in serum at a level of of >3 μg/mL.

The results of the experiments described above indicated that only a subpopulation of serum immunoglobulin was binding to the affinity column. To further characterize this subpopulation, preparations of affinity-purified immunoglobulin and samples of human serum were analyzed by Western blotting using antisera specific for IgG isotypes 1−4. The blots showed that IgG_2 was over-represented in the affinity-purified immunoglobulins relative to that present in serum (Figure 3), and IgG_1, the predominant isotype in serum, was under-represented. On the basis of densitometric analysis of the digitized blots, the IgG_2 was present in the affinity-purified sample in an approximately 1.5−2-fold excess relative to its prevalence in serum, IgG_3 was slightly under-represented, and IgG_1 and IgG_4 were under-represented by ~80 and ~70%, respectively.

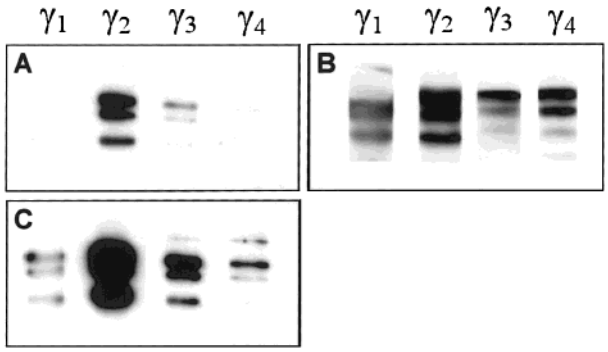

FIGURE 3: Isotype analysis of affinity-purified IgG. Proteins isolated by rECAR-affinity chromatography (A and C) or human serum (B) were applied across the width of a 5 to 15% SDS-PAGE gel without reduction. After electrophoresis, the proteins were blotted to PVDF. Strips cut from the blots were probed with antisera against IgG_1, IgG_2, IgG_3, or IgG_4, and then with an HRP-labeled secondary antibody, and developed by enhanced chemiluminescence. Panels A and B are from 5 s exposures, and panel C is from a 5 min exposure.

The ability of the rECAR to bind the IgG and IgM was demonstrated by methods independent of the affinity column to rule out the possibility that these immunoglobulins bound to the affinity column by nonspecific interactions. Using an ELISA format, wells of Immulon 2 plates were coated with rECAR and incubated with varied concentrations of affinity-purified immunoglobulin. Bound immunoglobulin was detected using the protein A−HRP conjugate. The results (Figure 4A) showed that immunoglobulin bound to rECAR-coated wells, but not to wells containing no rECAR. Immunoglobulins in dilutions of whole human serum also bound to rECAR-coated wells, but not to uncoated wells (Figure 4B). Dilutions of serum from a donor with antibodies against hemocyanin were mixed with HRP-coupled hemocyanin and added to wells coated with the rECAR or blocking reagent. The bound HRP−hemocyanin conjugate was detected only in rECAR-coated wells, and was dependent on the presence of serum containing antibodies against hemocyanin (Figure 4C). This experiment showed that antibodies of known specificity (i.e., anti-hemocyanin) bound the rECAR and that immunoglobulin binding to rECAR occurred even when antigen-binding sites were directed against a known antigen. This result indicates that the immunoglobulins bound to the rECAR are probably not antibodies against the rECAR.

In other experiments, affinity-purified IgG and IgM were transferred from SDS−polyacrylamide gels to PVDF and probed with the rECAR conjugated to HRP. The rECAR−HRP conjugate bound to IgG and IgM on the membrane, and no rECAR−HRP conjugate was detected in other regions of the blot (Figure 5). Intact IgM barely enters the resolving gel, and transfer to PVDF is less efficient than for IgG. Therefore, the relative intensities of ECAR binding to IgG and IgM (Figure 5D−F) are not directly comparable to the intensity of the Coomassie blue-stained IgG and IgM (Figure 5A−C). The rECAR−HRP conjugate did not bind to reduced immunoglobulin heavy or light chains.

To test the specificity of the interaction further, the rECAR−HRP conjugate was examined for its ability to bind affinity-purified immunoglobulins in immunoprecipitates formed by diffusion against specific antibodies. These experiments (Figure 6) showed that the rECAR−HRP conjugate

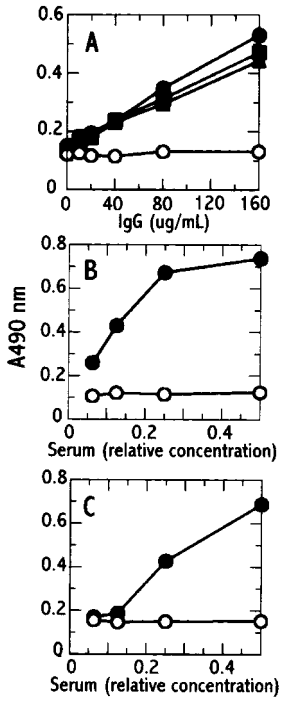

FIGURE 4: Immunoglobulin binding to the rECAR demonstrated by an ELISA. Immulon II plates were coated with the rECAR and then blocked with a TBS/BSA/Tween mixture (black symbols) or with the blocking solution alone (white circles). The rECAR was used at 5.6 μg/mL (B and C), and at 1.25, 2.5, and 5 μg/mL [●, ■, and ▲, respectively (A)]. In panel A, serial dilutions of affinity-purified immunoglobulin were added to the wells, and bound immunoglobulin was detected with the protein A−HRP conjugate. In panel B, serum was diluted in TBS and Tween (0.5 relative concentration corresponds to 50% serum). Bound immunoglobulin was detected with the protein A−HRP conjugate. In panel C, serum from a donor with antibodies to hemocyanin was diluted and added to the wells in the presence of HRP-labeled hemocyanin. Bound antibody−hemocyanin complexes were detected by the HRP activity.

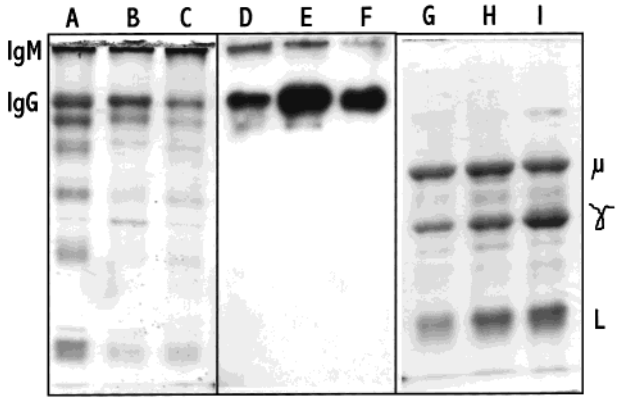

FIGURE 5: SDS−PAGE (5 to 10% gel) and ligand-overlay blot analysis of affinity-purified immunoglobulins. Three affinity-purified protein preparations were separated by SDS−PAGE, in triplicate, with one set reduced (lanes G−I). Lanes A−C and G−I were stained with Coomassie blue. Proteins in lanes D−F were transferred to PVDF and probed with HRP-labeled rECAR.

associated with immune complexes formed by affinity-purified immunoglobulins and antisera against human immunoglobulins, but was not associated with precipitates formed by the interaction between anti-mouse immunoglobulins and an irrelevant mouse monoclonal antibody. Other immunodiffusion experiments, using varied ratios of the anti-human

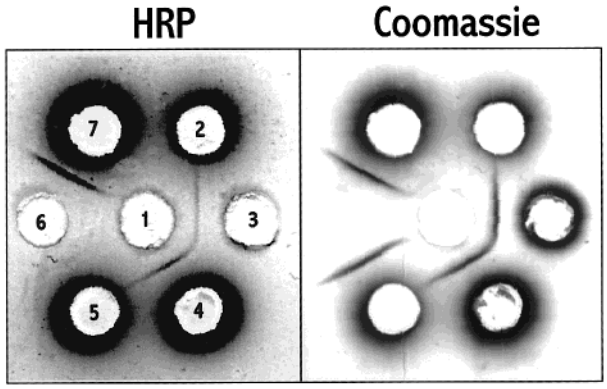

FIGURE 6: Immunodiffusion shows HRP-labeled rECAR association with affinity-purified immunoglobulins. HRP-labeled rECAR was added with samples in wells 2, 4, 5, and 7. In addition, the wells contained (1) rabbit anti-human immunoglobulins, (2) the rECAR−HRP conjugate alone, (3 and 4) affinity-purified immunoglobulins, (5) irrelevant IgG_1 mouse monoclonal immunoglobulin, (6) rabbit anti-mouse immunoglobulins, and (7) MoAb.E1 monoclonal antibody against the rECAR. After the wells had been washed to remove soluble protein, 4-chloro-1-naphthol was used to detect HRP-labeled rECAR (left panel), and then the gel was stained with Coomassie blue to detect all of the protein (right panel).

immunoglobulin and the rECAR−HRP conjugate, failed to form immunoprecipitates between the anti-human immunoglobulins and the rECAR−HRP conjugate in the absence of human immunoglobulins. The inability of the rECAR-binding immunoglobulins to form immunoprecipitates with the rECAR (Figure 6, wells 2 and 3, and titration results not shown) supports the argument that these are not antibodies against the rECAR, and is consistent with the rECAR binding of antibodies against hemocyanin (Figure 4C).

Additional experiments were conducted to determine whether the immunoglobulins isolated on the rECAR also bind the fully processed CAR (e.g., glycosylated and without the added amino-terminal extension) expressed by cultured cells. Affinity-purified immunoglobulins were transferred to PVDF from SDS−polyacrylamide gels run without reduction. Lanes of the blot were incubated with lysates of HeLa cells (source of the CAR), or lysates of RDt3 cells [which express a truncated CAR missing the native cytoplasmic domain, CARt3 (*14*)]. The IgG and IgM were located on control strips of the blot using either the protein A−HRP or rECAR−HRP conjugate, and corresponding regions of the experimental lanes were excised and eluted with SDS sample solvent containing DTT. To control for nonspecific binding of the CAR to the blocked membrane, regions of adjacent lanes in which protein was not loaded were also excised and eluted. Western blots of the eluted samples (Figure 7) showed that the CAR, or CARt3, was recovered from regions of the blot containing IgG and IgM. In multiple experiments, the CAR and CARt3 were either undetectable or greatly diminished in samples from control regions of the blot. IgG heavy chain (gamma) on the blots (Figure 7) was detected due to cross reactivity with the anti-mouse immunoglobulin antibodies (these anti-mouse antibodies do not detect the CAR or CARt3 in the absence of the monoclonal anti-CAR antibodies). These results confirmed that the authentic CAR expressed by human cells bound the IgG and IgM isolated using the recombinant extracellular domain of the CAR produced in *E. coli*.

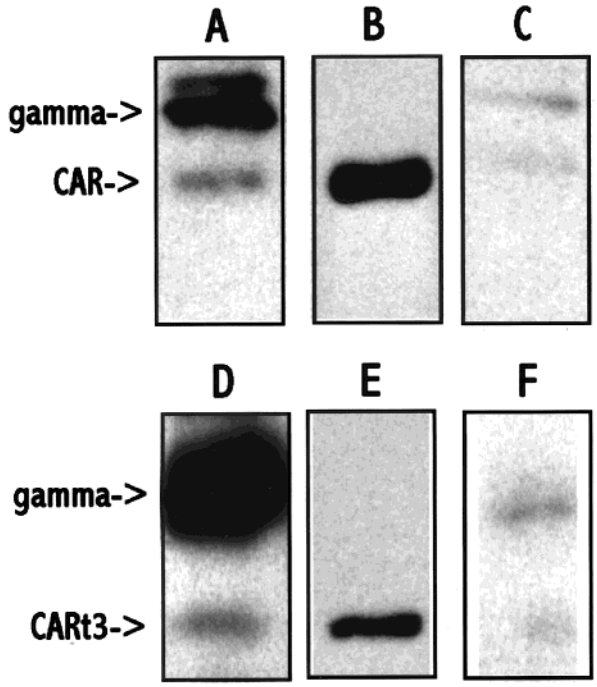

FIGURE 7: Western blot showing that the CAR from HeLa cells and CARt3 from RDt3 cells are eluted from affinity-purified immunoglobulins after ligand-overlay blotting. The CAR was eluted from the IgG and IgM regions of the blot (A), but not from the control regions of the blot (C) incubated with HeLa lysate. A blot of the HeLa cell lysate is shown for comparison in panel B. CARt3 was eluted from the IgG and IgM regions of the blot (D), but not from control regions of the same blot (F) incubated with RDt3 lysate. A blot of the RDt3 lysate is shown in panel E.

DISCUSSION

The CAR protein was identified due to its role as the receptor for group B coxsackieviruses and many of the adenoviruses, but its physiological function remains to be determined. Limited data support CAR function in cell−cell interactions, possibly as an adhesion protein, and apparently by homophilic interaction (*16*). A role in cell−cell adhesion would be consistent with the increased level of CAR expression by human umbilical vein endothelial cells as they become confluent in culture (*15*). Other studies found that the growth rate of prostate carcinoma cell lines was inversely related to the levels at which they expressed the CAR (*17*), which is again consistent with an increased level of cell−cell adhesion, and slower growth, of cells expressing high levels of the CAR. On the other hand, the CAR is maximally expressed in rodents in the latest stages of development, peaking near birth and declining thereafter in most tissues (*16*, *18*, *19*), which appears, at least conceptually, to contradict the high-level CAR−low-growth rate correlation. Clearly, understanding the physiological role of the CAR requires identification of its natural ligand(s) and elucidation of the biological sequelae to the CAR−ligand association.

We approached the identification of natural CAR ligands using affinity chromatography on the solid-phase rECAR to isolate CAR-binding proteins from various sources. When serum was used as the source material, three principal bands were detected on polyacrylamide gels. Given the absence of major serum proteins (e.g., albumin), it appeared that these three proteins were specifically bound to and eluted from the rECAR affinity column. The subsequent experiments conclusively identified these three proteins as the heavy chains of IgG and IgM and immunoglobulin light chains, and confirmed that IgG and IgM bind the rECAR as well as the CAR present in lysates of cultured human cells. CAR-binding immunoglobulins were present in every serum sample that was studied, indicating that the CAR has inherent immunoglobulin binding capacity, as it is unlikely that this number of healthy donors would all have antibodies against this cell surface protein. The recombinant rECAR is isolated from *E. coli*, so it is unlikely that the immunoglobulins which bind the rECAR and CAR are naturally occurring antibodies against carbohydrates. Moreover, antibodies against a known antigen (hemocyanin) bound the rECAR.

That the CAR binds immunoglobulins is particularly intriguing since strong evidence implicates pathological dysregulation of the immune system as an etiological factor in CVB-related diseases (*1*, *2*, *20*). A relationship between the CAR and the immune response has recently been suggested from studies showing increased levels of expression of the CAR at sites of inflammation (*21*, *22*). While we have established that the CAR binds immunoglobulins, there are multiple immunoglobulin-binding proteins and the CAR is not unique in this regard. Moreover, other immunoglobulin superfamily proteins have been found to bind multiple ligands, and there may be ligands for the CAR in addition to immunoglobulins. For example, CD4 shares some structural homology with the CAR and also binds immunoglobulins (*23*, *24*). CD4 also binds MHC II, which is probably its principal ligand (*25*). Notably, CD4 also serves as a virus receptor (*26*).

The basis for selective binding of IgG_2 and IgM, as well as the small amounts of immunoglobulin bound to rECAR, remains to be explained. The low recovery is not without precedent, however, as a recent report has shown that echicetin is bound by a fraction of IgM present in plasma at 2 μg/mL, and the IgM−echicetin complex can aggregate platelets (*27*). An increased level of expression of the CAR in response to experimentally induced inflammation (*22*) and the immunological processes documented in viral myocarditis suggest an association between CAR expression and the immune response (*21*). Results described in this study support this suggestion, and reveal that the relationship may be more intimate than anticipated.

ACKNOWLEDGMENT

We thank Mr. Justin Hobbs for technical assistance with this work.

REFERENCES

1. Gauntt, C. J. (1997) in *The Coxsackie B Viruses* (Tracy, S., Chapman, N. M., and Mahy, B. W. J., Eds.) pp 259−282, Springer-Verlag, Berlin.
2. Schwimmbeck, P. L., Huber, S. A., and Schultheiss, H.-P. (1997) in *The Coxsackie B Viruses* (Tracy, S., Chapman, N. M., and Mahy, B. W. J., Eds.) pp 283−303, Springer-Verlag, Berlin.
3. Chapman, N. M., Kim, K. S., Tracy, S., Jackson, J., Hofling, K., Leser, J. S., Malone, J., and Kolbeck, P. (2000) *J. Virol. 74*, 7952−7962.
4. Chapman, N. M., Ragland, A., Leser, J. S., Hofling, K., Willian, S., Semler, B. L., and Tracy, S. (2000) *J. Virol. 74*, 4047−4056.

5. Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997) *Science 275*, 1320−1323.
6. Tomko, R. P., Xu, R., and Philipson, L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3352−3356.
7. Carson, S. D., Chapman, N. M., and Tracy, S. M. (1997) *Biochem. Biophys. Res. Commun. 233*, 325−328.
8. Carson, S. D., Carson, S. M., and Konigsberg, W. H. (1983) *J. Biol. Chem. 258*, 9510−9513.
9. Engvall, E. (1980) *Methods Enzymol. 70*, 419−439.
10. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
11. Laemmli, U. K. (1970) *Nature 227*, 680−685.
12. Towbin, H., Theophil, S., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
13. Carson, S. D. (1985) *Anal. Biochem. 145*, 148−150.
14. Carson, S. D. (2000) *FEBS Lett. 484*, 149−152.
15. Carson, S. D., Hobbs, J. T., Tracy, S. M., and Chapman, N. M. (1999) *J. Virol. 73*, 7077−7079.
16. Honda, T., Saitoh, H., Masuko, M., Katagiri-Abe, T., Tominaga, K., Kozakai, I., Kobayashi, K., Kumanishi, T., Watanabe, Y. G., Odani, S., and Kuwano, R. (2000) *Mol. Brain Res. 77*, 19−28.
17. Okegawa, T., Li, Y., Pong, R.-C., Bergelson, J. M., Zhou, J., and Hsieh, J.-T. (2000) *Cancer Res. 60*, 5031−5036.
18. Tomko, R. P., Johansson, C. B., Totrov, M., Abagyan, R., Frisen, J., and Philipson, L. (2000) *Exp. Cell Res. 255*, 47−55.
19. Nalbantoglu, J., Pari, G., Karpati, G., and Holland, P. C. (1999) *Hum. Gene Ther. 10*, 1009−1019.
20. Huber, S. A., Gauntt, C. J., and Sakkinen, P. (1998) *Adv. Virus Res. 51*, 35−80.
21. Liu, P. P., and Opavsky, M. A. (2000) *Circ. Res. 86*, 253−254.
22. Ito, M., Kodama, M., Masuko, M., Yamaura, M., Fuse, K., Uesugi, Y., Hirono, S., Okura, Y., Kato, K., Hotta, Y., Honda, T., Kuwano, R., and Aizawa, Y. (2000) *Circ. Res. 86*, 275−280.
23. Bewley, M. C., Springer, K., Zhang, Y.-B., Freimuth, P., and Flanagan, J. M. (1999) *Science 286*, 1579−1583.
24. Lenert, P., and Zanetti, M. (1991) *Int. Rev. Immunol. 7*, 237−234.
25. Janeway, C. A. (1991) *Semin. Immunol. 3*, 153−160.
26. McDougal, J. S., Nicholson, J. K., Cross, G. D., Cort, S. P., Kennedy, M. S., and Mawle, A. C. (1986) *Nature 137*.
27. Navdaev, A., Dormann, D., Clemetson, J. M., and Clemetson, K. J. (2001) *Blood 97*, 2333−2341.

BI015571Y