Alternatively Spliced Soluble Coxsackie-adenovirus Receptors Inhibit Coxsackievirus Infection*

Received for publication, October 27, 2003, and in revised form, February 16, 2004 Published, JBC Papers in Press, February 21, 2004, DOI 10.1074/jbc.M311754200

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The coxsackie-adenovirus receptor (CAR) is a transmembrane receptor of the immunoglobulin superfamily whose expression is altered in myocardial and malignant diseases. Soluble isoforms of other adhesion molecules and cytokine receptors have been proven to have significant agonist and antagonist effects on their full-length receptors; however, little is known about soluble CAR receptors. Using reverse transcription-PCR, we identified three CAR isoforms that lack the transmembrane domain and are the result of alternative RNA splicing between exons IV and VII (CAR4/7), exons III and VII (CAR3/7), and exons II and VII (CAR2/7). The three CAR isoforms contain different regions of the extracellular domain of CAR and have C termini that are distinct from the full-length receptors. These alternatively spliced CAR proteins are released from transfected HeLa cells confirming that they are soluble proteins. In addition, the soluble CAR proteins are able to interact with the bacterially expressed extracellular domain of CAR. In addition, CAR4/7 but not CAR2/7 was found to bind to coxsackievirus B3 (CVB3). Each of the three soluble CAR isoforms is able to inhibit CVB3 infection of transfected HeLa cells. Given that soluble CAR isoforms can bind to the extracellular domain of CAR and the CVB3 capsid, they may have significant inhibitory or stimulatory effects on CAR signaling and may have an important role in the host defense against viral infection.

Coxsackie- and adenoviruses are well known pathogens in inflammatory and dilated cardiomyopathy (1, 2). Both of these structurally unrelated viruses use the coxsackie-adenovirus receptor (CAR)1 for cell attachment and internalization (3, 4). Human decay-accelerating factor (CD55) was also identified as an attachment but not entry receptor for CVB1, CVB3, and CVB5 (5, 6), and integrins αvβ5 and αvβ3 promote adenovirus internalization but not virus attachment (7). CAR is a glycoprotein composed of two extracellular immunoglobulin domains, a single hydrophobic α-helical transmembrane domain, and an intracellular region (8). The human CAR gene composed of at least eight exons is located on chromosome 21q11.2 (9). The extracellular domain of CAR is encoded by exons I–VI, whereas the cytosolic region of hCAR1 is encoded by exon VII. A second membranous CAR isoform, hCAR2, is produced by alternative RNA splicing between an internal splicing site of exon VII and exon VIII (10). Although the structure of CAR is fairly well described, the function of CAR, apart from its role as a viral receptor, is only recently being defined. There is evidence that CAR acts as a homophilic adhesion protein and that it is involved in the morphogenesis and differentiation of heart and brain (11, 12). Cohen et al. (13) described the colocalization of CAR with ZO-1 in epithelial tight junctions indicating that CAR is involved in the functional barrier to paracellular movement of macromolecules and ions.

It has been shown that CAR expression is altered in several disease states. For example, myocardial CAR protein is increased in heart diseases (14, 15), and a down-regulation of CAR expression in bladder cancer cells is associated with tumor growth (16).

CAR belongs to the immunoglobulin superfamily (17). Members of this protein group are implicated in embryonic development, immune response, and cell-cell contact. Several also act as viral receptors (18). One of the characteristics of the immunoglobulin domain family is that many of the genes produce soluble receptors that lack the transmembrane and cytosolic domains of the full-length receptor (19). The soluble receptors are produced by proteolytic cleavage of the original membrane-bound receptor or by de novo synthesis from alternatively spliced RNAs that are specific for the soluble receptor (20). The soluble receptors are released from the cell and appear in biological fluids or tissue culture supernatants (21). Because the ligand-binding site is highly conserved in the circulating receptors of different classes, they may act as antagonists in a manner similar to the soluble receptor of TNF-α that inhibits ligand binding to the membranous receptor (22), or they may act as agonists as occurs with the soluble interleukin-6 receptor that has a role in activation of interleukin-6-mediated signaling (23). Thus, the soluble receptors are involved in the regulation of the biological activity of the receptors and their ligands. Soluble receptors have been shown to play a significant role in various disorders, including heart disease (24), malignancies (25), and viral infection (26). Accordingly, there is increasing interest in the understanding of the function of the receptors for diagnostic, prognostic, and therapeutic purposes.

Given the importance of CAR in viral infection, heart disease, and cancer, we have identified and analyzed three alternatively spliced isoforms of CAR that lack the transmembrane region. These proteins can be secreted from cells, bind to the...
extracellular CAR domain, and inhibit coxsackievirus infection in vitro. These results demonstrate that CAR not only acts as a virus receptor during coxsackievirus infection but that soluble isoforms of the receptor may have a role in the antiviral defense of the host.

**EXPERIMENTAL PROCEDURES**

**Western Blot Analysis**—Protein was separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) under denaturing and reducing conditions and transferred onto polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Boston, MA). Native gels were run omitting SDS and reducing agents from the buffer system. Western blot was performed under standard conditions using anti-VP1 antibody (DAKO Corp., Botany, Australia) or affinity-purified, polyclonal CAR-specific antibodies. The CAR antibody was generated by immunization of rabbits with bacterially expressed extracellular domain of murine CAR. Bound antibodies were detected either with the ECL system (Amer sham Biosciences, Piscataway, NJ) and a peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or with alkaline phosphatase-conjugated goat anti-rabbit IgG (Invitrogen) and 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium (Promega, Madison, WI).

**Detection of CAR Isoforms**—1 μg of RNA extracted from HeLa cells was reverse transcribed with the SuperScript Amplification System (Invitrogen). PCR was performed with Elongase (Invitrogen), 5′-CAT-GCGGTCCTCGTGTGCTCGTCTCCGTG-3′ as primer P5′ and 5′-AGGCCTTATAGATAGACCCAT-3′ as P3′(1). PCR products were separated on a 1% low melting agarose gel, appropriate bands were excised, and CAR-specific sequences were re-amplified with P5′ and P3′(2) 5′-AGGTGGAGAGTCGGA-3′ directly on aliquots of melted gel. PCR products were treated with 5 units of Taq polymerase for 10 min at 72°C and were then cloned into the pcDNA2.1 TOPO vector (Invitrogen). All PCR products were sequenced.

**Transfection**—The coding sequences of human CAR2/7 (GenBank™AY072910), CAR3/7 (GenBank™AY072911), and CAR4/7 (AY072912), including their individual stop codons, were amplified by PCR using the corresponding plasmids that were produced as described above. Sequences were cloned between the EcoRI and XbaI site of the pEF6/V5-His A vector (Invitrogen). hCAR1 (GenBank™AY072912), CAR2/7, and CAR4/7 were cloned together with a FLAG tag sequence into a modified pEF6/V5-His A vector (27) using reverse-transcribed mRNA from HeLa cells as template. 40% confluent HeLa cells grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum were transfected with CAR isoform-specific expression vectors using Polyfect (Qiagen, Valencia, CA). Cells were harvested using a scraper 72 h after transfection, and protein was extracted. 150 μl of cell culture medium of the transfected cells was taken at the same time and centrifuged for 10 min at 15,000 × g. The supernatant was precipitated with 10% trichloroacetic acid and washed with ether/ethanol. Precipitates were resolved in 30 μl of SDS page-gel loading buffer. Specimens of culture medium and transfected cells extract were analyzed by Western blotting.

![Fig. 1](https://example.com/fig1.png) **Identification of alternatively spliced CAR isoforms.** A, RT-PCR of HeLa cell RNA using CAR-specific primers P5′ and P3′(1) resulted in four PCR products (a–d, left panel). PCR products were excised from the gel, re-amplified using primer P5′ and P3′(2) and sequenced (right panel). B, PCR product a consisted of the full-length CAR sequence with its 7 exons. Exon I encodes the N-terminal leader sequence of the hCAR1 protein, whereas exon II and III code for immunoglobulin domain, D1, and exon IV and V code for immunoglobulin domain, D2. The transmembrane domain is encoded by the first part of exon VI that contains together with exon VII the sequence for the cytosolic C terminus. The three CAR isoforms are the result of alternative splicing between exon IV (CAR4/7), exon III (CAR5/7), and exon II (CAR2/7), respectively, with exon VII. CAR4/7 contains D1, a part of D2 coded by exon IV, and a C terminus of 62 amino acids that is different from the C terminus of the full-length CAR. This is secondary to a frameshift in exon VII. CAR3/7 consists of D1 and contains the same C terminus as CAR4/7, whereas CAR2/7 does not have a full immunoglobulin-like domain and possesses a C terminus of 19 amino acids that is different from all the others forms of CAR identified. All CAR isoforms contain the N-terminal leader sequence but lack the transmembrane region.
Expression and Purification of CAR-specific Proteins—The coding sequence of the extracellular domain of human CAR (GenBank™ Y07593) and CAR4/7 were amplified by PCR and cloned into the NheI and HindIII restriction sites of the bacterial expression vector pRSET (Invitrogen). His-tagged proteins were expressed in BL21 bacteria at 32 °C for 4 h, extracted in 8 m urea, 100 mM NaCl, 20 mM Hepes, pH 8.0, and purified over a nickel-nitrilotriacetic acid resin (Qiagen). Proteins were dialyzed against 20 mM Hepes, pH 7.2, 137 mM NaCl. Soluble CAR was separated from precipitated protein by centrifugation.

Affinity Chromatography—A column of AminoLink Plus resin (Pierce, Rockford, IL) linked to the extracellular domain of human CAR was loaded with 500 μg of HeLa cell protein extract or 500 μl of cell culture medium from transfected HeLa cells containing CAR2/7, CAR3/7, or CAR4/7 protein and incubated for 1 h at 4 °C. The column was intensively washed with ice-cold lysis buffer and 20 mM Hepes, pH 7.2, respectively, containing 250 mM NaCl and proteinase inhibitors. Bound proteins were eluted with 100 mM glycine, pH 2.5, that were immediately neutralized with 1 x Tris-HCl, pH 8. Eluate was precipitated with 10% trichloroacetic acid, washed with ether/ethanol, and analyzed by Western blot using the CAR-specific antibody. The eluate from the affinity chromatography done with HeLa cell extract was additionally analyzed using antibodies against proteins not known to be associated with CAR, like the adenine nucleotide translocase (ANT), a highly expressed mitochondrial protein, and the elongation initiation factor (eIF4G).

Immunoprecipitation—200 μl of culture medium containing FLAG-labeled CAR2/7 or CAR4/7 from transfected HeLa cells was incubated with 20 μl (3.3 × 10^6 pfu/ml) cesium gradient-purified coxsackievirus B3 for 1 h at 4 °C. Medium from untransfected HeLa cells was used as control. Immunoprecipitation was performed with 20 μl of agarose-coupled anti-FLAG antibody (Sigma) in a total volume of 750 μl of PBS for 4 h at 4 °C. Precipitate was washed five times with PBS, 500 mM NaCl. Coprecipitation was analyzed by Western blot analysis using the anti-CAR or an anti-VP1 antibody (DAKO Corp., Botany, Australia).

100 ng of bacterially expressed and purified His-tagged CAR4/7 protein and 20 μl of CVB3 were incubated in PBS for 1 h at 4 °C. Immunoprecipitation was performed with 0.6 μg of mononclonal anti-His-antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 25 μl of protein G in a total volume of 750 μl of PBS for 2 h at 4 °C. Under shaking. Reactions without an antibody were used as controls. Protein G pellets were washed five times with PBS, 500 mM NaCl, and analyzed by Western blotting.

Coxsackievirus Inhibition Assay—Coxsackievirus B3 was derived from the infectious cDNA copy of the cardiotropic H3 strain of CVB3 (28). HeLa cells were grown in 6-well culture dishes and transfected with human full-length CAR and CAR isoform-specific eukaryotic pHE.parse(3.3 × 10^6 pfu/ml) CVB3 for 2 h at 4 °C. The cell extract was collected after 4 h. The cell extract was loaded with 10% trichloroacetic acid, washed with ether/ethanol, and analyzed by Western blot analysis using the CAR-specific antibody. Uninfected HeLa cells (−) were used as a control. Endogenous full-length CAR (hCAR) was identified in all cell extracts (left panel), but it was not present in the culture media (right panel). The alternatively spliced CAR isoforms, CAR2/7, CAR3/7, and CAR4/7, respectively, were detectable in both cell extracts and culture media. FLAG-tagged full-length CAR was not detected in the medium.

RESULTS

Identification of Alternatively Spliced CAR RNA—A previous publication has demonstrated distinct bands on Northern blots of HeLa cell RNA when probed with CAR cDNA (30). To further analyze these CAR-related RNA we amplified CAR-specific sequences using primers located in exon I (P5) and at the end of the coding sequence in exon VII (P3) (Fig. 1B) from HeLa cell cDNA. RT-PCR resulted primarily in the amplification of the full-length CAR sequence with the expected length of 1104 bp (Fig. 1A, left panel, band a). PCR products from three smaller bands (bands b–d) were re-amplified with primer P5′ and P3′ (2) (Fig. 1A, right panel) and sequenced. The PCR product in lane b was the result of an alternative splicing event between exon IV and exon VII, eliminating exons V and VI. This product was designated as CAR4/7. PCR products in lanes c and d are results of alternative splicing between exons III and VII (CAR3/7) and between exons II and VII (CAR2/7), respectively. While these studies were in progress, Thoelen et al. (31) reported the RT-PCR amplification of CAR2/7 and CAR4/7 and labeled the sequences γ-mRNA and β-mRNA of CAR, respectively.

All of the alternatively spliced isoforms lack the transmembrane region encoded by exon VI but included the leader sequence that is primarily encoded by exon I. Different portions of immunoglobulin domains are represented in the alternatively spliced isoforms as shown in Fig. 1B. CAR4/7 contains immunoglobulin domain D1 and a part of immunoglobulin domain D2. CAR3/7 includes all of domain 1, and CAR2/7 contains only the first 70 amino acids of the extracellular region of full-length CAR. All splicing events identified result in a shift in the reading frame in exon VII compared with the full-length CAR. CAR4/7 and CAR3/7 have the same C termini as each other, whereas CAR2/7 is different than all of the others.

Alternatively Spliced CAR Isoforms Are Soluble Receptors—Because all of the CAR isoforms contain the leader sequence but lack the transmembrane domain, we hypothesized that the alternatively spliced CAR isoforms could be released from the cell. To address this question, all three human CAR isoforms and FLAG-labeled full-length CAR were expressed in HeLa cells. Proteins in the cell extracts and the cell culture media were evaluated by immunoblot using an anti-CAR antibody. The endogenous full-length CAR, as well as transfected CAR proteins were present in HeLa cells (Fig. 2, left panel). In addition, the alternatively spliced isoforms were also detected.

![Figure 2: Alternatively spliced CAR isoforms are released from the cell.](image-url)
CAR Isoforms Express and Isolated from Bacteria—An important characteristic of soluble receptors is their capability to mediate the interaction between the membranous receptor and its ligand by binding to one or both of them. A structural analysis has previously shown that the D1 domain of CAR is able to form an antiparallel homodimer (32). To determine whether CAR isoforms interact with the extracellular domain of CAR (exCAR), human exCAR was expressed in bacteria, purified, and covalently bound to affinity chromatography resin. The purified exCAR showed a high degree of dimerization under native conditions and reduced significantly CVB3 infection in a dose-dependent manner (Fig. 3A), indicating that its structure is intact. Affinity chromatography was performed with HeLa cell protein extract. Western blot analysis shown in Fig. 3B demonstrates that full-length CAR clearly bound to the exCAR-coupled column but did not interact with AminoLink resin in the absence of exCAR. To test the specificity of the affinity chromatography, peak fraction was also subjected to Western blot analysis using antibodies against proteins like ANT and eIF4G that are not known to interact with exCAR. None of these proteins were found in the eluted fraction. An example is for ANT in Fig. 3B. Medium containing native CAR2/7, CAR3/7, and CAR4/7, respectively, released from transfected HeLa cells was loaded to the column. A significant binding was detected for all CAR isoforms, whereas they did not interact with unlinked resin (Fig. 3C). Medium from untransfected cells did not show CAR antibody-reacting proteins with the size of the soluble CAR isoforms (data not shown).

**CAR4/7 but Not CAR2/7 Interacts with CVB3—**Previous experiments have shown that the D1 domain of CAR interacts with coxsackievirus. Because the soluble CAR receptors contained at least a portion of D1, and because they bind to the exCAR, we hypothesized that soluble CAR proteins could also bind to CVB3. To address this question, CAR2/7 and CAR4/7 were expressed as FLAG-labeled proteins in HeLa cells and...
released into the medium. Purified CVB3 was added to the CAR isoform containing cell culture medium, and precipitation was performed with agarose-coupled anti-FLAG antibody. CAR2/7 and CAR4/7 were each detected in the precipitate and CVB3 coprecipitated with CAR4/7 (Fig. 4B) but not with CAR2/7 (Fig. 4A).

Transfection of HeLa Cells with Soluble CAR Isoforms Inhibits CVB3 Infection—To prove the biological activity of the soluble CAR isoforms, we transfected HeLa cells with either full-length CAR or one of each of the CAR isoforms and analyzed their effect on CVB3 infection. The transfection efficiency for full-length CAR, CAR2/7, CAR3/7, and CAR4/7 (19.3 ± 0.8, 28.7 ± 5.8, 18.9 ± 4.0, and 27.1 ± 0.8% of all cells, respectively) was similar for all isoforms. The cell growth and viability were not negatively affected by the transfection. 48 h after transfection, cells were infected with CVB3 without changing the medium that had been shown to contain the soluble CAR proteins. Overexpression of each of the alternatively spliced soluble CAR isoforms led to a significant inhibition of viral infection (Fig. 5A). The expression of full-length CAR did not significantly affect the efficiency of viral infection when compared with cells that were transfected with an empty vector (Fig. 5B). The elimination of CAR isoforms from the cell culture medium prior to CVB3 infection resulted in a consistent, but statistically insignificant reduction of the inhibitory effect of CAR3/7 and CAR4/7, whereas viral infection of CAR2/7-transfected cells was not changed.

External Treatment with Recombinant CAR4/7 Protects HeLa Cells from CVB3 Infection—We further analyzed whether soluble CAR protein has an impact on viral infection...
when it is externally added to HeLa cells. For this purpose, CAR4/7 was bacterially expressed and purified. It was shown to dimerize, interact with the extracellular CAR domain in the exCAR-specific affinity chromatography, and coprecipitate with CVB3 during immunoprecipitation (Fig. 6, A–C). In addition, CAR4/7 was able to significantly reduce viral infection in a dose-dependent manner when externally added to the cell culture medium of untransfected HeLa cells before and during CVB3 incubation (Fig. 6D). However, the inhibitory effect was lost in a time-dependent manner when CAR4/7 was eliminated from the medium before CVB3 was added (Fig. 6E). Thus, binding of CAR4/7 to CVB3 and reversible extracellular contact of CAR4/7 to the cell surface are both likely to be mechanisms by which viral infection of the cells can be inhibited.

**DISCUSSION**

These results demonstrate expression of three truncated CAR isoforms produced by alternative splicing. These CAR isoforms can be released from HeLa cells as soluble receptors. Importantly, soluble CAR proteins are able to bind to a bacterially expressed extracellular domain of CAR, and CAR4/7 but not CAR2/7 was found to interact with CVB3. In addition, soluble CAR isoforms showed biological activity inhibiting virus infection in *vitro*. Therefore, they fulfill typical characteristics of soluble receptors; i.e. binding to their membranous counterpart and/or to the ligand of the membrane receptor, thus affecting the function of the full-length CAR as a receptor for coxsackievirus.

CAR gene produces a number of alternatively spliced mRNA resulting in protein products with considerable variability in the predicted protein structure. They include membrane-bound protein receptors with different cytoplasmic domains (33), soluble proteins that preserve the majority of the extracellular domains of CAR, but have distinct C termini, and smaller polypeptides such as CAR2/7 that lack complete immunoglobulin domains and have a unique C-terminal sequence. Therefore, CAR meets a common feature of the immunoglobulin family to which it belongs: diversity produced by limited genetic material (34). We recognize that more alternatively spliced isoforms may exist, because 3' rapid amplification of cDNA ends analysis identified an additional splicing site in exon VII and an extra exon VIII with at least two more alternative splicing sites, which lies about 26 kb upstream from exon VII.²

²A. Dörner, D. Xiong, K. Couch, T. Yajima, and K. U. Knowlton, unpublished data.

Our experiments confirm the prediction that the alternatively spliced CAR isoforms are soluble receptors that can be released from the cell. Comparing the intracellular and extracellular amount of each transfected isoform (Fig. 2), CAR2/7 appears to be very efficiently released from the cell. Our observations fit a computer-based prediction (PSORT) that CAR2/7 is found in the extracellular compartment with a probability of 77.8%. An extracellular localization of 66.7% probability was predicted for CAR4/7 and 55.6% for CAR3/7. Signals for intracellular traffic have been identified in the C terminus of full-length CAR (35, 36). CAR3/7 and CAR4/7 contain three dileucines in their C-terminal sequence that might belong to dileucine motifs that are involved in lysosomal sorting, endocytosis, and basolateral targeting (37). Dileucines are not found in the CAR2/7 sequence.

Soluble receptors usually interact with their membranous receptor or with receptor ligands and in this way regulate the reactivity of their membranous counterparts. We were able to show that the soluble isoforms bind to the extracellular domain of CAR expressed in bacteria and purified by affinity chromatography. These findings are consistent with structural analysis of immunoglobulin domain D1 of CAR demonstrating that they form antiparallel homodimers (32), suggesting that CAR proteins may be important for cell-cell contact. CAR2/7 that has only the first 70 amino acids of the D1 domain is also able to interact with bacterially expressed exCAR. To what extent the various C termini of soluble receptors are involved in this interaction is not known. Even though CAR2/7 can bind to exCAR, it is not able to interact with CVB3. It has been shown that CAR uses different structural features within D1 for CVB3- and D1-D1 interaction. D1 is however fully conserved in the CAR4/7 isoform that was found to bind to exCAR and the virus (4, 32).

It is interesting that all of the described, soluble CAR isoforms protected transfected HeLa cells from CVB3 infection even though we could not demonstrate that CAR2/7 bound to CVB3. In the cases of CAR3/7 and CAR4/7, it is likely that binding of soluble CAR to the receptor and to the virus contributes to the inhibition. This is indicated in Fig. 6B wherein exchange of the CAR isoform-containing media had a small, but incomplete effect on viral infection. However, the elimination of CAR2/7 had no significant effect on viral inhibition, consistent with the fact that CAR2/7 does not bind to coxsackievirus. In addition, the inhibitory effect of CAR2/7 was slightly lower than that of CAR3/7 and CAR4/7 both of which should be able to bind to the receptor and the virus. Furthermore, bacterially expressed, purified CAR4/7 is able to inhibit virus infection when in the media and for a short time after CAR4/7 has been eliminated from the cell medium. Based on these data, our current model is that each of the CAR isoforms released from the cell can bind to the cell surface, likely through the membranous CAR and inhibit viral infection. In addition, an internal cellular effect of the soluble CAR isoforms blocking viral replication cannot be excluded. The inhibitory impact is, however, specific for the soluble form of CAR, because overexpression of membranous CAR did not appreciably affect CVB3 infection. The significance of soluble receptors in virus inhibition was recently supported by the observation that a CAR antibody-reacting protein of ~31 kDa found in the malignant pleural effusions could inhibit adenovirus infection (38).

CAR was shown to be important in tight junction formation, and the CAR-mediated junctional barrier could be disrupted by the addition of the extracellular domain of CAR (13). Adenovirus appears to use its knob protein similarly to disturb the intercellular CAR-CAR interaction in epithelia destabilizing the tissue integrity and facilitating the virus spreading (39). This suggests the possibility that the soluble CAR isoforms may function as natural modulators of epithelial tissue permeability by influencing the intercellular interaction of their membranous counterpart. An elevated CAR expression has been seen in the subendothelial region of vessels in hearts of patients with dilated cardiomyopathy (14). It is so far unclear whether this alteration is due to an increase in the membranous receptor and/or its soluble isoforms. In conclusion, CAR serves not only as a receptor for the virus, but expression of alternatively spliced isoforms of CAR may play a role in the antiviral defense system of the host and may have a regulatory effect on the membranous receptor.

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J. Biol. Chem. 2004, 279:18497-18503.
doi: 10.1074/jbc.M311754200 originally published online February 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311754200

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