Evidence that the α^2HS glycoprotein receptor and the Epstein–Barr virus nuclear antigen (EBNA) are identical

J.G. Lewis and C.M. André

Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

Received 26 June 1983; revised version received 8 August 1983

Evidence is presented which suggests that the α_2HS glycoprotein (α_2HS) receptor on lymphocytes transformed by Epstein–Barr virus (EBV) and the EBV-determined nuclear antigen (EBNA) are immunologically related, and may be identical. Western blotting, using both anti-EBNA and anti-α_2HS receptor serum identified a similar M_r 48 000 component in labelled detergent-solubilized transformed cell supernatants. The M_r 48 000 component recognized by anti-EBNA serum had a pI between 7–8, similar to that reported for the α_2HS receptor.

α_2HS glycoprotein receptor *Epstein–Barr virus* *Epstein–Barr virus nuclear antigen (EBNA)* *Lymphocyte*

1. INTRODUCTION

The infection of B lymphocytes with Epstein–Barr virus (EBV) leads to cellular transformation and the expression of the EBV-determined nuclear antigen (EBNA) [1]. It is suspected that EBNA may play an essential role in the transformation process and maintenance of the transformed state [2]. Whether this suggested regulatory role of EBNA is related to its ability to bind specific DNA sequences is unknown [3]. Recently, EBNA has been shown to possess protein kinase activity [4], and this has strengthened the possibility that it may be a regulatory protein involved in the gene expression of transformed cells.

As EBNA is thought to play a role in the transformation process, it may also be of prime importance in immune defense mechanisms against infection by EBV and, as such, the interaction of EBNA with serum components may prove to be relevant. We have shown that the immunomodulatory serum protein α_2HS [5] binds to lymphocytes transformed by EBV, but not to normal autologous non-transformed lymphocytes [6]. The α_2HS receptor, like EBNA, has a monomeric M_r 48 000 and the ability to bind DNA [7]. This suggests some relationship between the α_2HS receptor and EBNA.

Here, we report evidence which implies that the α_2HS receptor on lymphocytes transformed by EBV and EBNA may be identical. Antiserum raised against the α_2HS receptor immunoprecipitated the M_r 48 000 α_2HS receptor, as shown by SDS–PAGE. The use of both this antiserum and anti-EBNA serum also resulted in localization of this M_r 48 000 band from SDS–PAGE run in reducing conditions. Elution of the M_r 48 000 band, identified by anti-EBNA serum, from Western blots and subsequent isoelectric focusing resulted in a radioactive peak pI 7–8, similar to that of the α_2HS receptor. Furthermore, we show by indirect immunofluorescence that anti-EBNA serum blocks the binding of α_2HS to EBV-transformed lymphocytes. These results suggest that the α_2HS receptor and EBNA are immunologically related and may be identical.

2. MATERIALS AND METHODS

Peripheral blood lymphocytes were transformed with EBV culture supernatants derived from the B95-8 cell line [8], iodinated, and membranes

Published by Elsevier Science Publishers B.V.

solubilized as in [7]. Indirect immunofluorescence was performed using a 1:10 dilution of all antisera [7], and α_2HS was isolated [9] and used at a final physiological concentration of 0.8 mg/ml. EBNA positive and EBNA negative human sera were used with titres against EBNA 80, viral capsid antigen (VCA) <80, early antigen complex (EA) <10; and EBNA <2, VCA <10, EA <10, respectively. Antiserum to the α_2HS receptor was raised in New Zealand white rabbits by subcutaneous injection. Four weeks after the initial injection of the isolated α_2HS receptor [7], a similar booster was given (10–20 μg). Antiserum was harvested 10 days later.

SDS–PAGE was carried out according to Laemmli [10] in 5–20% gradient slab gels. Markers were phosphorylase B (M_r 97 000), bovine serum albumin (BSA, M_r 68 000), ovalbumin (M_r 44 000), carbonix anhydrase (M_r 29 000), and lysozyme (M_r 14 000): ^{125}I-α_1-antitrypsin (α_1AT) (M_r 54 000) was also used for autoradiography. For immuno-precipitation experiments 20 μl ^{125}I-labelled detergent solubilized cell supernatant was incubated with 5 μl rabbit anti-α_2HS receptor serum or 5 μl non-immune rabbit serum. Following incubation for 24 h at 4°C, 50 μl goat anti-rabbit serum (Wellcome) was added for a further 24 h at 4°C. After centrifugation, the resulting precipitate was washed 5 × with PBS, incubated with 2% SDS containing mercaptoethanol (95°C for 5 min) and subjected to SDS–PAGE. Following electrophoresis gels were fixed in trichloroacetic acid, dried and autoradiographed.

For Western blots, 100 μl aliquots of ^{125}I-labelled detergent solubilized cell supernatants were subjected to SDS–PAGE and then transferred to nitrocellulose (Millipore HAWP, 45 μm) by overnight electrophoresis in 25 mM Tris, 192 mM glycine containing 20% methanol. The nitrocellulose was then blocked with 5% BSA in Tris-buffered saline (TBS, pH 7.4) containing 0.05% NP40, after which the nitrocellulose was incubated (60 min at 20°C) in either EBNA positive, EBNA negative, rabbit anti-α_2HS receptor or non-immune rabbit serum, all diluted 1:50 with TBS containing 5% BSA and 0.05% NP40. Following thrice washing in TBS containing 0.05% NP40, the nitrocellulose was incubated (60 min at 20°C) in appropriate second antibody solution, either goat anti-human IgG-peroxidase labelled (1:3000, Tago) or goat anti-rabbit IgG-peroxidase labelled (1:3000, Tago) prepared in TBS containing 5% BSA and 0.05% NP40. After washing, the bands were visualised by incubation with 30 mg diaminobenzidine in 100 ml 0.1 M Tris–HCl (pH 7.6) containing 40 μl H_2O_2 (30% solution). The enzyme reaction was terminated by washing in water. The nitrocellulose was dried, photographed and autoradiographed, allowing exact identification of peroxidase-stained bands.

The peroxidase-labelled M_r 48 000 band, identified with anti-EBNA serum from SDS–PAGE of a sample run in reducing conditions, was carefully cut from the nitrocellulose sheet, together with the non-peroxidase labelled region of similar mobility of a sample run in non-reducing conditions. Each region was solubilized with acid–acetone and the precipitate resuspended in 8 M urea for isoelectric focusing in 4% polyacrylamide disc gels containing 8 M urea and 4% ampholine solution. After focusing, the gels were sliced into 2 mm segments and placed in tubes with 0.5 ml distilled water. Gel slices were counted and the pH of the eluted fractions determined.

3. RESULTS

The initial incubation of viable EBV-transformed lymphocytes with anti-EBNA serum prior to sequential incubations with α_2HS, rabbit anti-α_2HS serum and sheep anti-rabbit immunoglobulinfluorescein isothiocyanate (FITC), blocked fluorescence, compared to cells initially incubated with α_2HS followed by rabbit anti-α_2HS serum and sheep anti-rabbit immunoglobulin-FITC, or cells incubated initially with anti-EBNA serum, followed by rabbit anti-human IgG and sheep anti-rabbit immunoglobulin-FITC.

Immunoprecipitates produced by incubation of ^{125}I-labelled detergent solubilized cell supernatants with rabbit anti-α_2HS serum revealed a single M_r 48 000 component compared to non-immune rabbit serum (fig.1). This antiserum also revealed a major M_r 48 000 component from Western blots only when the labelled detergent solubilized cell supernatants were initially subjected to SDS–PAGE in reducing conditions (fig.2). Similarly, a M_r 48 000 component was identified from Western blots using anti-EBNA serum only when the labelled detergent solubilized cell supernatants were initially run by SDS–PAGE under reducing condi-

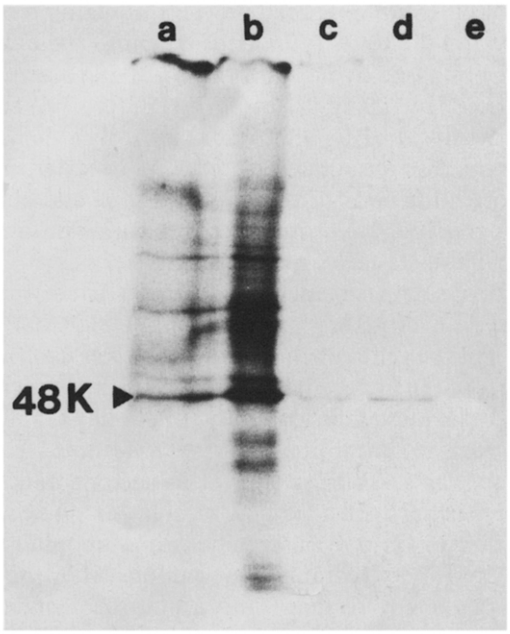

Fig.1. Autoradiograph of ^{125}I-labelled proteins analyzed by SDS–PAGE. Labelled detergent solubilized EBV-transformed lymphocyte supernatants (20 μl) with (a) and without (b) mercaptoethanol. Immunoprecipitates with rabbit anti-α_2HS receptor serum 1, rabbit anti-α_2HS receptor serum 2, and non-immune rabbit serum, c, d and e respectively. The M_r 48000 α_2HS receptor band is indicated.

tions (fig.3). Minor components of M_r 54000 and M_r 71000 were also identified. The use of EBNA-negative serum revealed no peroxidase staining bands.

The isoelectric focusing profiles of the extracted peroxidase-labelled M_r 48000 component identified by EBNA-positive serum in a sample run in reducing conditions, and the equivalent region from a sample run in non-reducing conditions, are shown in fig.4. The presence of an additional component, pI 7–8 in the reduced sample compared to

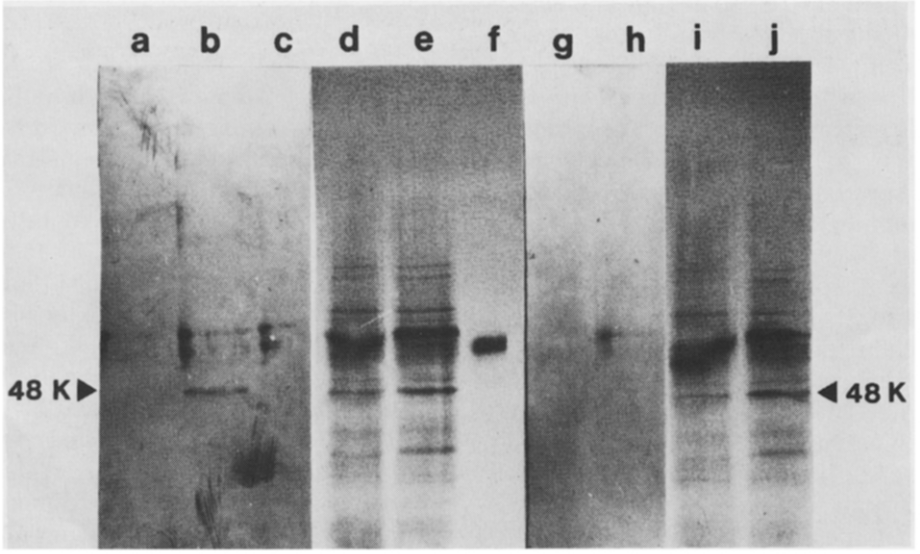

Fig.2. Western blots and autoradiographs of ^{125}I-labelled detergent-solubilized EBV-transformed lymphocyte supernatants (100 μl) after transfer to nitrocellulose. Lanes a, b and c are Western blots using rabbit anti-α_2HS receptor serum 1 of labelled detergent solubilized cell supernatants initially separated by SDS–PAGE in the absence (a) and presence (b) of mercaptoethanol. Lane c shows the ^{125}I-α_1AT marker (M_r 54000). Lanes d–f are autoradiographs of lanes a–c, respectively. Lanes g and h are Western blots using non-immune rabbit serum of labelled detergent solubilized cell supernatants initially separated by SDS–PAGE in the absence and presence of mercaptoethanol, respectively. Lanes i and j are autoradiographs of lanes g and h, respectively. The M_r 48000 band is indicated.

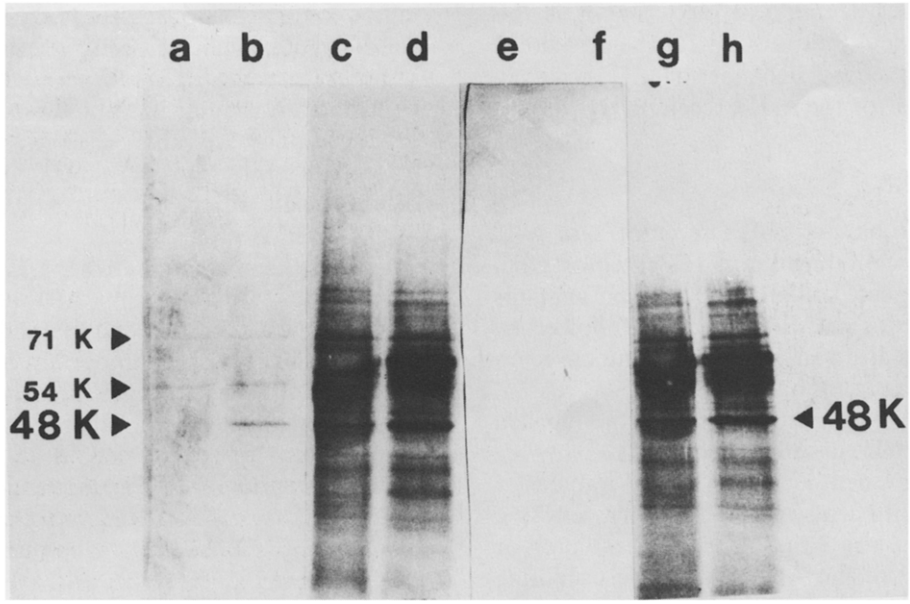

Fig.3. Western blots and autoradiographs of ^{125}I-labelled detergent solubilized EBV-transformed lymphocyte supernatants (100 μl) after transfer to nitrocellulose. Lanes a and b are Western blots using anti-EBNA serum of labelled detergent solubilized cell supernatants initially separated by SDS–PAGE in the absence (a) and presence (b) of mercaptoethanol. Lanes c and d are autoradiographs of a and b, respectively. Lanes e and f are Western blots using EBNA-negative serum of labelled detergent solubilized cell supernatants initially separated by SDS–PAGE in the absence (e) and presence (f) of mercaptoethanol. Lanes g and h are autoradiographs of e and f, respectively. The M_r 48000 band is indicated together with minor components M_r 54000 and M_r 71000.

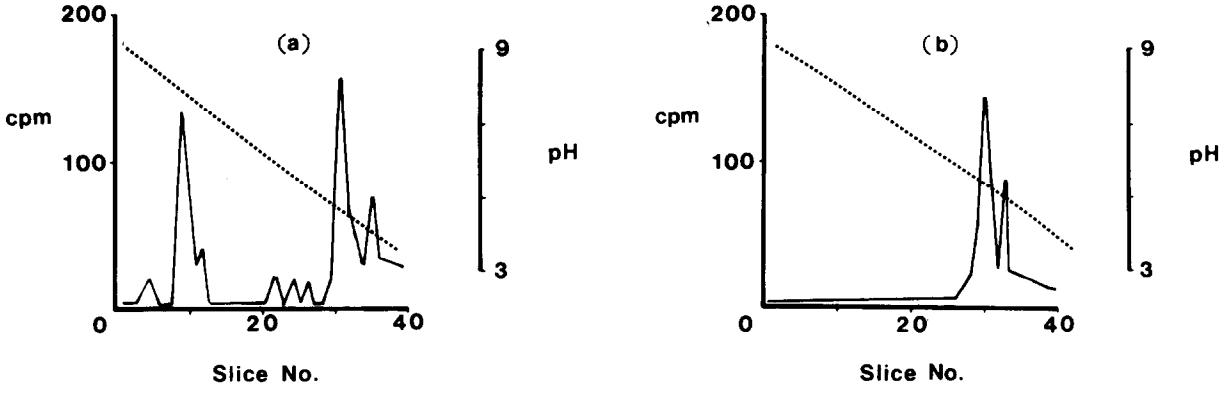

Fig.4. Isoelectric focusing of the eluted M_r 48000 peroxidase-staining band from nitrocellulose identified by anti-EBNA serum of a labelled detergent solubilized cell supernatant run in the presence of mercaptoethanol (a). The corresponding non-peroxidase-staining region of similar mobility (M_r 48000) was eluted from nitrocellulose of a labelled detergent-solubilized cell supernatant run in the absence of mercaptoethanol and also subjected to isoelectric focusing (b). After focusing, gels were sliced into 2 mm segments and counted. Radioactivity is expressed as cpm after background subtraction. The pH gradient is also indicated (– – –) using ampholines (pH 3–10).

the non-reduced sample, suggests that it is this component which is responsible for the peroxidase labelling using anti-EBNA serum. This corresponds to the pI of the α_2HS receptor [7].

4. DISCUSSION

Although rabbit anti-α_2HS receptor sera were capable of precipitating the α_2HS receptor from labelled detergent solubilized cell supernatants (fig.1), the use of anti-EBNA serum followed by rabbit anti-human IgG resulted in immunoprecipitation of all bands (not shown).

Electrotransfer to nitrocellulose of the labelled detergent solubilized cell supernatants following SDS–PAGE resulted in a slight loss of transfer of higher-M_r components (fig.1–3). The resulting Western blots using either anti-α_2HS receptor or anti-EBNA serum showed the peroxidase-staining M_r 48 000 band only when samples were run in reducing conditions (fig.2,3). This suggests that both these antisera are recognizing the same component. This component could be derived from a higher-M_r species which is not identified under non-reducing conditions, due to incomplete electrotransfer of higher-M_r components.

Isoelectric focusing confirms the suggestion that the anti-EBNA serum-identified M_r 48 000 component is the α_2HS receptor. The α_2HS receptor, like EBNA, exists in an aggregated form, and like EBNA, has a monomeric M_r 48 000 [7,11]. It therefore seems likely that the α_2HS receptor and EBNA are not only immunologically related but are identical. This is supported by the findings from indirect immunofluorescence, where anti-EBNA serum blocked the binding of α_2HS.

The anti-EBNA serum also recognized two minor components, M_r 54000 and M_r 71000 (fig.3). Whether these correspond to multiple EBNA forms is unknown. In non-producer cell lines, such as those derived from B95-8 supernatants, there is sufficient mRNA derived from EBV DNA to code for several medium size proteins [3]. Although the pI of EBNA has been reported, some uncertainty exists, as it is claimed by some to be an acidic protein, and by others to share biochemical properties with the histones [3]. The nature of this variation, together with the variation in M_r may reflect the existence of multiple EBNAs. Western blots using anti-α_2HS receptor serum showed minor components of higher M_r in both the labelled detergent solubilized cell supernatants and the ^{125}I-α_1AT marker (fig.2). Hence, these minor components are probably spurious, especially in view of the ability of this antibody to immunoprecipitate only the M_r 48000 from labelled detergent solubilized cell supernatants analyzed by SDS–PAGE (fig.1).

The results suggest that the α_2HS receptor on lymphocytes transformed by EBV and EBNA are immunologically related and may be identical. As EBNA seems to play an important regulatory role in EBV-transformed cells, the question as to whether α_2HS can directly alter transformation or proliferation of these EBV-transformed cells merits attention. In addition, the use of α_2HS–Sepharose 4B should prove useful for the isolation of EBNA for subsequent antiserum production.

ACKNOWLEDGEMENTS

The EBNA positive and negative sera were kindly provided by Dr George Klein, Karolinska Institute (Sweden). We thank the Medical Research Council of New Zealand for support.

REFERENCES

[1] Epstein, M.A. and Archong, B.G. (1979) in: The Epstein–Barr Virus (Epstein, M.A. and Archong, B.G. eds) pp. 1–22, Springer, Berlin, New York.
[2] Reedman, B.M. and Klein, G. (1973) Int. J. Cancer 11, 499–520.
[3] Vonka, V. and Hirsch, I. (1982) Prog. Med. Virol. 28, 145–179.
[4] Kamata, T., Takaki, K., Hinuma, Y. and Watanabe, Y. (1981) Virology 113, 512–520.
[5] Lewis, J.G. (1983) PhD Thesis, University of Otago.
[6] Lewis, J.G., Crosier, P.S. and André, C.M. (1982) FEBS Lett. 138, 37–39.
[7] Lewis, J.G. and André, C.M. (1982) FEBS Lett. 143, 332–336.
[8] Sugden, B. and Mark, W. (1977) J. Virol. 23, 503–508.
[9] Lewis, J.G. and André, C.M. (1980) Immunology 39, 317–322.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Luka, J., Lindahl, T. and Klein, G. (1978) J. Virol. 27, 604–611.