PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **182**, 564–567 (1986)

INTERFERON INDUCING TRANSFORMED CELL SURFACE GLYCOPROTEINS: PURIFICATION BY Ia ANTIGEN AFFINITY CHROMATOGRAPHY

THOMAS K. HUGHES, ERIC M. SMITH AND J. EDWIN BLALOCK

Department of Microbiology
University of Texas Medical Branch
Galveston, Texas 77550

Abstract. Ia antigens were previously shown to be B-cell receptors for transformed cell surface glycoproteins (TCSG). Ia antigen recognition of TCSG subsequently initiated interferon production. These findings have been exploited to highly purify TCSG from mouse L ($H-2^k$) cells in one step by Ia^k antigen Sepharose affinity chromatography. These results point to the highly specific recognition of TCSG by Ia antigens and the B-cells on which they reside. The implications and applications of these studies are discussed. © 1986 Society for Experimental Biology and Medicine

Introduction. Transformed, but not normal, syngeneic and allogeneic cells induce the production of interferon (IFN) α and α/β by nonsensitized human peripheral blood lymphocytes and mouse spleen cells, respectively (1-4). In both of these systems, B lymphocytes are apparently responsible for the bulk of IFN production (3,4). We have recently reported that 90K and 130K dalton transformed cell surface glycoproteins (TCSG) are the molecules responsible for IFN induction by mouse L and human WISH cells, respectively (5). Further, Ia antigens are apparently the B-cell receptors for these TCSG and Ia antigen recognition of TCSG initiates IFN production (6). In this report, we have tested and shown the specificity of this interaction by highly purifying the L-cell TCSG in one step on an Ia antigen Sepharose affinity column.

Materials and Methods. Mice: CBA/J ($H-2^k$) female mice were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were used between 6 and 12 weeks of age.

Cells: Mice were sacrificed by cervical dislocation, their spleens aseptically removed, and single cell suspensions were prepared by teasing and vigorous pipetting in the required buffer or medium. Cell numbers were determined by counting with a hemocytometer. Mouse L-929 ($H-2^k$) cells were obtained from American Type Culture Collection. Cells were routinely grown to confluency in Costar 32 oz. plastic culture flasks or microtiter plates (Cambridge, MA) in a 37°C, 4% CO_2 incubator. For propagation of L-cells, Eagles minimal essential medium (EMEM), with Earle's salts, supplemented with 10% fetal bovine serum (FBS), penicillin (100 units) and streptomycin (100 ug/ml) was used. Mouse spleen cell suspensions, inducer and IFN assays were done with EMEM containing 2% FBS.

Preparation and Assay of TCSG IFN Inducer: Mouse L-929 cells ($2-4x10^7$ cells), grown to confluence in 32 oz. tissue culture flasks, were scraped from flasks with rubber policemen and resuspended in 10 ml. of the required buffer or EMEM with 2% FBS. The cells were then sonicated at 40 KHz (Branson E-Module) at room temperature for 1 minute and centrifuged at 1000 xg for 5 minutes. The supernatant fluid was used as the IFN inducing TCSG. Inducer activity was measured by

0037-9727/86 $1.50
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incubating 0.1 ml of TCSG with 0.1 ml ($1.5 - 3x10^6$) CBA/J($H-2^k$) mouse spleen cells for 18-24 hrs in a microtiter plate at 37°C, 4% CO_2. Duplicate samples were then harvested and assayed for IFNα/β activity. Serial dilutions of IFN were made in EMEM with 2% FBS. One-tenth ml of each dilution was incubated in duplicate on mouse L-929 cells grown to confluency in microtiter plates. After 18-24 hours incubation at 37°C, 4% CO_2, interferon was decanted and the cells were challenged with 30-40 plaque forming units of VSV (7).

Ia^k Antigen Sepharose Affinity Column Chromatography of TCSG: Affinity chromatography was performed on a 5.0 ml column containing Sepharose 4B (Pharmacia) to which approximately 3.0 ug of anti-Ia^k antibody affinity chromatography purified Ia^k antigen was attached (purification described in 6). Ia^k antigen was bound to Sepharose by cyanogen bromide activation. Columns were run at a flow rate of approximately 1.6 ml/minute using PBS. Five mls of crude TCSG was loaded on the column. 4 M $MgCl_2$ was used for elution. Samples were dialyzed against PBS and concentrated prior to the assay for IFN induction.

Polyacrylamide Gel Electrophoresis (PAGE) of Ia^k Antigen Sepharose Affinity Column Purified TCSG: Electrophoresis (8) was performed on 7% vertical 0.75 mm x 140 mm x 160 mm polyacrylamide slab gels containing 0.1% SDS and 0.5 M urea utilizing a discontinuous buffer system of 0.6 M Tris-HCl (pH 6.6-6.8) in the stacking gel and 0.6M Tris-HCl (pH 8.8-9.0) in the separating gel. A constant amperage of 15 mA/gel was used for an approximate run of 4-6 hours or when the tracking dye migrated to the end of the gel. Gels were then silver stained (Bio Rad, Richmond, CA) to detect protein bands.

Preparation of Antiserum to TCSG: Purified TCSG in complete Freunds adjuvant was initially injected into the footpads of a 4 month old New Zealand white rabbit. Subsequent injections were given intramuscularly in the hip. A total of approximately 15 µg was injected into the rabbit over a 1-1/2 month period in roughly 1-1/2 week intervals. Bleeding was from the ear vein. The blood was allowed to clot overnight at 4°C in a test tube, after which the serum was collected.

Results and Discussion. Table 1 shows that when a crude preparation of TCSG was passed over an Ia^k antigen affinity column, the bulk of the protein washed through while the vast majority of the IFN inducing activity was retained by the column and eluted with 4 M $MgCl_2$. SDS-PAGE was then used to determine the number of proteins which bound to the affinity column. Figure 1 shows that the precolumn (lane A)

Table 1

Binding of the IFN inducing TCSG to an Ia^k antigen Sepharose affinity column

Sample	Protein Concentration (ug/ml)[b]	A_{280}	IFN (U/ml) Induced[a]
Starting material	130	N.D.	200
Wash through	108	1.55	50
Eluate	2.9	0.075	500

Five mls. of crude TCSG were loaded onto the affinity column. 4 M $MgCl_2$ was used for elution; N.D., not done.

[a]Amount of IFN induced in a mouse spleen cell culture by 0.1 ml of sample.

[b]Protein concentrations corrected to 5 ml volume for wash through and eluate to compare to volume of starting material.

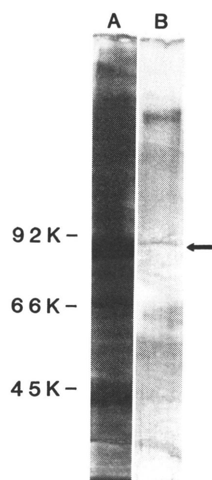

Figure 1. SDS-PAGE of Ia^k antigen Sepharose affinity column purified TCSG. A, precolumn; B, eluate.

stains darkly due to the large amount of protein detected by the sensitive silver staining technique. However, in the eluate (lane B), one band corresponding to a molecule weight of 90K was detected by silver stain. This molecular weight is the same as we have previously reported for IFN inducing TCSG by gel filtration (5). Only one other higher molecular weight band was present in the affinity column purified TCSG and it disappeared following reduction with 2 mercaptoethanol (data not shown). Since it disappeared, our assumption is that the high molecular weight band may be an aggregate of 90K TCSG, but no other experiments have been performed to confirm this. Since these gels were silver stained, it seems reasonable to conclude that the specificity of the Ia affinity column was extremely high. Therefore, it appears that the IFN inducing TCSG from L cells can be highly purified in one step on an Ia^k antigen Sepharose column.

Since the biological activity of the TCSG was destroyed by SDS-PAGE, a question arises as to whether the material that bound to the affinity column and migrated as a single band during SDS-PAGE was the same as that which is present in a crude preparation. In order to address this question, we made antiserum to the purified TCSG and attempted to block the activity of the crude IFN inducer from L cells. Table 2 shows that this antiserum almost completely inhibited the crude inducer activity while preimmunization serum had no effect. The degree of inhibition was dependent on both the IFN inducer and antiserum concentration. Thus, these data show

Table 2

Antiserum to Ia antigen Sepharose affinity column purified TCSG blocks the IFN inducing activity of a crude L-cell inducer preparation [a]

Dilution of			
IFN Inducer	Antiserum	IFN Titer(U/ml)	% Inhibition
1:3	-	200	-
1:3	1:30	<10	>95
1:3	1:100	160	20
1:10	-	100-	
1:10	1:30	<10	>90
1:10	1:100	100	0
1:30	-	65	-
1:30	1:30	<10	>85
1:30	1:100	<10	>85
1:3	-	250	-
1:3	1:30 (preimmunization)	250	0

[a]Dilutions of the crude L-cell inducer were incubated with antiserum at the indicated concentrations for 1 hour at 37°C prior to assay for IFN inducing activity.

that the IFN inducing TCSG in the crude preparation is the same as the material that binds to the affinity column and migrates as a single band during SDS-PAGE.

In addition to their passive role in antigen presentation (for review see 9), we have shown that Ia antigens have a more active function in serving as the B cell receptor which initiates IFN production in response to TCSG (6). Our present findings that an Ia^k antigen Sepharose affinity column binds a single protein (TCSG) from a crude preparation which contains well over one hundred, seems to point to the exquisite specificity of the B cell recognition of TCSG. Since the affinity column used in these studies was made with antigens from the entire I region of $H-2^k$, we do not know at present whether a particular subregion of I (IA, IB, etc.) is responsible for TCSG recognition. However, this question should be answered by purifying the products of I subregions with monoclonal antibodies and using these I antigens as affinity absorbents for TCSG. Perhaps more importantly, by using homogeneous TCSG from different transformed cell types as affinity absorbents one should be able to determine whether a given Ia antigen has a unique recognition function. Thus, the Ia antigen TCSG system could be studied in a fashion analogous to antibody antigen reactions. Ia antigen recognition of TCSG may also be exploited in the isolation and characterization of these possibly specific markers of transformation. This prospect would be particularly attractive since there are pre-existing affinity absorbents (i.e. Ia or DR antigens).

ACKNOWLEDGEMENTS. This work was supported by a grant from the State of Texas and NIH Research Grant AM30046. T.K. Hughes was supported by a James W. McLaughlin Postdoctoral Fellowship.

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Received March 4, 1986.
P.S.E.B.M. 1986, Vol. 182.
Accepted May 23, 1986.