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Anti-idiotypic Antibody Identifies the Cellular Receptor of Reovirus Type 3

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The binding and subsequent infectivity of reovirus to target cells are mediated by interaction with specific cell surface viral receptors. To gain a more detailed understanding of the biochemistry of the reovirus receptor and the cellular consequences of viral attachment, we have studied the binding of type 3 reovirus (Dearing strain) in a quantitative manner utilizing an antiidiotypic antibody probe. A syngeneic monoclonal antiidiotypic antibody (87.92.6) was prepared by immunization with hybridoma cells which secrete an antireovirus hemagglutinin-specific antibody. This antiidiotypic antibody was previously shown to specifically recognize the cell surface receptor for reovirus type 3. In this report, we demonstrate that antiidiotype mimicked reovirus tropism in binding to murine thymomas; antiidiotype inhibited the binding of reovirus to specific targets, but not the binding of anti-H-2; and cross linking of receptor-bound antiidiotype by antiimmunoglobulin induced patching, but not capping of reovirus receptors. Utilizing radiolabeled antiidiotype, we next quantitate the number of reovirus receptors on R1.1 and YAC thymoma cells and, finally, report on the preliminary identification of the reovirus receptor as a 67,000-Da membrane glycoprotein.

Key words: reovirus receptors, antiidiotype antibody, fluorescence analysis, Western gel analysis

The mammalian reoviridae include three serotypes, each composed of a segmented ds RNA genome enclosed in an icosahedral double capsid. Of the three outer capsid proteins (σ1, σ3, and μ1c), the binding of reovirus to its cellular receptor is mediated by the σ1 viral hemagglutinin (HA) [1–3]. Binding of reovirus to target cells is specific and inhibitable by HA3, and shows linear and saturable binding kinetics [4,5]. The nature of the immune response following viral inoculation is also directed by the viral HA. Using recombinant and mutant viruses that differ only at the HA site, both the specificity and complexity of the antiviral response have been mapped to the HA [6–10].

Work in our laboratory has centered on the utilization of antiidiotypic antibodies in the study of the reovirus cellular receptor. A monoclonal antiidiotypic antibody with specificity to the reovirus receptor was constructed by immunization of syngeneic mice with antibody-secreting hybridoma cells which produce an anti-HA type 3

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(HA3)-neutralizing antibody [11,12]. Antiidiotypic antibodies were initially screened for the ability both to bind idiotype (i.e., the anti-HA3 monoclone) and to mimic the tissue tropism of reovirus type 3 binding [12,13]. The specificity of one antiidiotypic antibody (87.92.6) for the reovirus cell surface receptor was subsequently confirmed by its ability to inhibit the lytic activity of reovirus HA3-specific cytotoxic T lymphocytes and by the observation that reovirus HA3-specific cytotoxic T cells lyse 87.92.6 hybridoma cells [8.9]. In this report, we utilize antiidiotypic antibody 87.92.6 to further biochemically characterize the type 3 reovirus receptor on murine thymoma cells.

MATERIALS AND METHODS

Cell Lines and Virus

R1.1 and YAC thymoma cells were maintained in RPMI 1640 media (K.C. Bilogicals, Lenexa, KA) containing 10% fetal bovine serum, 2 mM 1-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, and 100 U/ml streptomycin as described previously [14]. Reovirus type 3 (Dearing strain) and type 1 (Lang strain) were generously supplied by Dr. B. Fields (Harvard Medical School). Virus was purified from mouse L929 cells infected in vitro. Virus was extracted with freon (Sigma, St. Louis, MO) followed by CsC1 equilibrium centrifugation as described by Ramig et al [15].

Fluorescence Analysis

Visualization of reovirus receptors in thymoma cells was achieved by incubation of 10^6 cells in 50 μl with either 10^{11} reovirus particles or 10 μg of purified antiidiotype for 45 min at 4°C, followed after extensive washing by either biotinylated antireovirus antibody (15 μg 9BG5) and a 1:100 dilution of fluorescein-conjugated avidin (Vector, Burlingame, CA) or 50 μg of fluorescein-conjugated $(Fab')_2$ goat antimouse IgM (Cappel, West Chester, PA), respectively. Cells were then washed and fixed in 2% paraformaldehyde, then analyzed either visually using the neofluar 40/0.75 lens of a Zeiss fluorescence-activated cell sorter (FACS). Inhibition of reovirus binding to R1.1 cells by antiidiotype was determined by incubation of 10^6 R1.1 cells with antiidiotype (10 μg) in phosphate-buffered saline (PBS) containing 2% fetal bovine serum and 20 mM Hepes for 45 min at 4°C. Cells were then extensively washed and incubated with either 10^{11} biotinylated viral particles, 50 μg biotinylated anti-H-2^k, or 50 μg biotinylated anti-Thy 1.2 for 45 min at 4°C followed by the addition of a 1:100 dilution of fluorescein-conjugated avidin. Cells were fixed and analyzed as described above.

Purification and Biotinylation of Immunoglobulins

Syngeneic monoclonal antiidiotypic antibody was purified from cell-free supernatants of 87.92.6 cells grown in HB101 synthetic media (Hana, Berkeley, CA) as described previously [11]. Briefly, the supernatants were sequentially precipitated at 30–50% ammonium sulfate, then passed over a Sephadex G-100 column (Pharmacia), followed by chromatography on DEAE-Affi Gel Blue (BioRad, Richmond, CA). Anti-Thy 1.2 (monoclonal HO13.4, a gift of Dr. J. Kung, NIH) was purified by chromatography on Bet-2 Sepharose as described by Kung et al [16]. Anti-H-2^k (monoclone 15-5-5P, a gift of Dr. D. Sachs, NIH) was purified by chromatography

on Protein A–Sepharose (Pharmacia). Purified immunoglobulins and reovirus were biotinylated by incubation with biotin succinimide ester (120 μg/ml) in 0.1 M $NaHCO_3$ (pH 8.4) for 4 hr at room temperature, then extensively dialyzed to remove unbound biotin [17].

Iodination and Radiolabeled Binding Assays

Purified immunoglobins and viral particles were labeled with $Na^{125}I$ (NEN, Boston, MA, carrier-free) using lactoperoxidase-coupled enzymobeads [18]. Labeled antibody and virus were separated from unbound label by passage over G-25 Sephadex prewashed with 2% bovine serum albumin in PBS, and run in PBS. Antiidiotype and reovirus were labeled to a specific activity of 6.2 μCi/μg and 8.3 μCi/μg, respectively. In both instances, > 94% of label was precipitated by the addition of cold 10% trichloroacetic acid.

Binding studies were conducted in 50-μl aliquots each containing 10^6 cells in PBS with 0.5% bovine albumin and 0.2% NaN_3. Labeled antiidiotype was added in increasing amounts (0–100 nM) to cells and incubated at 4°C for 60 min. Cells were then layered over 250 μl of phthalate oils in conical microfuge tubes, and the cells were pelleted by centrifugation as described by Dower et al [18]. The tips of the tubes were excised, and the amount of ^{125}I-anti-idiotype bound was determined by counting in a gamma counter. Results were analyzed according to the methods of Scatchard [10] using the criteria of Klotz [20]. Binding values were corrected for pentameric IgM which was verified by both molecular sieve and sucrose gradient analysis.

Identification of Reovirus Receptors by Western Gel Analysis

Reovirus receptors were identified by the binding of ^{125}I-labeled antiidiotype and ^{125}I-labeled reovirus to purified cell membrane extracts run of SDS-polyacrylamide gels and transferred to nitrocellulose paper. Membranes were purified from hypotonically lysed cells by a combination of differential centrifugation and discontinuous sucrose density gradients [21]. Membranes from 5×10^7 cells were loaded per lane and electrophoresed under reducing conditions on 5–10% discontinuous gels. Proteins were transferred to nitrocellulose paper at 250 mA for 18 hr at 4°C using a Hoefer electrophoresis TE series transphor unit. The nitrocellulose paper was blocked with 0.1 M Tris (pH 7.5), 0.25% gelatin, 0.5% NP40 at 37°C for 2 hr, then hybridized with ^{125}I-labeled probes ($2–5 \times 10^6$ cpm/10 ml) for 1 hr at 20°C. The paper was then washed thoroughly in Tris-saline, air-dried, and exposed to Kodak X-OMAT AR film for 12 hr.

RESULTS

Specificity of Antiidiotype Binding

The specificity of antiidiotype binding to reovirus receptors on thymoma cells was evaluated utilizing FACS analysis. Figure 1 depicts the parallel binding of antiidiotype and reovirus particles on R1.1 cells (Fig. 1A,B) in contrast to the absence of antiidiotype binding to reovirus receptor-negative YAC thymoma cells (Fig. 1C,D). Confirmation that antiidiotypic antibodies recognize the cell surface receptor for reovirus was achieved by the observation that antiidiotype specifically inhibited the binding of reovirus type 3 to R1.1 cells. As shown in Figure 2, incubation of R1.1 cells with antiidiotype prior to reovirus exposure resulted in a 62% decrease in the

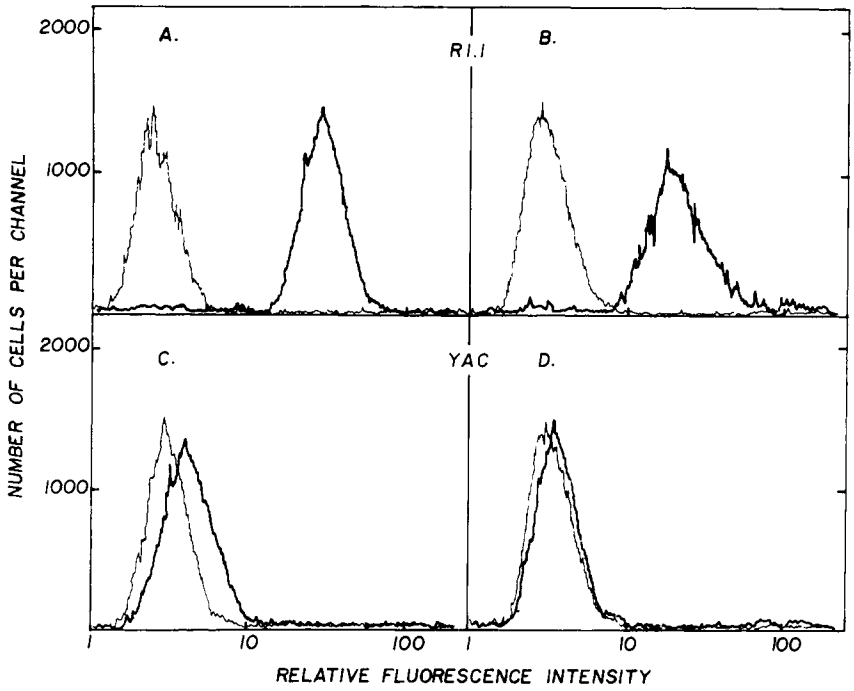

Fig. 1. Binding of reovirus and antiidiotype to thymoma cells. 10^6 thymoma cells, R1.1 (A,B) or YAC (C,D), were incubated either with 10^{11} reovirus particles followed by staining with biotinylated antireovirus HA (9BG5) and avidin-fluorescein (A,C), or with antiidiotype followed by fluoresceinated goat anti mouse IgM (B,D). Fifty thousand cells/sample were analyzed using the fluorescence-activated cell sorter for the intensity of viral or antiidiotype staining. All samples were analyzed for background staining by the addition of only secondary staining reagents (light lines) and for staining in the presence of either reovirus or antiidiotype (bold lines). Results are presented as the number of cells per fluorescence channel vs the relative fluorescence intensity on a log scale.

peak mean channel fluorescence (Fig. 2A). Preincubation with antiidiotype had no effect on the subsequent binding of either anti-H2 or anti-Thy (Fig. 2B,C). Similar incubations conducted with control IgM (monoclonal HO 13.4) and with type 1 reovirus failed to demonstrate nonspecific inhibition either of reovirus 3 binding by IgM antibody or of type 1 virus by antiidiotype (data not shown).

Direct visualization of antidiotype binding on the surface R1.1 cells was accomplished by the addition of purified antiidiotype followed by fluorescein-conjugated goat antimouse IgM (Fig. 3). Immediately following the binding of antiidiotype, a bright ring fluorescence was detected, indicating that receptors were equally dispersed throughout the cell membrane (Fig. 3A). Incubation of antiimmunoglobulin cross-linked receptors at 37°C stimulated a transition of receptors from a dispersed to an aggregate phase. So-called patching of reovirus receptors was detected as early as 5 min of incubation. Further aggregation of patched receptors into polar caps was not detected upon prolonged incubation for up to 60 min, although a gradual reduction in patched-fluorescence was noticed. These results are in marked contrast to those reported by Epstein et al [22] for reovirus-mediated receptor redistribution on non-transformed murine thymocytes.

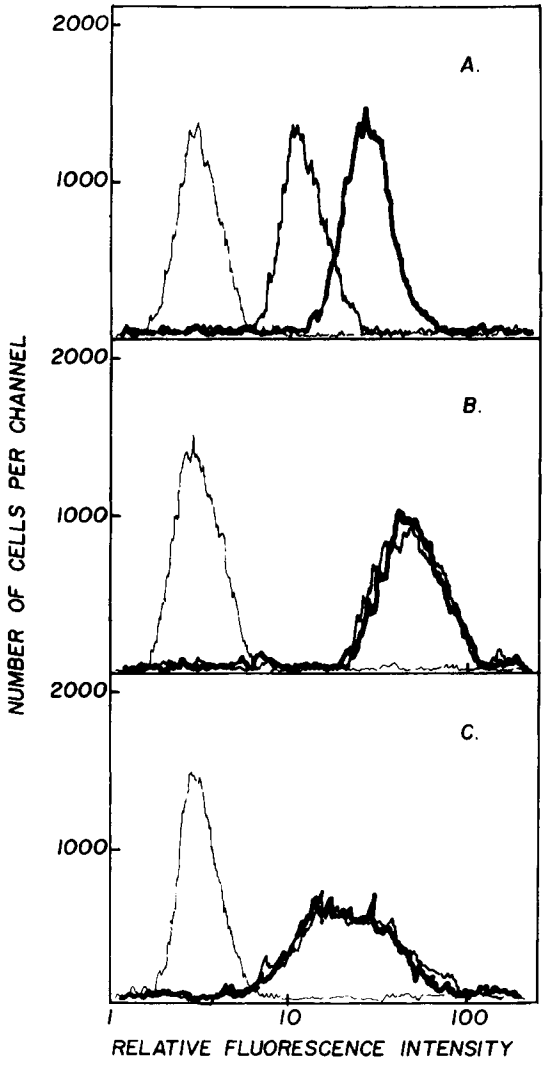

Fig. 2. Antiidiotype inhibits the binding of reovirus to its cellular receptor. 10^6 R1.1 thycoma cells were incubated with either (A) biotinylated-reovirus, (B) biotinylated anti-H-2, or (C) biotinylated anti-Thy followed by staining with fluorescein-conjugated avidin (bold lines). To demonstrate inhibition of binding by antiidiotype, cells were preincubated with antiidiotype for 45 min at 4°C prior to washing and further incubation with each of the three reagents listed above (medium lines). As a control, R1.1 cells were incubated with fluorescein-conjugated avidin alone (light lines). Cells were fixed and analyzed as described for Figure 1. The degree of antiidiotype inhibition is reflected in a leftward displacement of the mean fluorescence intensity.

Binding and Scatchard Analysis of Reovirus Receptors

Radioiodinated antiidiotype was used to determine the number and affinity of reovirus binding sites on thymoma cells. Antiidiotype was labeled in vitro with Na ^{125}I using a recently described iodination procedure which employs lactoperoxidase-coupled enzymobeads [18]. This procedure allows for high specific activity of iodi-

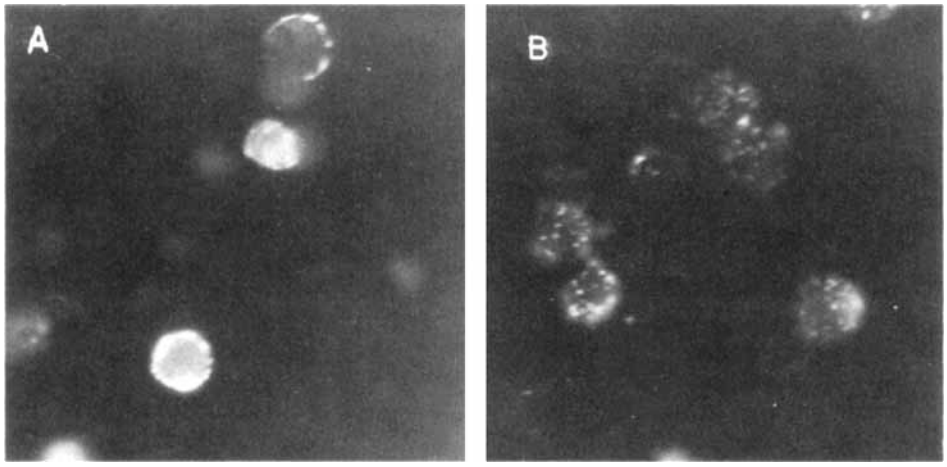

Fig. 3. Aggregation of reovirus receptors following cross linking of bound antiidiotype. R1.1 cells were incubated with antiidiotype, and stained with fluoresceinated goat anti mouse IgM as described in Materials and Methods. (A) Fluorescence image of cells immediately following staining at 4°C. (B) Fluorescence image of cells following incubation of stained cells for 30 min at 37°C prior to fixation.

nation yet under gentle conditions which discourage denaturation. To quantitate binding, increasing amounts of ^{125}I-labeled antiidiotype were added to a fixed number of thymoma cells. Results presented in Figure 4 demonstrate that, in agreement with previous studies using labeled viral particles [4,5], the binding of antiidiotype to R1.1 cells (reovirus type 3–positive) displayed linear, saturable kinetics, and that R1.1 cells possess an average of 79,000 reovirus binding sites with an apparent K_d of 9.6×10^{-10} M for antiidiotype. In contrast, the binding of labeled antiidiotype to YAC cells (reovirus type 3–negative) never exceeded background levels. The nonspecific component of binding was assessed by measuring the amount of ^{125}I-antiidiotype bound in the presence of a 100-fold excess of unlabeled antiidiotype. Nonspecific binding never exceeded 6% of the total bound antiidiotype. To accurately conduct Scatchard analysis, binding was conducted over a 3-log range of antiidiotype concentrations to plateau levels. Only a portion of this data is presented.

Identification of the Reovirus Cell Surface Receptor by Western Blot Gel Analysis

Preliminary biochemical characterization of the cell surface reovirus receptor on R1.1 cells was accomplished using Western blot gel analysis. R1.1 cell membranes were first purified using differential and equilibrium gradient centrifugation [17]. Membrane extracts were then run under reduced conditions on discontinuous 5–10% SDS-polyacrylamide gels, and the separated proteins were electrically transferred to nitrocellulose paper. Identification of the reovirus membrane receptor was achieved by independent incubation of Western blots with ^{125}I-labeled antiidiotype or ^{125}I-labeled reovirus. Figure 5 is an autoradiograph of the results of these incubations. Labeled antiidiotype bound to a single 67,000-Da band (Fig. 5A) on R1.1 membrane extracts, whereas no prominent band was detected on YAC membranes. Confirmation of this 67,000-Da band as the reovirus receptor was achieved by the observation that labeled reovirus bound to this same band in a separate incubation (Fig. 5B). Binding

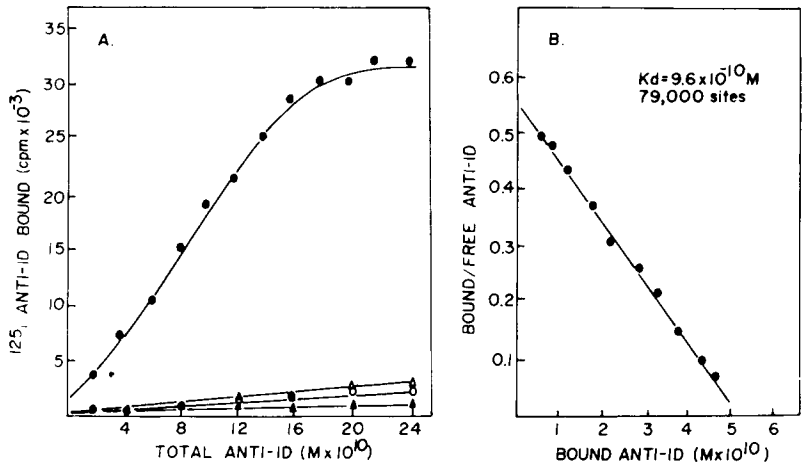

Fig. 4. Binding and scatchard analysis of antiidiotype on thycoma cells. (A) Binding of radiolabeled antiidiotype to R1.1 and YAC cells. 10^6 cells in 50μl aliquots containing PBS–0.5% bovine albumin–0.2% NaN_3 were incubated with increasing amounts of ^{125}I-labeled antiidiotype for 60 min at 4°C. Cells were washed free of unbound label by centrifugation through phathlate oils as described in Materials and Methods. Values presented represent the means of triplicate samples. The specific binding component of R1.1 (●—●) and YAC (▲—▲) cells was determined by subtracting the nonspecific component (○—○) and (△—△) from the amount of total bound. (B) Scatchard analysis of the binding data presented in A.

or radiolabeled type 1 virus or control IgM (HOB. 4) to the 67,000-Da band was not detected (data not shown).

DISCUSSION

The recent literature indicates that binding of the majority, if not all, of the mammalian viruses to target cells is mediated by interaction at specific cell surface sites. Membrane binding proteins for lactate dehydrogenase virus [23], influenza virus [24], Sendai virus [25], Moloney leukemia virus [26], Friend leukemia virus [27], Rauscher murine leukemia virus [28], and adenovirus type 2 [29] have been recently described and partially characterized biochemically. Previous work has demonstrated that the tropism of the mammalian reoviruses is also mediated by specific receptor recognition [4,5,7,8,12,13,30,31]. To a large degree this work has been made possible by the isolation of antiidiotype antibody probes which interact with reovirus receptors in a manner indistinguishable from that of virus. The use of antiidiotypic antibodies as specific receptor probes was first made by Sege and Peterson [32] on the insulin system. Subsequently, a number of laboratories have applied antiidiotypic probes in receptor studies.

The construction of a syngeneic monoclonal antiidiotype reactive with the reovirus receptor has enabled a more detailed cellular and biochemical analysis of reovirus receptors than was previously possible. In this report we have demonstrated the specificity of 87.92.6 antiidiotype binding to cells that bear type 3 reovirus membrane receptors. This is in agreement with previously published reports of the blocking ability of xenogeneic rabbit antiidiotype [30]. The binding and cross linking

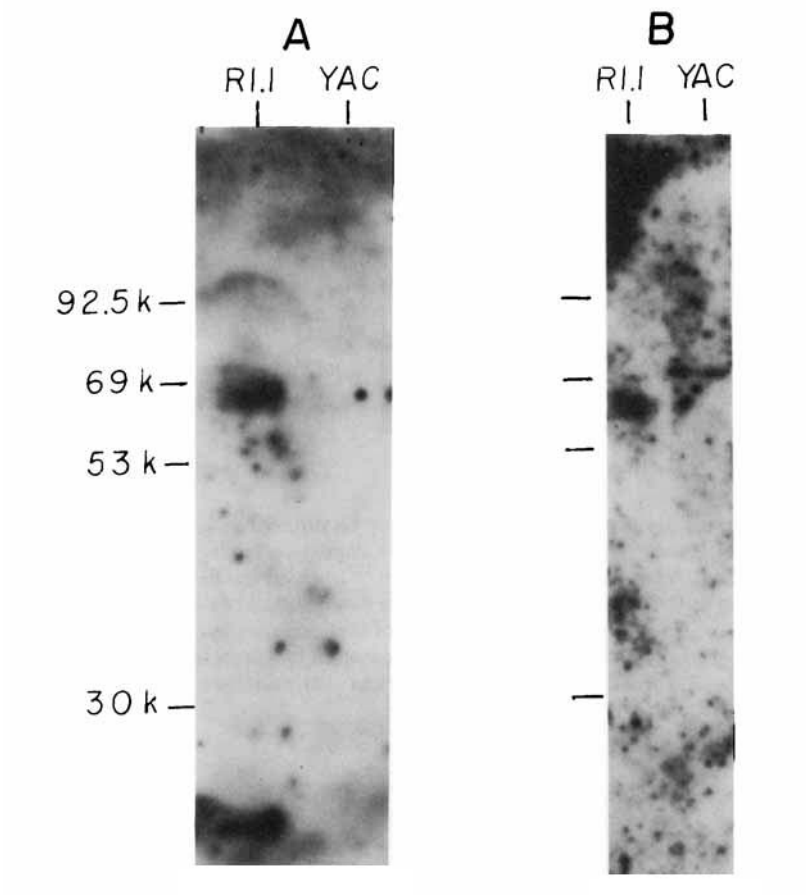

Fig. 5. Identification of the mammalian reovirus by Western blot analysis. (A) Binding of ^{125}I-monoclonal antiidiotype to Western blots. (B) Binding of ^{125}I-reovirus to Western blots. Purified membranes from 5×10^7 R1.1 and YAC cells were run under reducing conditions on discontinuous 5–10% SDS-polyacrylamide gels, then transferred to nitrocellulose paper. The nitrocellulose was then blocked with gelatin, and labeled antiidiotype or reovirus was added and allowed to bind to transferred membrane proteins. Dried blots were developed by autoradiography.

of antiidiotype on the surface of R1.1 cells was shown to stimulate the aggregation, but not capping, of reovirus receptors. Capping of reovirus receptors on murine T cells following cross linking of bound reovirus by antiimmunoglobulin has been reported by Epstein et al [22]. Our results confirm the linkage of reovirus receptor to microfilament arrays and are consistent with a loss of submembrane cytoskeletal associations in transformed cells [34].

Binding analysis of reovirus receptors using radiolabeled antiidiotype indicated that R1.1 cells possess an average of 79,000 receptor sites per cell. Labeled antiidiotype and labeled virus were also used to molecularly characterize the membrane receptor for reovirus type 3. Together with previous reports on the sensitivity of reovirus receptors to protease and tunicamycin treatment [13], these results indicate

that the reovirus receptor is a 67,000-Da glycoprotein. More detailed biochemical characterization of receptors from a number of different cells is currently under way.

The attachment of reovirus type 3 to its cellular receptor has profound effects on cell function. Binding of UV-irradiated virus to murine T cells stimulates the activation of nonspecific suppressor T cells [35], and binding to murine L929 cells results in a potent inhibition of cellular DNA synthesis [36]. The mechanism(s) of these receptor mediated events remains wholly undetermined. The presence of a large number of viral receptors on R1.1 thymoma cells, which are amenable to study using antiidiotype probes, should provide a useful model for analysis of the effects of receptor perturbation in the absence of viral infectivity.

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