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IDENTIFICATION OF A CELL MEMBRANE RECEPTOR FOR INTERFERON INDUCTION BY POLY rI:rC

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Summary. - PR-RK, a cell line derived from rabbit kidney cells (RK-13), was insensitive to the cytotoxic effect and interferon (IFN) inducing activity of the copolymer of riboinosinic and ribocytidylic acid (poly rI:rC). However, PR-RK was sensitive to the cytotoxic effect of the copolymer of riboadenylic and ribouridylic acid (poly rA:rU). Comparison of PR-RK cells and RK-13 cells by cytofluorometric analysis revealed that the binding of poly rI:rC was considerably reduced on PR-RK cells. These results suggested that the receptor for poly rI:rC might be different from the receptor for poly rA:rU, and this difference could provide a basis for the identification of the dsRNA receptor on cell surface. Western blot analysis of the components of cell membrane fraction prepared from RK-13 cells was performed by using a monoclonal antibody, which binds to cell membrane of RK-13 cells but not to PR-RK cells, and which blocks IFN induction by poly rI:rC in RK-13 cells. The 60K protein was identified as one of the poly rI:rC receptor protein.

Key words: interferon induction; poly rI:rC; dsRNA receptor

Introduction

The copolymer of riboinosinic and ribocytidylic acid (poly rI:rC) has been widely used as an IFN inducer in animal cells with or without polycations such as DEAE-dextran (Vilček *et al.,* 1968). Nevertheless, the mechanism of interferon induction by poly rI:rC is not yet understood. Although an earlier report (Colby and Chamberlin, 1969) suggested the presence of a specific receptor for poly rI:rC inside the cell, the nature of the receptor is unknown. Even though the synthesis of interferon in induced cells has been discussed (Burke, 1982; Burke, 1983), the signal-transducing system from cell surface to the nucleus, required for the derepression of interferon genes, remains to be elucidated.

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In our previous experiments (Yoshida and Azuma, 1985), we observed that only a part of the poly rI:rC bound to RK-13 cells played a role in IFN induction, indicating the presence of specific poly rI:rC receptors on cell membranes for IFN induction as well as nonspecific binding. The possibility that an interaction between viral surface component(s) and cell surface membrane may trigger type 1 IFN induction, has also been reported (Capobianchi *et al.*, 1985; Ito and Hosaka, 1983; Kohase and Vilček, 1979; Yoshida, 1985). On the other hand, Kelley and Pitha (1985) demonstrated the adsorption and internalization of poly rI:rC into murine cells, and suggested that internalization is required for the induction of IFN. One way to examine the mechanism of IFN induction is to isolate the cellular factors that mediate the induction signal (Riordan and Pitha-Rowe, 1985).

In this paper, we report on the identification and isolation of specific poly rI:rC receptors participating in the IFN induction from the cytoplasmic membranes of cultured cells.

Materials and Methods

Chemicals. Poly rI:rC and poly rA:rU were obtained from Yamasa Shoyu Co., agarose-poly rI:rC type 6 from Pharmacia, octylphenoxypolyethoxyethanol (Nonidet P40) and ethidium bromide from Sigma, peroxidase-conjugated anti-mouse IgG from Cappel Laboratories, ^{125}I-labelled anti-mouse IgG goat F(ab')$_2$ and adenosine 5'-[gamma-^{32}P]triphosphate ([gamma-^{32}P]ATP, 185 TBq/mmol) from Amersham International plc., T4 polynucleotide kinase from Promega Biotec, and Eagle's minimum essential medium (MEM) from Nissui Seiyaku Co.

^{32}P-poly rI:rC and ^{32}P-poly rA:rU were prepared by 5'-phosphorylation of these polynucleotides with [gamma-^{32}P]ATP and T4 polynucleotide kinase by using a slight modification of the method described by Lillehaug and Kleppe (1975). Briefly, the reaction mixture consisted of the following components, in a total volume of 1000 μl: 40 mmol/l Tris-HCl (pH 8.0), 10 mmol/l $MgCl_2$, 5 mmol/l dithiothreitol, 14 units of T4 polynucleotide kinase, 3.7 MBq of [gamma-^{32}P]ATP, and 1 mg of poly rI:rC or poly rA:rU. After incubation at 37 °C for 6 hr with shaking, the mixture was applied on the column of Sephadex G-25 (Column PD-10, Pharmacia) equilibrated with 10 mmol/l phosphate buffered saline (PBS, pH 7.4), and eluted with PBS to remove low molecular weight substances. ^{32}P-labelled poly rI:rC and poly rA:rU were diluted with MEM, and then used for binding assay.

Cell cultures. RK-13 and PR-RK cells, derived from RK-13 cells by repeated treatment with poly rI:rC (Yoshida and Azuma, 1985), were cultivated in MEM supplemented with 10 % calf serum (MEM-CS10). After a confluent cell sheet was formed, the serum concentration was decreased to 5 % (MEM-CS5) for experiments.

Cytofluorometric analysis of poly I:C binding to cells. Confluent monolayer cultures of RK-13 and PR-RK cells were dispersed by treatment with 0.02 % EDTA in PBS, fixed with cold 70 % ethanol, and then washed 5 times with PBS. Cells were immediately treated with 100 μg/ml of poly rI:rC or control buffer at 37 °C for 30 min, washed 3 times with PBS and stained with 0.001 % ethidium bromide. The stained cells (10^6/ml) were run in a cytofluorometric analyzer, Shomedic Cell Sorter CS-20 (Showa Denko Co.).

Preparation of antiserum to RK-13 cell specific antigens. BALB/c mice were immunized with RK-13 cells and the antiserum obtained was adsorbed with PR-RK cells to remove antibodies against common antigens of PR-RK and RK-13 cells, as described previously (Yoshida and Azuma, 1985). The antibody titer of anti-RK-13 serum to RK-13 and PR-RK cells were assayed by

enzyme-linked immunosorbent assay (ELISA) (Nakane, 1975). The titer was 800 units/ml to RK-cells and less than 20 units/ml to PR-RK cells.

Preparation of monoclonal antibody against poly rI:rC receptor. Anti-poly rI:rC receptor monoclonal antibody was prepared by the method of Köhler and Milstein (1975). Briefly, BALB/c mice were immunized with cytoplasmic membrane fractions of RK-13 cells twice at a week interval. The spleen cells were separated 4 days after the last immunization and hybridized with mouse myeloma cells (P3X63-Ag8-653). Hybridomas secreting antibody against poly rI:rC receptor protein were screened as follows: RK-13 and PR-RK cell sheets in 96-well culture plate (Corning Glass Works) were washed twice with PBS, treated with culture fluids of hybridomas at 37 °C for 30 min and washed with PBS. Antibody positive wells were detected by ELISA. Then, antibody containing culture fluids, which were positive in wells of RK-13 cells but negative in wells of PR-RK cells, were screened by inhibition assay of IFN production. The monoclonal antibody, 6D1, which reacts with RK-13 cells but not with PR-RK cells and inhibits IFN production in RK-13 cells by poly rI:rC, was obtained and used for the determination of poly rI:rC receptor protein.

Detection of specific poly rI:rC binding activity of fractionated cell components. Detection of poly rI:rC binding activity of subcellular components was carried out by radioimmuno assay (RIA) as follows: 50 μl subcellular samples, which were obtained from fractions of cytoplasmic membrane by Nonidet P40 lysis, affinity chromatography and fast protein liquid chromatography (FPLC) as described below, were placed into 96-wells of Millititer HE plate (sealed with nitrocellulose membrane, Millipore Co.), incubated at 4 °C overnight and washed with PBS–10% foetal bovine serum (PBS-FS10). Blocking of nitrocellulose membrane was carried out by incubation of the plate with PBS-FS10 at 37 °C for at least 2 hr. Then, each well was washed and treated with 100 μg/ml poly rI:rC or control buffer at 37 °C for 1 hr. After washing, 50 μl of PR-RK cell-absorbed anti-RK-13 serum, diluted to 1:4 was added to each well and incubated at 37 °C for 1.5 hr. After washing, ^{125}I-labelled anti-mouse IgG goat F(ab')$_2$ was added and incubated at 37 °C for 1.5 hr. After washing, membranes of each well were dried, punched out and the radioactivity was counted in a Packard Prias PGD Autogamma Counter.

The difference in radioactivities between untreated and poly rI:rC-treated well indicates the amount of components to which poly rI:rC binds. Specific poly rI:rC binding activity was defined as the cpm of untreated well minus the cpm of poly rI:rC-treated well.

Preparation of cytoplasmic membrane lysate. Cytoplasmic membrane fractions of RK-13 cells and PR-RK cells were prepared by aqueous two phase method described by Brunette and Till (1971) with modifications described in our earlier report (Yoshida and Azuma, 1985). Collected cytoplasmic membrane fractions were sonicated with Kontes Microsonicator at maximum power for 1 min and treated with 0.5 % Nonidet P40 at 4 °C overnight. Supernatant fluid was recovered by centrifugation at 8000 x g for 30 min and used as cytoplasmic membrane lysate.

Affinity chromatography of poly rI:rC binding components. 1 ml of agarose-poly rI:rC was packed in a plastic column and equilibrated with PBS. Cytoplasmic membrane lysate was diluted tenfold with PBS and applied onto the column at a flow rate of 0.5 ml/min. Then the column was washed with PBS and the components retained on the column were eluted with 1:100-diluted PBS (1/100x PBS). Elution was monitored by absorbance at 280 nm with an ISCO UA-5 UV-monitor.

FPLC fractionation of poly rI:rC binding components. Poly rI:rC binding components eluted from agarose-poly rI:rC column were applied directly to the FPLC system using Mono Q column. Elution was carried out using a linear gradient from 1/100x PBS to 10x-concentrated PBS (10x PBS) at a flow rate of 2 ml/min. When rechromatography was performed, the concentration of the buffer of the sample was adjusted to 1/100x PBS. The elution pattern was monitored by absorbance at 280 nm.

SDS-PAGE analysis of poly rI:rC receptor. FPLC fractions showing the specific poly rI:rC binding activity were dialyzed against distilled water and concentrated by lyophilization. Slab electrophoresis of the concentrated samples in 10 % polyacrylamide gel containing 0.1 % SDS (SDS-PAGE) was carried out according to the method described by Laemmli (1970). Gel was fixed with 10 % TCA and stained with a silver staining kit (Daiichi Chemicals Co.).

Western blot analysis of poly rI:rC receptor. The analysis was carried out by the method described by Ey and Ashman (1986). Briefly, the protein bands separated by SDS-PAGE were transferred to

nitrocellulose membrane (Toyo Roshi Co.) in a buffer containing 25 mmol/l Tris, 192 mmol/l glycine (pH 8.3)-methanol, 4:1 (v/v) in a transfer cell (Nihon Eido Co.) at 4 v/cm for 2 hr. After transfer, the nitrocellulose membrane was placed in 2 ml of PBS-FS10 and then in 2 ml of PBS containing 50μ l of 6D1 monoclonal antibody. The protein bands which bound to the 6D1 were developed by ELISA.

Results

The time course of binding of ^{32}P-poly rI:rC to cells was examined. As can be seen in Fig. 1, ^{32}P-poly rI:rC binds to PR-RK and RK-13 cells maximally 10 to 30 min after addition. Binding of ^{32}P-poly rI:rC to PR-RK cells was only 60 % of that to RK-13 cells. Relative amounts of poly rI:rC bound to RK-13 and PR-RK cells were compared by the method of cytofluorometric analysis. Ethidium bromide can interact not only with the double stranded DNA but also with the double stranded RNA, so that the adsorption of poly rI:rC to a cell increases the

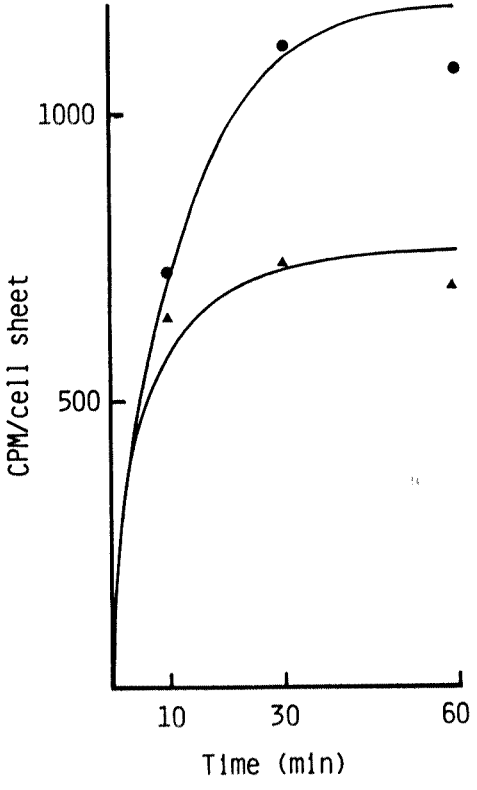

Fig. 1
Binding of ^{32}P-poly rI:rC to RK-13 and PR-RK cells
^{32}P-poly rI:rC was added to RK-13 (●) and PR-RK (▲) cell sheets grown in wells of plate and incubated for desired periods at 37 °C. The cell sheets were washed with PBS and the radioactivity of cell sheets was counted.

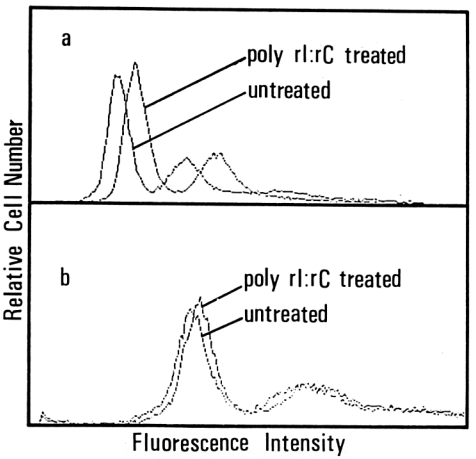

Fig. 2
Cytofluorometric analysis of poly rI:rC adsorption on RK-13 and PR-RK cells
RK-13 (a) and PR-RK (b) cells were dispersed, fixed, and treated with 100 μg/ml poly rI:rC or with a control buffer. After treatment, cells were washed and stained with ethidium bromide and the increase of fluorescence intensity caused by the adsorption of poly rI:rC on cells was measured.

fluorescence intensity of the cell after staining with ethidium bromide. As shown in Fig. 2, the increase of fluorescence intensity of RK-13 cells after treatment with poly rI:rC was higher than that of PR-RK cells. These results directly indicate the decreased amounts of poly rI:rC bound to PR-RK cells and also confirm our earlier results (Yoshida and Azuma, 1985) that poly rI:rC binds to PR-RK cells without IFN induction, indicating the specific and non-specific binding of poly rI:rC to cell membrane.

To test the sensitivity of RK-13 and PR-RK cells to the cytotoxic effect of poly rI:rC and poly rA:rU, the relative plating efficiency of these cells was measured. As shown in Fig. 3, RK-13 cells were sensitive to both poly rI:rC and poly rA:rU, however, PR-RK cells were sensitive only to poly rA:rU. Then, the binding of ^{32}P-poly rA:rU to RK-13 and PR-RK cells was compared with that of ^{32}P-poly rI:rC. Table 1 shows no difference between the binding of poly rA:rU

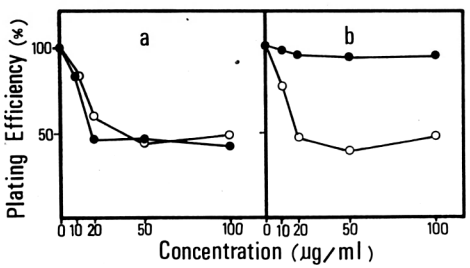

Fig. 3
Sensitivity of RK-13 and PR-RK cells to the cytotoxic effect of poly rI:rC and poly rA:rU
RK-13 (a) and PR-RK (b) cells were seeded in 24 well plate at a concentration of 100 cells/well, and incubated in the presence of various concentrations of poly rI:rC (●—●) or poly rA:rU (○—○) at 37 °C for 2 weeks. Cell colonies were fixed with ethanol, stained with crystal violet and counted. The relative plating efficiency (%) at each concentration of copolymer was calculated relative to the colony count in the control culture medium.

to RK-13 cells and PR-RK cells, though the binding of poly rI:rC to PR-RK cells was again lower than that to RK-13 cells. Competition experiments of binding between ^{32}P-poly rI:rC and cold poly rI:rC or poly rA:rU revealed that (a) the binding of ^{32}P-poly rI:rC competed with cold poly rI:rC on RK-13 cells but not on PR-RK cells, and (b) the binding of ^{32}P-poly rI:rC did not compete with cold poly rA:rU on RK-13 and PR-RK cells (Table 2). These results show that poly rA:rU can bind to PR-RK cells and that poly rI:rC binds to the sites of cell membrane different from the sites binding with poly rA:rU. They also confirm our previous conclusion (Yoshida and Azuma, 1985) that the resistance of PR-RK cells to poly rI:rC is not due to the increased RNase activity which degrades dsRNAs. In our previous report (Yoshida and Azuma, 1985), we have also shown that the antiserum to RK-13 cells which had been adsorbed with

Table 1. Binding of ^{32}P-polynucleotides on RK-13 and PR-RK cells

Polynucleotides	Binding (cpm/μg protein) on		
	RK-13	PR-RK	PR-RK/RK-13
^{32}P-poly rI:rC	1941 ± 106	1364 ± 159	0.70
^{32}P-poly rA:rU	624 ± 46	686 ± 82	1.10

Conditions as Fig. 1, except that ^{32}P-poly rA:rU was also used and incubation time was 1 hr.

Table 2. Competition between ^{32}P-poly rI:rC and cold polynucleotides in binding to RK-13 and PR-RK cells

Cold polynucleotides		Binding (cpm/μg protein) of ^{32}P-poly rI:rC on			
		RK-13	%	PR-RK	%
None		1941 ± 106	(100)	1364 ± 159	(100)
Poly rI:rC	1000 μg/ml	1110 ± 157	(57)	1242 ± 63	(91)
Poly rI:rC	500 μg/ml	1277 ± 179	(66)	1104 ± 75	(81)
Poly rA:rU	1000 μg/ml	2017 ± 161	(104)	1845 ± 234	(135)
Poly rA:rU	500 μg/ml	2017 ± 223	(104)	1516 ± 259	(111)

Cold polynucleotides were added to the cell sheet as in Fig. 1 and incubated at 37 °C for 1 hr and washed with PBS. ^{32}P-poly rI:rC was then added to the cultures, which were then incubated at 37 °C for 1 hr, washed, and the radioactivity was counted.

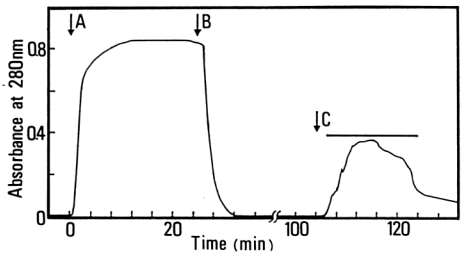

Fig. 4
Agarose-poly rI:rC affinity chromatography of poly rI:rC binding components
The cytoplasmic membrane lysate from RK-13 cells was applied (arrow A) to an agarose-poly rI:rC column equilibrated with PBS and the column was washed with PBS (arrow B). Elution of the components retained in the column was carried out by dissociation of double stranded RNA of agarose-poly rI:rC with a decreased ionic strength, 1/100x PBS (arrow C). Pooled fractions were indicated with the bar.

PR-RK cells, inhibits IFN induction by poly rI:rC in RK-13 cells pretreated with the antiserum. All these results indicate that the receptors specific for poly rI:rC are present on the surface of RK-13 cells and absent on PR-RK cells. Since RK-13 cells in contrary to PR-RK cells can produce IFN in response to poly rI:rC (Yoshida and Azuma, 1985), the receptor to poly rI:rC present on RK-13 cells seems to play an intrinsic role in the IFN induction.

A cytoplasmic membrane fraction was prepared, lysed and fractionated by an agarose-poly rI:rC affinity chromatography. Fig. 4 shows that the components bound to agarose-poly rI:rC were eluted from the column with 1/100x PBS according to the dissociation of dsRNA. Eluted fractions were pooled and further fractionated by FPLC. Poly rI and poly rC contaminants in the pooled

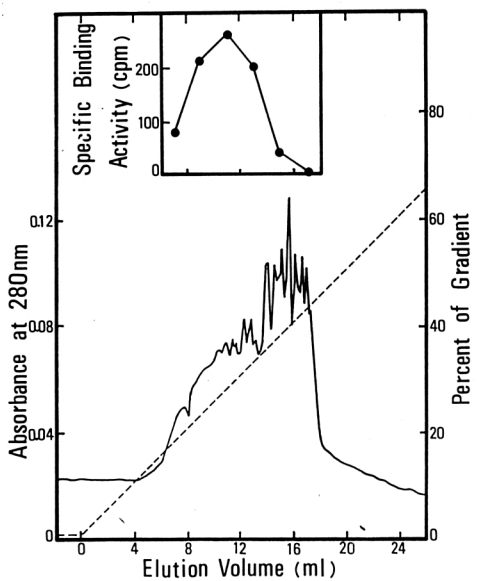

Fig. 5
Fast protein liquid chromatography of poly rI:rC binding components
The pooled fractions from affinity chromatography (Fig. 3) were loaded a Mono Q column of the FPLC system equilibrated with 1/100x PBS. Elution was carried out by linear gradient from 1/100x PBS to 10x PBS (broken line) monitored by absorbance at 280 nm (solid line) and 2 ml fractions were collected. Each fraction was concentrated and assayed for specific poly rI:rC binding activity by the RIA method (insert, ●—●).

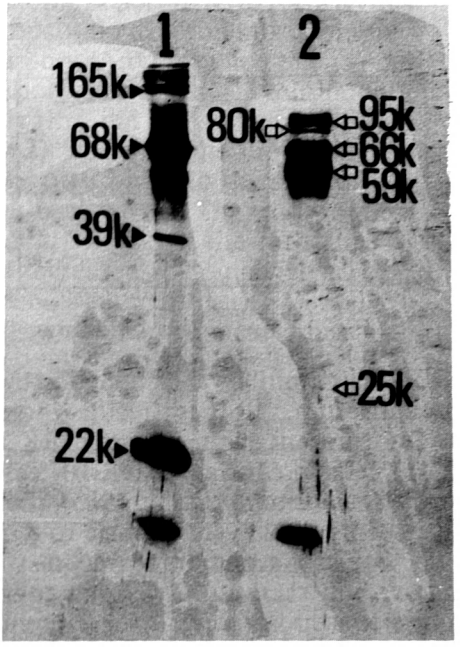

Fig. 6
SDS-polyacrylamide gel electrophoresis of poly rI:rC receptor
Peak fractions of specific poly rI:rC binding activity (Fig. 5) were concentrated and analyzed by electrophoresis in 10 % polyacrylamide slab gel followed by the silver staining. Lane 1: the molecular weight marker; lane 2: the poly rI:rC receptor proteins. Arrows in the lane 2 indicate the main bands of the receptor proteins.

fractions were removed by the first FPLC fractionation, and then the second FPLC was carried out. Fig. 5 shows the elution pattern of the second FPLC of poly rI:rC binding components derived from RK-13 cells. The second FPLC fractions were concentrated and used for detection of specific poly rI:rC binding activity by RIA method (insert in Fig. 5). The peak of the specific binding activity which eluted at 28 % of the gradient and was not detected in the case of PR-RK cells, represents the poly rI:rC binding components which react specifically with the anti-RK-13 cell serum adsorbed with PR-RK cells.

The peak fraction of the specific binding activity of RK-13 cells (shown in Fig. 5) was analyzed by SDS-PAGE and silver staining method. As shown in Fig. 6, 5 main bands (95K, 80K, 66K, 59K, and 25K) and a few minor bands were detected. The protein bands of polyacrylamide gel were transferred to nitrocellulose membrane, and the specific protein bands reacting with the 6D1 monoclonal antibody on the membrane were developed by ELISA. As shown in Fig. 7, 60K protein band was detected in the membrane fraction of RK-13 cells.

Discussion

Recently, many kinds of receptors have been identified in mammalian cells and their structure and role in cells responding to biologically active

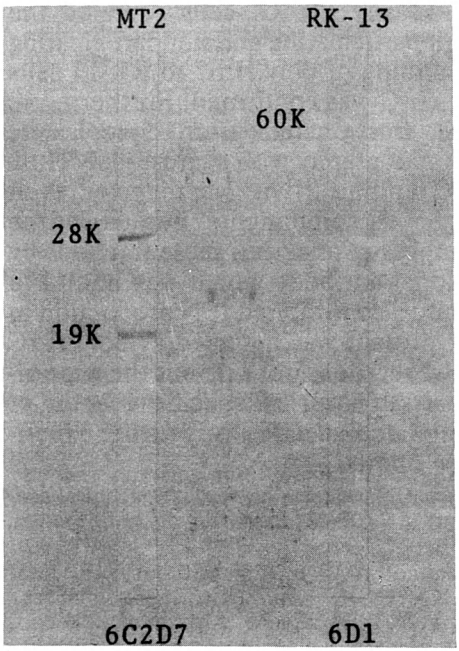

Fig. 7
Western blot analysis of poly rI:rC receptor protein
The bands of poly rI:rC-binding proteins (Fig. 6) were transferred to nitrocellulose membrane and treated with monoclonal antibody 6D1. 6D1-specific protein band was developed by ELISA. 28K and 19K protein bands of MT2 are the molecular weight markers developed by 6C2D7 monoclonal antibody specific for MT2.

substances, such as glucocorticoid hormone (Hollenberg *et al.,* 1985; Weinberger *et al.,* 1985), insulin (Ebina *et al.,* 1985; Graves *et al.,* 1985), adrenalin (Lefkowitz *et al.,* 1985), epidermal growth factor (Friedman *et al.,* 1984), T cell growth factor (interleukin-2) (Kondo *et al.,* 1986), fibronectin (Patel and Lodish, 1986), and interferons (Aguet, 1980; Aguet *et al.,* 1988; Feinstein *et al.,* 1985; Orchansky *et al.,* 1984; Pestka *et al.,* 1987; Yonehara *et al.,* 1983) have been discussed. Most of the receptors reported exist on the surface of cytoplasmic membrane and mediate signals to nucleus by internalization of specific substances or by formation of additional mediator molecules. Poly rI:rC is also a biologically active substance and the presence of a receptor to poly rI:rC has been postulated in early investigations. Many attempts have been made to analyze the interaction between poly rI:rC and its postulated receptors (Bachner *et al.,* 1975; DeClercq and DeSomer, 1974; DeClercq *et al.,* 1973; Hutchinson and Merigan, 1975; Magee *et al.,* 1976; Mayhew *et al.,* 1977; Taylor-Papadimitriou and Kallos, 1975). However, kinetic analysis of poly rI:rC binding to cell surface could not provide any direct evidence of the presence of specific poly rI:rC receptors required for IFN induction, probably because of nonspecific adsorption of poly rI:rC to cells, which did not participate in IFN induction.

In our previous studies (Yoshida and Azuma, 1985), poly rI:rC resistant PR-RK cells were used as a negative control for specific poly rI:rC binding.

Analysis of the difference between RK-13 and PR-RK cells revealed the adsorption of poly rI:rC on cell surface both participating and nonparticipating in IFN induction. In the present study, binding of poly rI:rC to RK-13 cells attributing to the biological effect of poly rI:rC was confirmed. Furthermore, poly rI:rC binding components were fractionated from cytoplasmic membranes of RK-13 cells by affinity chromatography with agarose-poly rI:rC and FPLC. In the FPLC fractions, poly rI:rC binding activity was detected, even though the fractions consisted of heterogenous components. Because of the heterogeneity of fractionated receptor sample, we analyzed these fractions by Western blotting method with a monoclonal antibody which was bound to surface of RK-13 but not to PR-RK cells, and also inhibited IFN production in RK-13 cells by poly rI:rC. Only one 60K protein was detected as poly rI:rC specific receptor protein. This finding, however, does not rule out the possibility that the other proteins (Fig. 6) are the parts of poly rI:rC specific receptor, or that these are the proteins binding poly rI:rC non-specifically. Further experiments are now in progress to clarify these points.

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