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Presence of insulin binding sites on viral particles

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Summary

Using a standard radio-receptor assay, we have demonstrated that [^{125}I]insulin can bind specifically to each of two types of purified enveloped viruses, influenza A virus and Rous sarcoma virus. A non-enveloped icosahedral virus (echovirus 11) and herpes simplex virus type 2, which acquires its envelope from the nuclear membrane of the cell, did not possess insulin receptor activity. Displacement of specifically bound radiolabelled insulin from the viral surface was achieved by addition of an excess of unlabelled insulin but not by addition of another unrelated protein, cytochrome C. We conclude that certain types of enveloped viruses may acquire insulin binding sites from the plasma membrane of their host cell.

Insulin; Influenza virus; Rous sarcoma virus; Binding activity

Introduction

It has often been suggested that viral particles, which bud from cell surfaces, acquire some host cell proteins as part of their outer envelopes.

Thus, reports indicate that a small quantity, i.e. less than 1%, of the protein in influenza virus envelopes is of host cell origin (Holland and Kiehn, 1970). Also histocompatibility antigens from infected cells have been reported to be incorporated into the vesicular stomatitis virus (VSV) envelope (Hechtland Summers,

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1972). Recent findings indicate that a small amount of cellular protein may even be found in highly purified VSV particles (Lodish and Porter, 1980). It remains to be determined which types of cellular proteins a virus particle may acquire in this manner, and whether these proteins are altered in any way.

A line of evidence supporting the notion that virus infection may alter the immunogenicity of cell membrane antigens comes from studies on viral oncolysis. It has been demonstrated that immunization of animals with virus-infected cancer cells can improve immune responses to tumor-associated antigens (Lindenmann and Klein, 1967; Haller and Lindenmann, 1975; Wise, 1977). In addition, infection of mouse thymus cells with Newcastle disease virus (NDV), herpes simplex (HSV) or vaccina virus has been found to lead to a 30-fold increase in antibody reactions against the membrane alloantigen Thy-1 (Wise, 1977).

In this study, we present evidence for the presence of insulin binding sites on the surface of influenza virus and Rous sarcoma virus but not on herpes simplex virus or echovirus 11. Demonstration of the ability of virions to acquire functional hormone receptors is important because it may provide greater insight into mechanisms of viral budding at the host cell surface (Bromberg et al., 1982) and may explain the involvement of viruses in autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM). Autoantibodies against insulin (Atkinson et al., 1986), islet cell surface proteins (Onodera et al., 1982) and the insulin receptor (Shimoyama et al., 1986) have all been associated with IDDM. We were interested in understanding whether these antibodies might have been induced by the viral infections that have epidemiologically been associated with IDDM (Notkins, 1984).

Materials and Methods

Preparation of [^{125}I]*insulin*

[^{125}I]insulin was prepared by a modification of a chloramine T labelling procedure (Cuatrecasas and Hollenberg, 1976) and had a specific activity of 1625 cpm/fmol.

Chicken embryo fibroblasts (CEF)

Avian leukosis virus-free fertilized eggs were purchased from the Institut Armand Frappier, Laval, Québec, Canada. The CEF cells, used in this study, were secondary cultures (2–3 days old), derived from the enzymatic digestion of 11-day-old embryos. They were grown in minimum essential medium (MEM) supplemented with 4% fetal calf serum (FCS) (Microbiological Associates, Bethesda, MD), penicillin (100 U/ml) and streptomycin (100 μg/ml), as previously described (Wainberg et al., 1982).

Human fibroblast cultures

Human diploid fibroblasts were established from a deltoid skin biopsy and were grown in antibiotic-free Eagle's minimal essential medium (Eagle, 1959) supple-

mented with 1 mM pyruvate and 10% (v/v) FCS. The cells were grown under 5% CO_2, the medium being changed three times weekly. Cells were plated at 10^4 cells/cm^2 in 35 mm plastic Petri dishes and grown to confluence (usually 6–7 days). Cell monolayers were rinsed free of serum-containing medium and were maintained for 30–48 h in serum-free medium containing 1 mg/ml bovine serum albumin (BSA) prior to insulin binding studies. Additional details can be found elsewhere (Germinario and Michaelidou, 1986; Germinario et al., 1984).

Viruses

HSV-2 was propagated in cultures of CEF cells in MEM supplemented with 5% FCS. The infected cells were incubated at 37°C under 5% CO_2 for 4–5 days. The B77 strain (subgroup C) of Rous sarcoma virus (RSV) was also grown in cultures of CEF under conditions similar to those used for HSV-2. The *Enterovirus* echo 11 strain was grown in cultures of Rhesus monkey kidney cells (MKC) (Connaught Laboratories, Toronto, Canada) or HEP-2 cells, as indicated in the text. Influenza A virus (serotype H3N2) was grown in Rhesus monkey kidney cells or in CEF.

In the case of HSV-2 and echovirus 11, culture medium was harvested from infected cells at the time of maximum cytopathic effect. Influenza virus was harvested from infected cultures after 4 days, by which time the cells could be shown to be positive by hemadsorption assay. RSV was harvested after 4–5 days, when cultures of CEF cells could be shown to be almost completely transformed (Wainberg et al., 1982). All virus-containing fluids were clarified by centrifugation at 5000 $\times$ *g* for 20 min at 4°C. Virus was then pelleted from supernatant fluids by centrifugation at 25000 $\times$ *g* for 2 h at 4°C. The viral pellets were resuspended in phosphate-buffered saline (PBS), pH 7.4. Virus preparations were then purified by further centrifugation through a sucrose gradient at 80000 $\times$ *g* for 75 min at 4°C. The appropriate viral bands were harvested by aspiration and dialyzed against PBS. Finally, virus was repelleted and resuspended in PBS. Viral preparations were quantitated in terms of total protein by use of Folin-phenol reagent (Lowry et al., 1950). Levels of viral protein obtained were as follows: CEF-grown RSV, 4.1 mg/ml, at an estimated virus particle concentration of 1.3×10^{11}/ml, as determined by viral focus formation assay (Wainberg et al., 1982); CEF-grown influenza A virus, 2.6 mg/ml, at an estimated infectious particle concentration of 1.8×10^{10}/ml (limiting dilution hemadsorption assay (Eagle, 1959); CEF-grown HSV-2, 1.9 mg/ml, estimated concentration of 1.6×10^9 particles/ml, as determined by plaque assay (Eagle, 1959); HEP-2 grown echovirus 11, 3.1 mg/ml, at a concentration of 2.3×10^{10} virus particles/ml, as determined by plaque assay (Eagle, 1959).

Insulin binding studies

The viral particles described above were incubated in suspension with 1.67×10^{-10} M [^{125}I]insulin in the presence and absence of excess unlabelled insulin or cytochrome C for 2 h at 4°C. Viral particles were then pelleted by centrifugation at 100000 $\times$ *g* for 15 min, washed with PBS, recentrifuged, and counted in a LKB gamma counter. Levels of specific insulin binding were determined by calculating the difference between that amount of [^{125}I]insulin bound to cells either in the ab-

sence or presence of excess unlabelled insulin (Cuatrecasas, 1971), i.e. specific binding is that amount which is displaced by excess unlabelled ligand.

With regard to human fibroblast cultures, the cell monolayers were incubated at 22°C with 1 ml of PBS, pH 7.5, containing 0.2% (w/v) BSA and 1.67×10^{-10} M [^{125}I]insulin. As in the case of viruses, unlabelled insulin was used to displace radiolabelled insulin. At the end of the incubation period, the cell monolayers were rinsed four times with PBS at 4°C and dissolved in 1 N NaOH for determination of radioactivity and protein (Lowry et al., 1950). All experiments were performed using four replicate samples.

Results

Our initial experiments involved the study of specific binding of [^{125}I]insulin (1.67×10^{-10} M) to 168 μg protein of each of influenza A virus and RSV. Human diploid fibroblasts were employed as a positive control. The results of Fig. 1 show that ^{125}I-labelled insulin could be specifically bound to these viruses, because the use of an excess quantity of unlabelled insulin could displace such specifically bound insulin from both these two types of viruses as well as from the surface of the fibroblasts. The percentages of specific [^{125}I]insulin binding, i.e. amount of displaced ^{125}I when compared to numbers of cpm initially bound, were found to be 70 ± 6% for the human diploid fibroblasts, 59% ± 5% for RSV and 79% ± 4% for influenza virus (Table 1). These figures represent that amount of label which was specifically bound, i.e. which could be displaced by an excess of unlabelled insulin (15 μg/ml). In contrast, the data of Fig. 2 indicate that little or no specific [^{125}I]insulin binding occurred at the surfaces of equivalent amounts (168 μg pro-

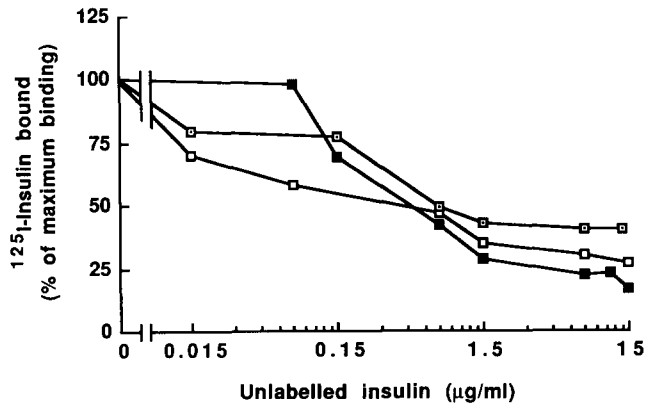

Fig. 1. Displacement of bound [^{125}I]insulin by unlabelled insulin. [^{125}I]insulin was studied for ability to specifically bind to Rous sarcoma virus (⊡), influenza A virus (■) and human diploid fibroblasts (□). Number of cpm maximally bound to human diploid fibroblasts was 3428 per mg protein. For Rous sarcoma virus and influenza virus, maximal binding was 3087 and 3861 cpm per mg protein, respectively. A total of 168 μg viral protein was used in these studies.

TABLE 1

Percentage of [^{125}I]insulin bound after attempted displacement by excess unlabelled insulin (15 μg/ml)

Target of [^{125}I]insulin	Cell used for virus propagation	No. cpm initially bound*	No. cpm remaining after displacement	Specific binding (%)**
Human deltoid fibroblasts	–	3428	1028	70.0
Influenza A	CEF	2982	926	68.9
Influenza A	Rhesus kidney	3861	803	79.2
Rous sarcoma virus	CEF	2350	949	59.6
Rous sarcoma virus	CEF	3087	1303	57.8
Echovirus 11	Rhesus kidney	4561	3416	25.1
Echovirus 11	HEP-2	3752	3226	14.0
HSV-2	CEF	2859	2610	8.7
HSV-2	CEF	1673	1601	4.3

* A total of 285,462 cpm of [^{125}]insulin was used as ligand in each study.

** % specific binding is that % of bound [^{125}]insulin that was displaced by excess unlabelled insulin (i.e. 15 μg/ml).

tein) of each of two viruses which do not bud from the plasma membrane of infected cells, echovirus 11 and HSV-2. In these instances, the percentages of specific binding were 14 ± 2% and 4 ± 1.2%, respectively (Table 1).

We next asked whether the specific binding of [^{125}I]insulin to each of RSV and influenza virus might be displaced by the addition of high concentrations of an unrelated polypeptide. For this purpose, [^{125}I]insulin binding studies were performed as described in Materials and Methods, and displacement was attempted with each of unlabelled insulin (molecular weight ≈ 6 kDa) and cytochrome C (molecular weight ≈12 kDa). The results of Figs. 3 and 4 show that, in this instance, the per-

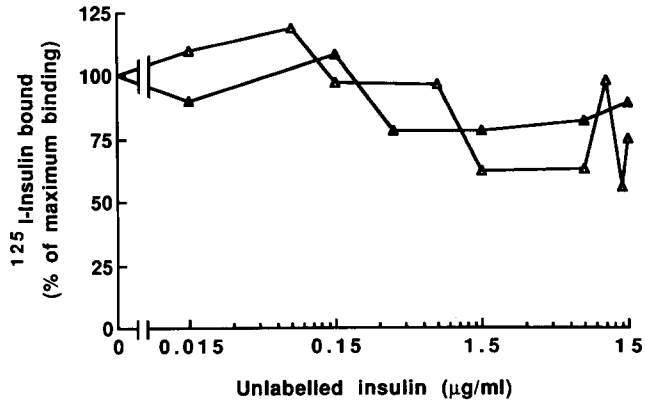

Fig. 2. Displacement of [^{125}I]insulin which had been bound to each of echovirus-11 (△) and HSV-2 (▲) by excess unlabelled insulin. Maximal binding for echovirus-11 and HSV-2 was 3752 and 2859 cpm per mg protein, respectively. A total of 168 μg viral protein was used in these studies.

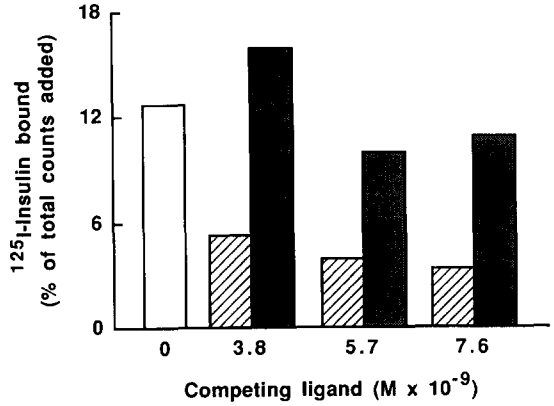

Fig. 3. Displacement of binding to Rous sarcoma virus of [^{125}I]insulin by excess unlabelled insulin (▨) and cytochrome C (■). Total radioactivity of [^{125}I]insulin added was 330000 cpm to 524 μg of viral protein.

centage of [^{125}I]insulin cpm bound to virus was considerably higher than that observed in the experiments of Figs. 1 and 2. The higher percentage of total binding in the case of RSV, as opposed to influenza A, may be the consequence of a greater number of insulinbinding sites on the former as opposed to the latter virus. The data of Fig. 3 further show that the use of cytochrome C, as a displacement molecule, had little effect on that amount of [^{125}I]insulin which remained specifically bound to RSV, while unlabelled insulin, in contrast, proved to be an effective displacement vehicle. Similar findings were obtained with regard to the attempted use of cytochrome C to displace specific [^{125}I]insulin binding from influenza virus (Fig. 4).

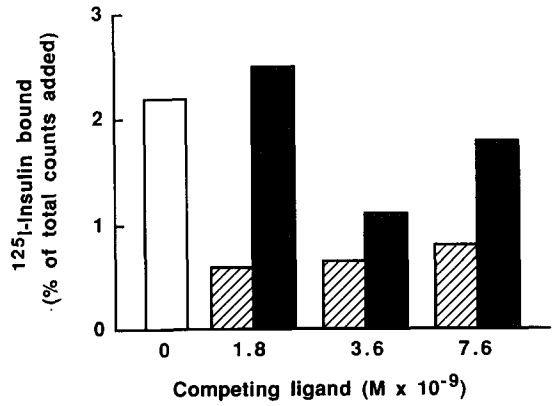

Fig. 4. Displacement of binding to influenza virus of [^{125}I]insulin by excess unlabelled insulin (▨) and cytochrome C (■). Total radioactivity of [^{125}I]insulin added was 330000 cpm to 524 μg of viral protein.

Discussion

We have demonstrated that [^{125}I]insulin can bind specifically to the surface of several purified enveloped viruses. A similar degree of specific binding was not observed with a non-enveloped enterovirus nor with a virus which acquires its envelope from the nuclear membrane. These findings suggest that enveloped viruses can acquire insulin binding sites from their host cells through budding. Efforts to carry out immunoprecipitation of the insulin receptor at the viral surface would probably prove unsuccessful, because of the low level of insulin binding activity present, nor could a Western blot be expected to work for the same reason.

An alternative approach to prove the presence of insulin receptors on viral surfaces might consist in the cross-linking of [^{125}I]insulin by means of covalent linkage to virions, followed by analysis of putative ligand–receptor complexes by polyacrylamide gel electrophoresis and radioautography (Pilch and Czech, 1979). Unfortunately, the results obtained in our laboratory with RSV and influenza virus were indeterminate. A current approach in our laboratory consists in raising monoclonal antibodies against the insulin receptor in order to determine whether they might be able to block specific insulin binding to viral surfaces.

Insulin-dependent diabetes mellitus (IDDM) has long been suspected of having an autoimmune etiology. Autoantibodies against antigens on the surface of islet cells have been detected as long as several years before the onset of clinical IDDM. Such antibodies are being used as markers for likelihood of development of severe IDDM (Lernmark et al., 1980). In addition, autoantibodies against insulin have been demonstrated in the sera of patients with newly diagnosed IDDM, and there is evidence to suggest that they too may serve as markers for this condition (Atkinson et al., 1986). It is believed that anti-insulin autoantibodies are probably produced as a result of treatment with exogenous insulin (Craighead, 1975). However, the appearance of anti-insulin autoantibodies in some individuals prior to administration of exogenous insulin remains to be definitively explained. Finally, autoantibodies against insulin receptors have been discovered in the sera of some IDDM patients (Shimoyama et al., 1986). The origin and role of these anti-receptor antibodies in the pathophysiology of IDDM has yet to be determined.

Epidemiological studies have linked each of mengovirus, measles virus (Notkins, 1984) and influenza virus (McEvoy et al., 1986) to IDDM. Encephalomyocarditis virus (Yoon et al., 1985) and coxsackie B virus (Yoon et al., 1986) have been shown to induce IDDM in animals. However, viral infection is generally not believed to be the immediate cause of IDDM (Yoon and Ray, 1985). Rather, most investigators believe that virus-induced beta-cell injury might lead to autoimmune manifestations, which, in turn could eventually result in IDDM. Evidence in support of this theory is that Reovirus infection in mice can induce autoantibodies against insulin and ultimately bring about an IDDM-like condition (Onodera et al., 1981).

Our findings may help to bridge the gap between what is known about infectious processes and autoimmune reactions in the etiology of IDDM. The data of this manuscript provide one potential explanation for the presence of anti-insulin re-

ceptor antibodies and anti-insulin antibodies in individuals never exposed to exogenous insulin. We believe that synthesis of such immunoglobulins might be attributable to viral acquisition of insulin receptors, followed by development of an anti-viral immune response. Our data also allow us to propose an explanation for the presence of anti-insulin autoantibodies in virus-infected individuals. Namely, it is possible that autoantibodies might originally be produced against insulin receptors and that anti-idiotypic antibodies, which are made in turn, could be directed against insulin itself. Finally, we should emphasize that we are not proposing that all cases of IDDM follow from either viral infections or from the possibility that insulin binding sites might be present on the surfaces of viruses which bud from infected beta cells under physiological conditions. Clearly, our data do not support such a conclusion. Moreover, it is apparent that certain types of non-enveloped viruses, such as mengovirus, which do not bud from the plasma membrane, have been linked sero-epidemiologically to IDDM. Thus, we can only speculate that insulin binding sites, present at the surfaces of some viruses, might play a role in development of some cases of IDDM and/or other diseases. Further studies on this subject are in progress in our laboratory.

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