Restricted replication of vesicular stomatitis virus in T lymphocytes is coincident with a deficiency in a cellular protein kinase required for viral transcription

David E. Sleat, Nathaniel F. Chikkala, Subhash Gautam and Amiya K. Banerjee

1 Department of Molecular Biology and 2 Section of Immunology, Department of General Medical Sciences, Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, U.S.A.

Vesicular stomatitis virus (VSV) fails to replicate in mouse T lymphocytes unless the cells have been mitogenically stimulated with concanavalin A (Con A). We have examined the possibility that the failure of VSV to replicate in unstimulated T lymphocytes can be attributed to a deficiency in a host protein kinase which activates the viral P protein by phosphorylation, thus rendering it transcriptionally competent. Soluble extracts were prepared from purified mouse T lymphocytes, with or without prior treatment with Con A. The ability of these extracts to phosphorylate bacterially synthesized P protein of two VSV serotypes was measured in vitro. Activity of the protein kinase on the P proteins of the Indiana or New Jersey serotypes of VSV increased, on average 2.4- and 2.1-fold respectively, after treatment of the cells with 3 μg/ml Con A. Higher concentrations of Con A induced proportional increases (up to 10-fold) in the activity of the host protein kinase. Activities of the kinase phosphorylating the P protein in separate populations of CD4- and CD8-containing murine T lymphocytes increased similarly on mitogenic activation. No biochemical or immunological differences were observed between the T cell protein kinase and the previously characterized protein kinase (casein kinase II) from BHK-21 cells. The activity of the kinase that phosphorylates the P protein did not vary in CV-1 cells on treatment with α- or γ-interferon, both of which inhibited VSV replication. Similarly, casein kinase II activities in Raji and SIRC cells, which do not normally support VSV growth, were the same as in BHK-21 cells. Thus restriction of VSV replication in these cells, in contrast to T lymphocytes, was not associated with a deficiency in the host casein kinase II activity.

Introduction

Vesicular stomatitis virus (VSV) infects a particularly broad range of cells in tissue culture, including nearly all mammalian cell lines examined to date. However, some cell types do not support VSV replication and thus provide unique opportunities to examine the molecular basis for restriction of viral replication. Most of such cell lines are derived from Burkitt's lymphoma, e.g. cell lines Raji, EB-3 and Jiyoye (Henle & Henle, 1965, 1966; Epstein et al., 1966; Nowakowski et al., 1973). Other mammalian cell lines that completely or partially restrict VSV replication are derived from rabbit cornea (SIRC; Thacore & Youngner, 1973) and human peripheral blood leukocytes (E.H. IV). Most of these cell lines contain Epstein–Barr virus (EBV) which may account, at least in part, for their resistance to VSV (Epstein et al., 1966). However EBV has not been detected in SIRC and Raji cells, suggesting a different mechanism of resistance in these cells.

Some primary cell cultures also fail to support VSV replication; for example, T lymphocytes support only VSV replication following mitogenic stimulation by concanavalin A (Con A) or phytohaemagglutinin (Nowakowski et al., 1973; Webb et al., 1981), which has allowed quantification of lymphoblasts by viral plaque assay (Rup & Scott, 1987). VSV replication in stimulated primary T cells has also been examined by in vivo labelling of VSV RNAs within the infected cells (Webb et al., 1981). Whereas VSV subgenomic mRNAs are transcribed normally in restrictive cell lines, the 42S genomic RNA is not synthesized. Presumably the synthesis of viral mRNAs in these cells represents primary viral transcription of the infecting virus, as observed in vitro, indicating that VSV enters these cells and uncoats normally. The restricted step in the viral life cycle is therefore likely to be at the level of the
translation, modification or stability of one or more of the viral proteins, particularly those involved in transcription and hence replication.

Three VSV proteins are directly required for transcription and replication of the viral RNA (Banerjee, 1987a, b). These are the nucleocapsid (N) protein, the large (L) protein and the phospho (P) protein (also known as the NS protein). The catalytic functions of viral transcription, i.e. transcription initiation and elongation, capping, methylation and poladenylation are probably carried out by the multifunctional L protein. The N protein is required for the cotranscriptional encapsidation of the nascent genomic and antigenomic RNAs, which are, thus, not synthesized as naked RNAs. The P protein is a viral transcription factor which is essential for the L protein to transcribe the genome RNA. The precise mechanism of action of the P protein at the molecular level is currently a focus of study for many laboratories.

Phosphorylation of the VSV P protein has long been linked with transcriptional activity in vitro and/or in vivo (Barik & Banerjee, 1991, 1992a, b; Kingsford & Emerson, 1980; Witt & Summers, 1980; Hsu et al., 1982; Sinclair & Lucas-Lenard, 1982). The P protein is phosphorylated within two separate domains by at least two distinct kinases: casein kinase II, a ubiquitous protein kinase of a variety of cells (Barik & Banerjee, 1991, 1992a, b) which phosphorylates at an N-terminal domain and a kinase associated with (and which probably is) the L protein (Sánchez et al., 1985; Hammond et al., 1992), which phosphorylates two serine residues at the C terminus of the P protein (Chattoo padhyay & Banerjee, 1987). Studies of unphosphorylated bacterial P protein have demonstrated that phosphorylation by both kinase activities is probably required for the P protein to have transcriptional activity. Moreover, phosphorylation of the P protein to a transcriptionally active state is sequential: i.e. phosphorylation by the host protein kinase is necessary before phosphorylation by the L protein-associated kinase can take place (Barik & Banerjee, 1992a).

The reliance of VSV on a host protein kinase for the phosphorylation of the P protein to be transcriptionally active provides a potential step at which viral replication might be restricted. A deficiency in this host protein kinase would not prevent primary transcription of VSV as the P protein contained within the infecting virion is already phosphorylated and therefore transcriptionally active. However, P protein newly synthesized as a consequence of primary transcription would be unphosphorylated (or underphosphorylated) and thus incapable of participating in subsequent mRNA synthesis (secondary transcription) or replication of the viral genomic RNA.

Using unphosphorylated bacterial P protein (P0) as a substrate, we have determined the relative levels of the host protein kinase(s) in a number of cell types that vary in their respective abilities to support VSV replication. In one case, resting primary T lymphocytes, restriction of viral replication correlates with decreased activity of the host protein kinase.

**Methods**

**Cells and plasmids.** Baby hamster (Syrian) kidney cells (BHK-21; ATCC CCL 10), green monkey kidney cells (CV-1; ATCC CCL 70), human Burkitt's lymphoma cells (Raji; ATCC CCL 86) and rabbit cornea cells (SIRC; ATCC CCL 60) were obtained from the American Type Culture Collection. BHK-21 cells were maintained in Dulbecco's MEM containing 7% foetal bovine serum (FBS); CV-1 and SIRC cells were grown in Eagle's MEM containing 10% FBS and Raji cells were grown in RPMI-1640 with 10% FBS. All tissue culture reagents and media were obtained from Gibco.

The plasmid pET-3a-(P(I)) and bacterially synthesized P(NJ) protein (Barik & Banerjee, 1991) were kindly provided by Dr Sailen Barik. P(I) was expressed within and purified from Escherichia coli BL21 (DE3) as described (Barik & Banerjee, 1991).

**Virus.** VSV serotypes Indiana and New Jersey were isolated from BHK-21 cells as described previously (Banerjee et al., 1974; Barik & Banerjee, 1991).

**Preparation of cell extracts.** BHK-21, CV-1 and SIRC cells were grown to confluence in 150 ml culture flasks and the monolayers were washed twice with 25 ml sterile ice-cold PBS prior to harvesting. Flasks were drained and cells were scraped into 1 ml ice-cold homogenization buffer (20 mM-HEPES, pH 7.4, 10 mM-KCl, 1.5 mM-MgCl2, 0.5 mM-DTT), and were allowed to swell on ice for 5 min before disruption with 30 strokes in a 7 ml Dounce homogenizer. Nuclei and cell debris were removed by microcentrifugation for 1 min at 4 °C. The Raji suspension culture was harvested at an approximate cell density of 1 × 10^6 cells/ml, by centrifugation at 500 g for 10 min, washed twice with 25 ml PBS by pelleting, and was finally resuspended in 1 ml homogenization buffer and disrupted as described for other cell lines.

**Preparation and isolation of mouse spleen cells.** C3H/HeN mice were used throughout these studies. Spleen cells were isolated and enriched for T lymphocytes by adherence to nylon as described previously (Julius et al., 1973). Enriched T lymphocytes were cultured in RPMI-1640 supplemented with FBS, penicillin-streptomycin and L-glutamine. Con A was dissolved in RPMI-1640 before use at a final concentration of 3 to 6 µg/ml. After washing with PBS, T cells were resuspended in the hypotonic homogenization buffer (described previously) and disrupted by freezing and thawing at −80 °C. Nuclei and cell debris were removed by centrifugation as described earlier. The soluble supernatant fraction was used as a source of cellular P protein kinase. Infection of mouse T lymphocytes with VSV was as described previously (Webb et al., 1981) and infection was confirmed by detection of plus-strand genomic RNA by ribonuclease protection (Winter et al., 1985).

**Assay of cellular protein kinase.** The protein content of each cellular extract was determined in triplicate (Bradford, 1976). Extracts were then diluted with homogenization buffer to equivalent protein concentration, and protein content was again measured. In vitro phosphorylation of bacterially synthesized P protein was essentially as described (Barik & Banerjee, 1991). Fifty µl reactions contained up to 0.5 µg cell extract, 1 µg bacterial P protein, 10 µCi [γ-32P]ATP or -GTP and 20 µM-ATP or -GDP in VSV transcription buffer (50 mM-Tris-
Reactions were incubated at 30 °C for 60 min and terminated by the addition of 2 volumes (100 μl) Laemmli's protein gel loading buffer and incubation at 100 °C for 5 min. Proteins were then analysed on 10% polyacrylamide denaturing protein gels. Phosphorylation was quantified by excising each P band and solubilizing the rehydrated gel pieces in TS-2 tissue solubilizer (Research Products International) at 65 °C for 3 to 4 h before the addition of scintillation fluid and counting.

Western blot detection of the α subunit of murine casein kinase II. Soluble cell extracts were prepared from resting and stimulated murine T lymphocytes as described earlier, concentrated to 4 μg protein/μl and fractionated by 10% SDS-PAGE. Proteins were transferred to Genescreen (NEN) by electroblotting (Towbin et al., 1979). Blots were probed with a rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues 70 to 91 of the α (catalytic) subunit of human casein kinase II (Upstate Biotechnology). This antibody cross-reacts with the equivalent subunit of the rat enzyme which is completely homologous to its human counterpart at the amino acid level (Meisner et al., 1989). A goat anti-rabbit IgG antibody-peroxidase conjugate (Boehringer-Mannheim) was used for detection of the casein kinase II–primary antibody complex.

**Results**

**Activity of P protein kinase in T lymphocytes**

Unphosphorylated, bacterially synthesized P0(I) (Fig. 1a) or P0(NJ) (Fig. 1b) proteins (P proteins of the Indiana or New Jersey serotypes of VSV, respectively) were incubated *in vitro* with extracts of resting T lymphocytes, T lymphocytes stimulated with 3 μg/ml Con A, or with extracts of a host cell line for VSV, BHK-21.

Phosphorylation of P0(I) by cellular protein kinase was observed in the presence of [γ-32p]ATP (Fig. 1a, lanes 1 to 4) or [γ-32p]GTP (Fig. 1a, lanes 5 to 8) in both resting and Con A-stimulated T lymphocytes (lanes 3, 4, 7 and 8). It can be seen that phosphorylation of P0(I) was higher in extracts from cells which had been mitogenically stimulated (compare lanes 7 and 8). The extent of phosphorylation was, on average, about 2-4-fold higher than controls. This observation represents the results of nine separate protein kinase assays, performed with four different preparations of T lymphocytes. For P0(NJ), increase in protein kinase activity in mitogenically stimulated cells was observed although this was slightly less (2-1-fold) than that observed for P(I) (Fig. 1b, lanes 3 and 4). Levels of the P protein kinase in BHK-21 cells were found to be considerably higher (sevenfold) than in resting T lymphocytes (Fig. 1a, lanes 2 and 6, b, lane 2).

Phosphorylation of casein by T cell extracts was also measured, and was also found to increase (fourfold) with mitogenic stimulation (Fig. 1c, lanes 7 and 8), although the amount of phosphorylation by T cell extracts was still less than that by BHK-21 extracts. This similarity between the P protein kinase and the casein kinase activities suggests that the viral P protein is indeed phosphorylated by casein kinase II *in vitro*, as shown recently (Barik & Banerjee, 1992a), and presumably also *in vivo*.

**Biochemical properties of the P protein kinases from BHK-21 cells and T lymphocytes**

In order to determine whether the P protein kinase activities observed in BHK-21 cells and in T lymphocytes are the same, some biochemical properties of these two activities were compared. The casein kinase II of...
proteolysis. Two μg bacterial P(I) was phosphorylated in 100 μl reaction mixtures containing extracts from either BHK-21 cells or T cells with or without mitogenic activation. After incubation for 1 h at 30 °C, reaction mixes were placed on ice. Protease digestion of phosphorylated P(I) was in 10 μl containing 5 μl P(I) (0.1 μg) from the phosphorylation reaction, 50 mM-ammonium hydrogen carbonate pH 7-8 and chymotrypsin, trypsin or protease V8 at ratios of 1:100 to 1:20 (w/w) to their substrates. Reactions were incubated at 30 °C for 30 min then terminated by the addition of protein gel loading buffer and analysed by 10% SDS-PAGE. Gels were exposed to X-ray film for 3 days. Only those ratios of protease:substrate (100:1 for trypsin and V8; 20:1 for chymotrypsin) which gave the largest spectrum of digestion products are shown here. P(I) was phosphorylated by: lanes 1, BHK-21 extract; lanes 2, resting T cell extract; lanes 3, activated T cell extract, then incubated without protease, or with chymotrypsin (Chy), trypsin (Tryp) or protease V8 (V8).

BHK-21 cells can use [γ-32P]GTP as a substrate in addition to [γ-32P]ATP (Barik & Banerjee, 1992b). The protein kinase in T lymphocytes also utilizes GTP as a substrate, possibly more efficiently than ATP (Fig. 1, lanes 5 to 8). The P kinase activity using GTP also increases when T lymphocytes are subject to mitogenic stimulation.

Phosphorylation of the viral P protein to a transcriptionally active state occurs in two sequential steps catalysed by two distinct enzymes (Barik & Banerjee, 1991). The first phosphorylation reaction is catalysed by the host cell protein kinase examined here and, in the case of BHK-21, is not sensitive to either calf intestinal alkaline phosphatase (CIAP) or bacterial alkaline phosphatase. In contrast, phosphorylation by the L protein is phosphatase-sensitive (Barik & Banerjee, 1991). In the case of the T lymphocyte protein kinase, phosphorylation is also insensitive to CIAP (data not shown). The protein kinase of T cells, like the BHK-21 kinase, is also sensitive to heparin (data not shown) providing further evidence that the respective enzymes from BHK-21 cells and T lymphocytes perform the same catalytic function.

The differences observed in the activity of the P protein kinase in resting and mitogenically activated T lymphocytes may represent either an increase in the activity of a single kinase or could be the result of the induction of a separate kinase in activated T lymphocytes. These two possibilities were examined by looking for qualitative differences between the kinase activities in resting and activated T cells by partial proteolytic digestion analyses of the phosphorylated products (Fig. 2).

The overall proteolytic digestion patterns as well as the relative levels of phosphorylation of the digestion products are the same whether the kinase originated in BHK-21 cells, or in resting or mitogenically stimulated T cells. These results strongly suggest that the substrate specificity of the protein kinase from BHK-21 cells is similar, if not identical, to that of the T cells (Fig. 2). These observations not only confirm that the protein kinase of T cells and BHK-21 cells are similar or probably identical in terms of substrate specificity, but also show that mitogenic stimulation of T cells increases the activity of an existing kinase(s), rather than inducing a novel phosphorylating enzyme.

Detection of casein kinase II in T lymphocytes

The protein kinase in BHK-21 cells that is responsible for phosphorylation and thus activation of P0 was previously identified as casein kinase II (Barik & Banerjee, 1992b). Results presented here (Fig. 1 and 2) suggest that casein kinase II is also responsible for phosphorylating P0 in T lymphocytes. To confirm the identity of the T cell protein kinase, extracts of unstimulated and mitogen-activated T cells were subjected to Western blot analysis using a rabbit polyclonal antibody to a synthetic peptide corresponding to part of the human casein kinase II α subunit, which is completely homologous to the rat enzyme (Meisner et al., 1989) and therefore probably to the murine counterpart.

As shown in Fig. 3, the α subunit of mouse casein kinase was detected by this antibody, albeit inefficiently, at a size of approximately 40K to 45K which is similar to the analogous proteins of humans and rats (Fig. 3a). Moreover, levels of the α subunit of casein kinase II increased with mitogenic stimulation to approximately the same extent (threelfold) as the increase in enzyme activity (Fig. 3b), demonstrating that the T cell protein kinase is probably casein kinase II and that its activity in these cells is directly increased after mitogenic stimulation.

Casein kinase II activities in separate populations of CD4- and CD8-containing cells

T lymphocytes prepared by non-adherence to nylon wool (Julius et al., 1973) are a mixture of CD4-containing...
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helper T cells (T\textsubscript{H} cells) and CD8-containing cytotoxic T lymphocytes (CTLs). To preclude the possibility that casein kinase II activity, and thus VSV replication, were specific to only one cell type, we further fractionated this mixture of T lymphocytes by ‘panning’ using antibodies to murine CD4 and CD8 (Becton Dickinson) and separately determining casein kinase II activities in resting and Con A-stimulated cells (Fig. 4). Protein kinase activities were present in both cell types and increased with mitogenic stimulation in both cases, although the increase was slightly greater in CTLs. This finding would suggest that VSV replication occurs in both cell types after mitogen treatment.

**Casein kinase II activities in VSV-infected T lymphocytes**

Nylon wool non-adherent lymphocytes were exposed to increasing amounts of Con A (0, 3 or 6 \(\mu\)g/ml) for 18 h and then infected with VSV(I) or VSV(NJ) for 24 h. The activity of the protein kinase on both P\(_{0}\)(I) and P\(_{0}\)(NJ) was found to be directly proportional to the Con A concentration used to stimulate the T cells (Fig. 5). At 6 \(\mu\)g/ml Con A, the kinase activities in the activated cells were five to 10-fold higher than in the resting cells. Infection of the T cells with VSV was confirmed by protection of minus-strand RNA extracted from cells from nuclease digestion by a \(^{32}\)P-labelled P transcript (data not shown). Earlier observations (Webb \textit{et al.}, 1981) have also shown that VSV replication measured by
plaque assay is also directly dependent upon the Con A concentration. VSV infection had no effect on the levels of the P protein kinase (Fig. 5).

**Phosphorylation of P(I) by extracts from cell lines non-permissive for VSV**

Finally, we studied the relative activities of the host protein kinase activity in a number of stable cell lines which differ in their respective abilities to support VSV replication.

Interferon-treated extracts from CV-1 cells and extracts from SIRC and Raji cells were prepared and phosphorylation reactions were carried out as described for BHK-21 cells and T lymphocytes. Phosphorylation of P₀(I) or casein is shown in Fig. 6(a). In addition, CV-1 cells were exposed to β- and γ-interferon, thus inducing resistance to VSV (data not shown) and the levels of P phosphorylation by cell extracts using [γ-³²P]ATP or -GTP were measured (Fig. 6b). No significant difference in the degree of phosphorylation of P₀(I) by any of these extracts was observed, indicating that resistance to VSV in these cells appears not to be related to the phosphorylation status of de novo synthesized P protein.

**Discussion**

Phosphorylation of the bacterially synthesized P₀ protein of VSV strains Indiana or New Jersey by extracts from murine T lymphocytes correlates with mitogenic activation of the T cells; moreover, the degree of phosphorylation is directly dependent on the concentration of Con A used to stimulate the cells. Recent results from this laboratory demonstrate that transcription of VSV in vitro, and presumably transcription and replication in vivo, is completely dependent upon phosphorylation of the P protein by a cellular protein kinase, casein kinase II (Barik & Banerjee, 1992b), which allows further phosphorylation of the P protein by the viral L protein (or an L protein-associated protein kinase). In this communication, we have provided evidence that the protein kinase in resting T cells is casein kinase II, the activity of which is increased during mitogenic stimulation (like many other protein kinases; Rudd et al., 1989). Given the requirement for phosphorylation of the P protein by casein kinase II (or a related cellular enzyme) for transcriptional activity (Barik & Banerjee, 1992a) it is conceivable that the decreased activity of this enzyme in resting T lymphocytes may play a part in the restriction of VSV replication in these cells.

An alternative possibility is that the failure of VSV to replicate in resting T lymphocytes is a consequence of the overall metabolic inactivity of these cells, and that the low levels of protein kinase are simply a reflection of this metabolic inactivity. Reduced activities of 'housekeeping' functions, such as the translational machinery, could thus be responsible for reducing VSV replication, rather than the low activity of the protein kinase per se. However, an earlier study provides some support for a model for VSV restriction which pertains to the phosphorylation status of the P protein. The antiviral compound tricyclo-decane-9-yi-xanthogenate (D609) does not inhibit primary VSV transcription in NIH 3T3 cells but does prevent secondary transcription and replication, despite the synthesis of all five viral proteins at about 50% of their normal levels (Müller-Decker et al., 1987). However, phosphorylation of the P protein within these cells was decreased 17-fold by the presence of D609, again correlating viral replication to the phosphorylation status of the P protein. Xanthate compounds also inhibit replication of a number of other unrelated RNA and DNA viruses, which may indicate that they have several different modes of action (Sauer et al., 1984) or that this protein kinase phosphorylates a wide spectrum of viral proteins to active forms.

Finally, it is worth noting that the increase in VSV replication upon mitogenic stimulation of T lymphocytes observed previously (three- to 7.5-fold; Webb et al., 1981) is of a similar order of magnitude to the increase in casein
kinase II activity measured in the present study (three- to fivefold).

The exact mechanism by which casein kinase II activity increases upon mitogenic stimulation is not yet known. It is possible that this enzyme is activated by mitogenic stimulation (e.g. by phosphorylation) or it could be regulated at synthesis. Results presented here indicate that casein kinase II activities in T cells are not regulated post-translationally; in this respect, casein kinase II differs from tyrosine kinase which is activated by post-translational modification (reviewed in Rudd et al., 1989). However, we have examined only the catalytic α subunit in this study. Levels of the regulatory β subunit cannot currently be determined by Western blotting, so the possibility of direct activation of casein kinase II in T cells upon mitogenic activation cannot be completely dismissed. Nucleotide sequence data on the subunits of murine casein kinase II may assist such studies.

The lymphoblastoid Raji cell line and the interferon-treated CV-1 cells do not appear to be resistant to VSV by virtue of a deficiency in casein kinase II. This might be expected given the correlation between the activity of the catalytic kinase II and cell division. VSV resistance in Raji cells may actually be due to a number of factors, including instability of primary transcripts, underprocessing of transcripts (Johnson & Herman, 1984) and defective viral assembly (Wethers et al., 1985). The mechanisms of resistance to VSV in the SIRC cell line have not yet been determined.

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