Epstein-Barr virus binding to CD21, the virus receptor, activates resting B cells via an intracellular pathway that is linked to B cell infection

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Epstein-Barr virus (EBV) initiates infection of normal B lymphocytes by binding to CD21, a complement receptor. Since EBV, unlike most viruses, preferentially infects resting (non-activated) cells, the present studies were undertaken to evaluate the hypothesis that intracellular signalling pathway(s) triggered by EBV binding to CD21 activate the expression of certain cellular genes, as well as the initially expressed viral genes, and thus enable EBV to infect resting B cells. Experiments with non-transforming EBV, recombinant virus ligand and anti-CD21 MAb show that EBV binding to CD21 on resting B cells increases CD23 mRNA levels independently of viral gene expression. A panel of five protein kinase C (PKC) and tyrosine kinase (PTK) inhibitors, all with different modes of action, exhibited a distinctive pattern of effects on the EBV induced induction of CD23 expression, ranging from nearly complete inhibition to no influence. The results suggest that distinct PKC isoforms and PTKs are involved in the signalling pathway(s) triggered by EBV binding to CD21. Significantly, the five inhibitors showed the same pattern of effects on the earliest stages of infection (EBNA-2 transcription) and B cell transformation (mitogenesis and colony formation). The identical pattern of effects of these PKC and PTK inhibitors with diverse mechanisms of action on the EBV induced increase in both CD23 and EBNA-2 mRNA levels strongly suggests that their transcription is mediated by an intracellular signalling pathway which shares, at least in part, common members.

Introduction

Infection of normal human B lymphocytes by Epstein-Barr virus (EBV), a transforming human herpesvirus with oncogenic potential, is initiated by binding of the virus to a 145 kDa type I membrane glycoprotein termed CD21, or CR2 (complement receptor type 2), which also functions as the cellular receptor for the C3dg fragment of the complement system (Fearon & Carter, 1995; Nemerow et al., 1994). The major EBV glycoprotein, gp350/220, mediates virus binding to CD21; both gp350/220 and C3dg bind to the two amino-terminal short consensus repeats (SCRs) of CD21 via similar short primary sequence epitopes (Nemerow et al., 1989). After binding, passive virus endocytosis, de-envolvement, passage of the viral nucleocapsid into the cytoplasm and vectorial movement of the nucleocapsid to the nucleus follow within minutes (Nemerow & Cooper, 1984b).

Although the physiological functions of CD21 are incompletely understood, it likely modulates antigen (Ag)-specific B cell responses, since many types of Ags, including those of viral, bacterial and protozoal origin, activate complement and become coated with activation and processing fragments of several complement components, including C3 (Cooper, 1994); Ags bearing C3 fragments could simultaneously interact with the B cell Ag receptor (BCR) and CD21. Supporting a role for CD21 in modulating intracellular signals initiated by ligand binding to the BCR are studies which demonstrate that CD21 co-caps with the BCR, and experiments which show that CD21 ligands prime B cells for subsequent activation by Ab to membrane IgM, and synergize with anti-IgM in activating B cells (Tanner et al., 1987; Fearon & Carter, 1995). Such synergy markedly increases the transcription of the c-fos nuclear proto-oncogene (Luxembourg & Cooper, 1994b).

Multiple studies indicate that CD21 also possesses signal transducing functions which are independent of BCR ligation; in most cases, such stimulation has been found to be dependent on the presence of anti-ligand IgM and the presence of the Fc region of Ig class Ab. The signal transducing functions of CD21 are also independent of BCR ligation, since CD21 is found on all B cell subpopulations, and not just on Ag-activated B cells. It is possible that CD21 is required for the expression of certain cellular genes, which are necessary for the differentiation of B cells into plasma cells. Such a role for CD21 would be consistent with the observation that CD21 co-localizes with MHC class II molecules (Scheib et al., 1992), and that CD21+ B cells are more efficient than CD21- B cells in presenting Ag to CD4+ T cells (Tye et al., 1992).
on the presence of T cell derived cytokines, or to require pre-
activation of the B cells. These include the ability of CD21
ligands which bind to SCRs 1 and 2, including transforming
and non-transforming EBV, gp350/220, C3dg and MAbs to
CD21 to activate B cells, as assessed by cytokine secretion or
proliferation (Fearon & Carter, 1995; Nemerow et al., 1994).
EBV interaction with normal B cells has also been reported to
rapidly activate phospholipase C and protein kinase C (PKC)
(Guy & Gordon, 1989; Dosch et al., 1990; Dugas et al., 1988).
Very recently, EBV and gp350/220 binding to CD21 was
found to induce IL-6 transcription and synthesis (Tanner et al.,
1996). Additional evidence includes the ability of CD21
ligands to maintain the in vitro growth of B cell lines under
suboptimal culture conditions (Hatzfeld et al., 1988) and the
finding that a modest number of cellular proteins, including
CD21 and the lck tyrosine kinase, are phosphorylated after
binding of EBV and other CD21 ligands to B cells (Fearon &
Carter, 1995; Nemerow et al., 1994; Aquino et al., 1993;
Cheung & Dosch, 1991). CD21 ligands also induce homotypic
B cell aggregation (Tanner et al., 1987; Nemerow et al., 1994).
Finally, binding of CD23, another CD21 ligand, induces IgE
production by B cells in the presence of IL-4 (Aubry et al.,
1992), and rescues germinal centre B cells from apoptosis
together with IL-1x (Liu et al., 1991).

Some of the CD21 in the B cell membrane is physically
associated with CD19, CD81 and Leu-13 (Fearon & Carter,
1995). Of these proteins, studies using different approaches
have implicated CD19 as a key molecule in signal initiation via
co-stimulation of the BCR and anti-CD19 (Fearon &
Carter, 1995). CD19 is also associated with the tyrosine kinase lyn in
the B cell membrane (Fearon & Carter, 1995; Nemerow et al.,
1994). Whether CD19 functions in intracellular signalling
pathways triggered by CD21 ligands has not been elucidated.

The above cited data provide compelling evidence that
ligand binding to CD21 triggers an intracellular signalling
pathway(s). The focus of the current studies is the relevance of
these CD21 initiated signalling events to EBV infection, an
area which has not been clearly addressed. Although blocking
of intracellular Ca2+ changes has been reported to inhibit B cell
transformation and immortalization (Dugas et al., 1988; Dosch
et al., 1990), and interference with the Ca2+/calmodulin
pathway has been found to prevent virus endocytosis and thus
infection (Nemerow & Cooper, 1984a), these events have not
been directly linked to CD21 ligation, and the mechanisms
have not been evaluated. Similar comments pertain to the
reports that blocking the expression of the tyrosine kinase lck
by antisense oligonucleotides (Cheung & Dosch, 1991) and
inhibition of protein tyrosine kinase(s) (PTK) and phospha-
tidylinositol 3-kinase impair B cell immortalization (Sinclair &
Farrell, 1995).

EBV infects resting B cells, unlike most viruses which infect
activated cells. In addition, EBV is entirely dependent on the
cellular transcriptional machinery for the expression of viral
latent genes which mediate cellular transformation. In the
present study, we evaluated the hypothesis that an intracellular
signalling pathway(s) initiated by EBV binding to CD21
activates the cellular transcriptional machinery leading to the
expression of certain cellular genes, such as CD23, as well as
the initially expressed viral genes, and enables EBV to infect
resting B cells. The data support this hypothesis.

Methods

- Reagents, antibodies, EBV. AET (2-aminoethylisothiouronium
  bromide) and PMA were obtained from Sigma. Tyrphostin A25,
  herbestimycin A, H7, calphostin C and genistein were purchased from
  Calbiochem and dissolved in DMSO at concentrations of 49.4 uM
  (10 mg/ml), 1.74 mM (1 mg/ml), 27.5 mM (10 mg/ml), 50 uM
  (49.4 mg/ml), and 74 mM (20 mg/ml), respectively. Ficoll-Paque and Percoll
  were obtained from Pharmacia. MAb OKB7, directed against CD21, was
  purchased from Ortho Diagnostic Systems; the MAb was de-aggregated
  by centrifugation in a microfuge before use. Mouse IgG2b control Ab
  was purchased from Coulter. The recombinant gp105 fragment of
  gp350/220, prepared as described (Tanner et al., 1988), was a kind gift of
  G. Nemerow. EBV was obtained from B95–8 cells stimulated for 8 to 14
days with 30 ng/ml phorbol myristate acetate. EBV was pelleted from
  the culture supernatant, resuspended in RPMI containing 20% FCS, and
  stored in liquid nitrogen until use. Where indicated, the virus stock was
  irradiated for 30 min with a ultraviolet (uv) lamp (> 36 watts output in
  the uv range, 135 cm from the source) purchased from American
  Ultraviolet Company. All EBVuv preparations lacked mitogenic activity
  for isolated B cells ([3H]thymidine incorporation at 5 days) at the
  concentrations used in the experiments.

- B cell purification. Small, dense, resting B cells were purified from
  human tonsils and characterized by flow cytofluorometry as previously
  described (Luxembourg & Cooper, 1994a). The cells employed in these
  studies were consistently 95 to 98% CD19+, 50 to 80% IgM+ and 40 to
  70% IgD+, and they contained 0.5 to 3% CD3+ cells, less than 1% CD14+ cells
  and less than 1% CD56+ cells.

- Adsorption of gp105 to plastic culture wells. Gp105
  (50 mg/ml, 1 ml per well) was adsorbed to the wells of 6-well tissue
  culture plates (Becton Dickinson) overnight. After thorough washing,
  2 ml of purified B cells (10⁶/ml), which had been pre-treated with
  herbestimycin A or comparably diluted DMSO, were added to each well.

- Studies with EBV and PTK and PKC inhibitors. Purified resting
  B cells (10⁶/ml) were incubated with herbestimycin A (17.4 µM (10 µg/ml)
or 8.7 µM (5 µg/ml), Tyrphostin A25 (494 µM (10 µg/ml), genistein
  (185 µM (50 µg/ml) or H7 (27.5 µM (10 µg/ml), for 2 h at 37 °C; cells
  incubated with calphostin C (50 nM (40 ng/ml) were left for 2 h in the
  presence of light to activate the drug (Gopalakrishna et al., 1992). These
  concentrations were obtained by a large dilution of stock solutions of the
  inhibitors. The final concentrations of the inhibitors were chosen on the
  basis of the 50% inhibitory doses for enzyme inhibition, and doses
  conventionally used in the literature (Iwasaki et al., 1992; Fukazawa et
  al., 1991; Gazit et al., 1989; Akiyama et al., 1987; Takahashi et
  al., 1990; Gopalakrishna et al., 1992; Kobayashi et al., 1989; Luxembourg &
  Cooper, 1994 a, b). Genistein and H7 were both used at concentrations
  higher than their IC₅₀ values. In addition, after 24 h in culture, inhibitor
  treated and untreated cells exhibited comparable cell viability, as assessed
  by trypan blue exclusion. EBV was then added, at levels which had
  previously been shown to be optimally mitogenic for B cells, and
permitted to adsorb to the cells for 1 h on ice. After washing in PBS, the cells were resuspended in RPMI containing 10% FCS and placed in culture at a concentration of 10^5/ml. Controls in all experiments included samples containing virus and B cells, but not kinase inhibitor (positive control), and samples containing B cells, but neither virus nor kinase inhibitor (negative control). These contained appropriate amounts of DMSO.

- **B cell mitogenesis and colony formation assays.** Purified resting B cells were incubated with inhibitors, or a comparable DMSO dilution, followed by EBV, and then washed and resuspended, as described above. The cells were cultured (2 x 10^6 per well) in 96-well plates, in triplicate, in RPMI containing 10% FCS for either 5 or 14 days in a CO_2 incubator, after which they were pulsed for 4 h with 2 μCi [3H]thymidine (ICN); incorporation of [3H]thymidine was then assessed by liquid scintillation counting. In order to assess colony formation, purified resting B cells were incubated with inhibitors, or diluted DMSO, followed by EBV, and then washed and resuspended, as described above. Subsequent steps were as published (Nemerow & Cooper, 1981). Colony formation was assessed after 20 days of incubation.

- **Ribonuclease (RPA) assays.** Purified resting B cells were incubated with inhibitors, or diluted DMSO, followed by either EBV or EBVuv, and then washed, and resuspended, as described above. After 24 h in culture, the cells were separated on Ficoll-Paque, and dead cells discarded. For the studies with OKB7, the de-aggregated MAb was added (4 μg per 10^6 cells) 2 h prior to EBV. For the studies with gp105, resting B cells were incubated for 2 h either with DMSO or herbimycin A, washed and placed in culture for 24 h in the presence of gp105, previously adsorbed to the plate (Sinclair et al., 1994). RNA was extracted from equal numbers of live cells for each sample (generally 5 x 10^6 to 10^7), using the one-step method of Chomczynski & Sacchi (1987). RPA assays were performed essentially as previously described (Rochford et al., 1993). Bands were visualized by autoradiography and the levels of expression quantified on a phosphorimager (Bio-Rad), and expressed in pixel density units (PDU). The PDU values of the experimental samples were adjusted to variations in expression of the rpl32 housekeeping gene.

### Results

**EBV binding to CD21 on purified resting B cells increases CD23 mRNA levels independently of viral gene expression**

CD23, a B cell activation marker, is closely associated with EBV infection. Initially identified as a cell surface molecule induced early during EBV infection (Thorley-Lawson et al., 1985), CD23 expression was subsequently shown to be required for EBV induced immortalization (Hurley & Thorley-Lawson, 1988; Thorley-Lawson & Mann, 1985). CD23 has been demonstrated to be specifically upregulated by the two EBV latent gene products, EBNA-2 and LMP1 (Wang et al., 1990, 1991). In the present studies we found that increased CD23 mRNA levels, as assessed by quantitative RPA assays, were evident 24 h after incubation of EBV with highly purified resting human B cells (Fig. 1). Quantitative analyses of four such RPA studies, each in triplicate, revealed that EBV increased CD23 mRNA levels 2.0 to 4.5-fold over rpl32 levels. This induction was independent of viral gene expression, since EBVuv, which is not mitogenic, and does not induce viral gene expression (Gordon et al., 1980), comparably increased CD23 mRNA levels in this and other identical experiments (Fig. 1). EBVuv was unable to induce EBNA-2 expression, a finding in accord with the inability of EBVuv to transform B cells.

Two approaches were used to determine whether the EBV induced increase in CD23 mRNA levels was initiated by EBV binding to CD21 on the cell surface. First, the effect of the OKB7 anti-CD21 MAb, in de-aggregated form, on EBV induced CD23 expression was evaluated; OKB7 binds to the two amino-terminal SCRs of CD21 and inhibits EBV binding to, and infection of, B cells (Nemerow et al., 1985). As is apparent in Fig. 2(a), the EBV induced increase in CD23 mRNA levels was markedly reduced by pre-incubation of the purified B cells with the blocking OKB7 anti-CD21 MAb, but not with an isotype matched IgG2b control antibody. De-aggregated OKB7 alone did not increase CD23 mRNA levels (data not shown). These data indicate that the increase in CD23 mRNA levels is initiated by EBV binding to CD21, and not by an impurity in the virus preparation. As also shown in Fig. 2(a), EBNA-2 induction by EBV was also blocked by pre-incubation with OKB7, consistent with numerous earlier studies documenting the essential role of EBV binding to CD21 as the first step in EBV infection of B cells. In the second approach, CD23 mRNA levels were quantified in resting B cells incubated for 24 h with gp105, a recombinant truncated form of the major EBV glycoprotein, gp350/220, which, like the parent molecule, binds to CD21 (Tanner et al., 1988). As is apparent from Fig. 2(b), CD23 mRNA levels were increased in resting B cells incubated with gp105. Although rpl32 mRNA levels were also slightly increased by gp105, quantitative analyses indicate that gp105 increased CD23 mRNA levels 3-fold over rpl32 mRNA levels, a value which is entirely consistent with the 2 to 4.5-fold increase observed with infectious EBV (see above). These two lines of evidence show that CD21 ligation is necessary and sufficient to increase CD23 mRNA levels. The ability of gp105 to increase CD23 levels contrasts with the inability of OKB7 to do so. This difference undoubtedly stems from the fact that gp105 was presented to the B cells in aggregated form adsorbed to plastic, whereas OKB7 was added in soluble monomeric form; B cell stimulation through CD21 is known to be dependent on the aggregation state of the ligand (Bohnsack & Cooper, 1988; Melchers et al., 1985).

**The EBV induced increase in CD23 mRNA levels is mediated by an intracellular signalling pathway involving PKC and PTKs**

Intracellular signalling pathways are driven by sequential, highly regulated enzyme-substrate and protein-protein interactions typically involving de novo activation of intracellular protein tyrosine and serine-threonine kinases, and the formation of specific complexes mediated by protein-protein binding.
motifs. In order to determine whether the EBV induced increase in CD23 transcription in resting B cells was dependent on such intracellular enzyme-substrate interactions, we evaluated the effects of a panel of PKC and PTK inhibitors with different mechanisms of action on the EBV induced increase in CD23 mRNA levels. The inhibitors were selected on the basis of our previous studies which showed that c-fos activation induced by cross-linking CD21 with the BCR on resting B cells was PKC and PTK dependent (Luxembourg & Cooper, 1994b). With regard to PKC inhibitors, we found that EBV failed to induce an increase in CD23 mRNA levels in resting B cells which had been pre-incubated with 50 nm-calphostin C, while pre-incubating the cells with 17.4 μM-H7 was without effect, as shown in Fig. 3(a). In identical experiments, we evaluated the effects of three PTK inhibitors with different modes of action on the EBV induced increase in CD23 mRNA levels. As shown in Fig. 3(b), EBV failed to increase CD23 mRNA levels in resting B cells which had been pre-incubated for 2 h with 17.4 μM-herbimycin A, and comparable inhibition was also obtained with 8.7 μM-herbimycin A (data not shown). Of interest, 8.7 μM-herbimycin A also markedly inhibited CD23 mRNA induction in resting B cells incubated with EBVuv or gp105 (data not shown). It also completely inhibited the appearance of tyrosine phosphorylated proteins 5 min after

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**Fig. 1**

EBV and EBVuv mediated induction of CD23 (and EBNA-2) transcription. Transcription of CD23, EBNA-2 and rpL32 was assessed in a multiprobe RPA assay 24 h after addition of EBV or EBVuv to purified resting human B cells.

**Fig. 2**

(a) Inhibition of EBV induced CD23 and EBNA-2 transcription by OKB7. Transcription of CD23, EBNA-2 and rpL32 was assessed in a multiprobe RPA assay 24 h after addition of EBV to purified resting B cells pre-incubated with media, OKB7 or an IgG2b isotype matched control MAb. (b) Gp105 mediated induction of CD23. Transcription of CD23 and rpL32 was assessed in a multiprobe RPA assay 24 h after incubation with gp105.
EBV addition to resting B cells, as assessed by Western blotting studies (data not shown). Pre-incubation of the cells with 49·4 μM-tyrphostin A25 moderately inhibited (27%) the EBV induced increase in CD23 mRNA levels, as seen in Fig. 3(b), while, in identical studies, 185 μM-genistein showed no inhibition of the EBV induced increase in CD23 mRNA levels (data not shown). These results are representative of three similar studies. Thus, of the two PKC inhibitors tested, calphostin C markedly inhibited, but H7 had no effect on the EBV induced increase in CD23 levels, while among the PTK inhibitors, herbimycin A markedly inhibited, tyrphostin A25 moderately inhibited, and genistein had no influence on the EBV triggered induction of CD23 transcription. The concentrations of the PKC and PTK inhibitors used in these studies were not toxic, since resting B cells treated with the inhibitors for 24 h retained the ability to exclude trypan blue (not shown). Although the turnover rates of CD23 and rpL32, the housekeeping gene, are not known, the lack of significant effects of the PTK and PKC inhibitors on rpL32 mRNA levels suggests that the observed effects of the inhibitors on CD23 expression did not result from general alterations in RNA synthesis, reduced mRNA stability or toxic effects. These data with a panel of inhibitors with different mechanisms of action indicate that EBV binding to CD21 on the cell surface triggers an intracellular signal transduction pathway(s) involving PKC and PTK(s) which leads to increased CD23 transcription.

Effects of PTK and PKC inhibitors on EBV infection

In order to assess whether the signalling pathway described above was also involved in EBV infection, we evaluated the effects of the PKC and PTK inhibitors on EBNA-2 mRNA levels. The effects of the panel of PKC and PTK inhibitors on the initial stages of EBV infection, as assessed by EBNA-2 mRNA levels, were evaluated in quantitative RPA assays; EBNA-2 expression is required for B cell transformation.
As is apparent from the quantitative analysis of an RPA assay depicted in Fig. 4 (a), B cells exposed to calphostin C (50 nM) prior to virus addition exhibited a marked reduction (55%) in EBNA-2 expression, while H7 pre-treatment (27.4 μM) did not reduce EBNA-2 mRNA levels. With regard to PTK inhibitors, pre-incubation of B cells with herbimycin A (17.4 μM), before EBV addition, completely blocked EBNA-2 transcription as shown in the RPA assay depicted in Fig. 3 (b), and in the quantitative analysis of EBNA-2 mRNA levels in the same gel adjusted for changes in rpL32 expression in the presence of the inhibitors, shown in Fig. 4 (b). Pre-incubation with tyrphostin A25 (49.4 μM) reduced EBNA-2 levels by 48%, while pre-treatment with genistein (185 μM) had no effect (data not shown). These data are representative of three similar studies. The inhibitors exhibited comparable effects on EBV transformation, whether assessed by [³H]thymidine incorporation 5 or 14 days after infection, or by the formation of transformed colonies in semi-soft agarose 20 days after infection (data not shown). Thus the five PKC and PTK inhibitors showed the same pattern of effects on EBV infection as observed for CD23 transcription.

Discussion

Studies from several laboratories using different approaches have unequivocally shown that infection of normal human B cells is initiated by the specific binding of EBV, via the gp350/220 envelope glycoprotein, to CD21 on the cell surface, as reviewed earlier (Introduction). Other approaches indicate that CD21 is a component of an incompletely characterized B cell activation pathway which modulates intracellular signalling, particularly when co-ligated with the BCR or CD19 (Introduction). However, the role of the CD21 signalling pathway in EBV induced B cell activation leading to EBV infection, which represents the focus of the present studies, has not been evaluated.

The data presented here indicate that EBV binding to CD21 on purified resting B cells increases CD23 mRNA levels independently of viral gene expression. EBV induced CD23 expression is also independent of virus endocytosis, which is clearly required for infection with virus. Therefore, the inhibitory effects of certain PKC and PTK inhibitors on the EBV induced increase in CD23 mRNA levels implicates intracellular protein kinases in the events leading to increased CD23 transcription. The concentrations of inhibitors used in these studies were not overtly toxic to the resting B cells as they retained the ability to transcribe the cellular housekeeping gene rpL32, and they retained the ability to exclude trypan blue.

Significantly, the PTK and PKC inhibitors exhibited an unusual spectrum of activities ranging from nearly complete inhibition to no influence on EBV induction of CD23 expression. Thus, the two PKC inhibitors tested exhibited opposite effects, since calphostin C markedly inhibited, while H7 had no effect on EBV induced CD23 transcription. Among the PTK inhibitors, herbimycin A markedly inhibited, tyrphostin A25 slightly to moderately inhibited, and genistein was without effect on the EBV induced increase in CD23 mRNA levels. Of considerable interest, the five PKC and PTK inhibitors exhibited exactly the same pattern of effects on the earliest stages of EBV infection, as assessed by EBNA-2 transcription, which is, unlike CD23 expression, dependent on virus
endocytosis. Thus, EBNA-2 mRNA levels 24 h after infection were markedly inhibited (calphostin C), or unaltered (H7), by pre-incubation with the two PKC inhibitors, and markedly impaired (herbimycin A), slightly to moderately inhibited ( tyrphostin A25) or unaffected (genistein) by pre-incubation with the three PTK inhibitors; a comparable lack of effect of genistein on EBNA-2 transcription, was reported recently (Sinclair & Farrell, 1995). The identical pattern of effects of these PTK and PKC inhibitors with diverse mechanisms of action on the EBV induced increase in both CD23 and EBNA-2 mRNA levels strongly suggests that increased CD23 and EBNA-2 transcription is mediated by an intracellular signalling pathway which shares, at least in part, common constituents. The data also indicate that triggering of this signalling pathway is essential for transformation, since herbimycin A, tyrphostin A25, calphostin C and H7 exhibited the same pattern of effects on EBV induced [3H]thymidine incorporation, and on the formation of EBV transformed colonies in soft agarose.

In addition, the distinctive pattern of inhibition, or lack thereof, with five inhibitors which function via different mechanisms, suggests that distinct PKC isoforms and PTKs are participants in the intracellular signalling pathway(s) triggered by EBV binding to CD21. Because of recent advances in the understanding of the properties and activation mechanisms of PKC isoforms, the current data permit some insight into the isoforms involved. In the present studies, H7 had no effect on CD23 and EBNA-2 induction. H7, which is a competitive inhibitor of the catalytic domain of PKC enzymes, is more active against the Ca\(^{2+}\) dependent \(\alpha, \beta, \gamma\), and \(\delta\) PKC isoforms than the Ca\(^{2+}\) independent \(\eta, \theta, \xi, \xi \alpha, \xi \beta, \xi \gamma\) PKC isoforms (50% inhibitory concentrations of approximately 20 \(\mu\)M and 60 \(\mu\)M, respectively) (Simpson et al., 1993; Oudinet et al., 1992). B cells are reported to contain the \(\alpha, \beta, \delta, \eta, \xi\) PKC isoforms (Misichak et al., 1991). In addition to PKC isoforms, H7 also inhibits cyclic nucleotide dependent kinases (Gordge & Ryves, 1994). Calphostin C, which markedly inhibits CD23 and EBNA-2 induction, is a highly specific and powerful irreversible inhibitor of all of the PKC isoforms (Gopalakrishna et al., 1992) which are activated by diacylglycerol (DAG) (IC\(_{50}\) = 50 nM), since it binds to the DAG binding site of the enzyme (Gordge & Ryves, 1994) thus it inhibits all of the PKC isoforms except \(\xi\) (Nakanishi & Exton, 1992). Calphostin C has no activity against cyclic nucleotide dependent kinases at the 50 nM concentration used in the present studies (50% inhibitory concentration > 50 \(\mu\)M) (Kobayashi et al., 1989). These data strongly suggest that a Ca\(^{2+}\) independent form(s) of PKC, likely PKC \(\delta\) or \(\eta\), is required for EBV infection of B cells. The present studies, which suggest the involvement of a Ca\(^{2+}\) resistant form of PKC in EBV infection, are in accord with earlier evidence showing that transforming, as well as non-transforming EBV induces translocation of PKC to the membrane (Dugas et al., 1988), a measure of PKC activation, and with suggestive evidence of activation of a Ca\(^{2+}\) independent PKC by EBV (Guy & Gordon, 1989).

Only general statements can be made about the PTK(s) involved in EBV induction of CD23 and EBNA-2 since the inhibition characteristics are not well defined. Herbimycin A, which inhibited CD23 and EBNA-2 induction, binds to sulphhydryl groups on the catalytic domain of src family cytoplasmic PTKs and irreversibly blocks their activity (Fukazawa et al., 1991); it has no activity on PKC or on cAMP dependent protein kinase (Fukazawa et al., 1991), and it is particularly active on non-receptor PTKs (Okabe & Uehara, 1993). The tyrphostins, including A25, which moderately inhibited EBV induced transcription of CD23 and EBNA-2, are low molecular mass synthetic compounds which act as substrate homologues of the kinase domain of PTKs and, thereby, competitively inhibit the activity of PTKs (Gazit et al., 1989). Genistein, which had no influence on CD23 and EBNA-2 mRNA levels, binds to, and competitively inhibits, the ATP binding site of PTKs; it has no activity against serine and threonine kinases and PKC (Akiyama et al., 1987). Although published data document the preferential activity of genistein for receptor PTKs (Okabe & Uehara, 1993), it also inhibits non-receptor src family PTKs such as lyn and lck (Uckun et al., 1995). The differential effects of the three PTK inhibitors will prove to be most useful in ongoing studies to identify the PTK(s) required for infection.

The effects of H7 and genistein on the signalling pathway triggered by EBV binding to CD21 described here differ from their actions on intracellular signalling initiated by ligation of the BCR, CD19, or co-ligation of the BCR and CD21. Thus H7, which had no significant effects on CD23 or EBNA-2 induction in the present studies, markedly inhibits B cell activation induced by ligation of the BCR, and by co-ligation of the BCR and CD21; this was observed in studies from this laboratory using the same experimental conditions (Luxembourg & Cooper, 1994a, b). Similarly, other studies have shown that BCR cross-linking leads to activation and membrane translocation of a Ca\(^{2+}\) dependent and phospholipid dependent PKC isoform(s) (Gold & DeFranco, 1994): this signalling pathway is required for B cell proliferation in response to such cross-linking (Gold & DeFranco, 1994; Luxembourg & Cooper, 1994a). In addition, genistein, which was without effect in the current experiments, blocks signalling initiated by co-ligation of the BCR and CD21, as well as CD19 signalling in similar concentrations to those used here (Uckun et al., 1993; Carter et al., 1991). The current results thus suggest that the EBV initiated pathway involves activation of a Ca\(^{2+}\) insensitive PKC isoform and a genistein resistant PTK(s), whereas the BCR, CD19 and BCR plus CD21 initiated pathways involve activation of a Ca\(^{2+}\) dependent PKC isoform and a genistein sensitive PTK(s). The simplest interpretation is that divergent signalling pathways mediate intracellular events when CD21 alone is engaged, compared to co-ligation of CD21 and the BCR, when the BCR intracellular pathway appears to be dominant. Current studies are experimentally addressing this hypothesis by focusing on the identification and charac-
terization of the PTK(s), and the PKC isoform involved in the EBV triggered CD21 intracellular signalling pathway.

The CD21 dependent signalling pathway engaged by EBV clearly activates the transcriptional machinery of the resting B cell, as shown by the augmented transcription of CD23, and as very recently shown, IL-6 (Tanner et al., 1996). Whether or not these cellular gene products play critical roles in the early stages of infection, or alternatively, and more likely, are regulated by transcription factors activated by the CD21 signalling pathway, and thus are indirect markers of engagement of the pathway, remains to be determined. Regardless, by showing that EBV binding to CD21 induces the transcription of certain genes, the data provide clues to the mechanisms which enable EBV, in contrast to most viruses, to infect non-activated and non-cycling cells.

These studies were supported by USPHS grants CA52241 and AI33244, USPHS Training Grant T32 HL07195–19 and General Clinical Research Center Grant 2M01 RR00833. We thank Guillermo Verduzco for excellent technical assistance, Dr Glen Nemerow for providing recombinant gp105, and Catalina Hope and Joan Gausepohl for assistance with the manuscript. We also thank the Department of Pathology and the operating room staff of San Diego Children's Hospital for cooperation in obtaining tonsil surgical specimens used in these studies. Some tonsils were also obtained from the Cooperative Human Tissue Network.

References


Misichak, H., Kolch, W., Goodnight, J., Davidson, W. F., Rapp, U., Rose-


Received 25 March 1996; Accepted 6 August 1996