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Bovine leukemia virus gp30 transmembrane (TM) protein is not tyrosine phosphorylated: examining potential interactions with host tyrosine-mediated signaling

Valerie T. Hamilton *, Diana M. Stone, Suzanne M. Pritchard, Glenn H. Cantor [1]

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA

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Abstract

Bovine leukemia virus (BLV) causes persistent lymphocytosis, a preneoplastic, polyclonal expansion of B lymphocytes. The expansion increases viral transmission to new hosts, but the mechanisms of this expansion have not been determined. We hypothesized that BLV infection contributes to B-cell expansion by signaling initiated via viral transmembrane protein motifs undergoing tyrosine phosphorylation. Viral mimicry of host cell proteins is a well-demonstrated mechanism by which viruses may increase propagation or decrease recognition by the host immune system. The cytoplasmic tail of BLV transmembrane protein gp30 (TM) has multiple areas of homology to motifs of host cell signaling proteins, including two immunoreceptor tyrosine-based activation motifs (ITAMs) and two immunoreceptor tyrosine-based inhibition motifs (ITIMs), which are homologous to B-cell receptor and inhibitory co-receptor motifs. Signaling by these motifs in B cells typically relies on tyrosine phosphorylation, followed by interactions with Src-homology-2 (SH2) domains of nonreceptor protein tyrosine kinases or phosphatases. Phosphorylation of tyrosine residues in the cytoplasmic tail of TM was tested in four systems including ex vivo cultured peripheral blood mononuclear cells from BLV infected cows, BLV-expressing fetal lamb kidney cell and bat lung cell lines, and DT40 B cells transfected with a fusion of mouse extracellular CD8α and cytoplasmic TM. No phosphorylation of TM was detected in our experiments in any of the cell types utilized, or with various stimulation methods. Detection was attempted by immunoblotting for phosphotyrosines, or by metabolic labeling of cells. Thus BLV TM is not likely to modify host signal pathways through interactions between phosphorylated tyrosines of the ITAM or ITIM motifs and host-cell tyrosine kinases or phosphatases.

Keywords: Bovine leukemia virus; B lymphocyte; BCR; Tyrosine kinase; Phosphorylation; Signaling; ITAM; ITIM

Bovine leukemia virus (BLV) is an oncogenic retrovirus of the family Retroviridae and genus *Deltaretrovirus*, closely related to the human T-cell leukemia virus types 1 and 2 (HTLV-1 and -2)

* Corresponding author. Tel.: +1-509-335-6055; fax: +1-509-335-8529

[1] Present address: Pharmacia Corp., 7228-300-226.1, 301 Henrietta Street, Kalamazoo, MI 49007, USA.

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(Kettmann et al., 1994). Approximately 30% of infected cattle develop persistent lymphocytosis, a polyclonal, preneoplastic expansion primarily of CD5+ B lymphocytes (Letesson et al., 1991). The expansion significantly increases the probability of viral transmission from infected to naive animals (Ferrer et al., 1975); however, the mechanisms of this expansion have not been determined. Two viral proteins hypothesized to be involved with BLV-induced leukemogenesis include Tax and G4. HTLV and BLV Tax activate the viral LTR promotor, as well as cellular genes involved in activation and apoptosis (Jin et al., 1999; Neuveut and Jeang, 2002; Tajima and Aida, 2002), and BLV Tax has been shown to have in vitro transforming properties (Willems et al., 1990, 1998). Less is known about the role of the G4 protein, though recent studies show it can also cause transformation of cells in vitro (Kerkhofs et al., 1998).

The transmembrane protein (TM) of BLV has also been implicated in the preneoplastic B-cell expansion of BLV. Our laboratory has proposed a role for TM in the B lymphocyte expansion of persistent lymphocytosis via alteration or upregulation of B-cell signaling. Retroviral envelope proteins, including those of BLV, are involved in binding and fusion to the host cell surface and initial infectivity (Kettmann et al., 1994). The surface unit (SU) of BLV envelope, gp51, has been shown to associate with the transmembrane unit of BLV envelope, gp30, by disulfide bonding (Johnston and Radke, 2000) and molecular modeling suggests that the extracellular Env complex has a trimeric organization (Callebaut et al., 1994). Like many retroviruses, BLV TM has a relatively long cytoplasmic tail with several defined and postulated functions. BLV TM has a key role in host cell-to-cell fusion (Voneche et al., 1992), viral entry, and incorporation of envelope protein into virions (Inabe et al., 1999). A YXXϕ motif (where Y = tyrosine, X = any amino acid and ϕ = hydrophobic amino acids) in the cytoplasmic tail of BLV TM, also present in other retroviral transmembrane proteins, including those of human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and Rous sarcoma virus, (Berlioz-Torrent et al., 1999; Egan et al., 1996; Ochsenbauer et al., 2000; Sauter et al., 1996) can initiate rapid endocytosis of viral envelope from the cell surface, which would reduce the chance of detection by the immune system (Grange et al., 2000; Lodge et al., 1997). Our overall hypothesis is that in addition to its role in viral entry and infectivity, BLV TM also has a role in dysregulating B-cell proliferation and/or apoptosis that significantly contributes to the preneoplastic B-cell expansion observed in infected cattle.

Evidence for the involvement of BLV TM in dysregulation of B cell signaling includes sequences in TM that are homologous to B-cell signaling motifs (Reth, 1989; Songyang et al., 1994), the ability of chimeric TM proteins to alter signaling in B-cell lines (Alber et al., 1993; Beaufils et al., 1993), and co-immunoprecipitation of TM with SH2-containing protein tyrosine phosphatase-1 (SHP-1), an important B-cell signaling protein (Cantor et al., 1999). There are multiple motifs homologous to B-cell signaling motifs within the cytoplasmic tail of BLV TM. These include two overlapping immunoreceptor tyrosine-based activation motifs (ITAMs) with the consensus D/E-X_2-YX_2L/I-$X_{6-8}YX_2$L/I (Reth, 1989), two immunoreceptor tyrosine-based inhibition motif (ITIMs) with the sequences S-X-YX_2L (Sinclair, 2000) or IYSXL (Vivier and Daeron, 1997), and a proline-rich region upstream of the ITAMs and ITIMs, which has several specific motifs including $PX_2PX_{4-5}P$, PX_4PX_2P and RX_2PX_2P (Cantor, 1996; Alexandropoulos et al., 1995; Macias et al., 2002; Ren et al., 1993) (see Fig. 1). The ITAMs and ITIMs are completely conserved in geographically diverse isolates of BLV, as are forms of the proline-rich motifs (Mamoun et al., 1990). One of the ITAM YXXL sequences in BLV TM has the consensus sequence of the binding site for Syk, a significant kinase in early activation of antigen-mediated stimulation through the B cell receptor (BCR) (Songyang et al., 1994). B-cell activation is regulated by multiple molecular interactions initiated at the cell membrane by antigen and co-receptor binding, and transmitted by the addition or subtraction of phosphates onto signaling proteins within the cytosol (Reth and Wienands, 1997). In the mature B cell, the BCR and its main associated inhibitory

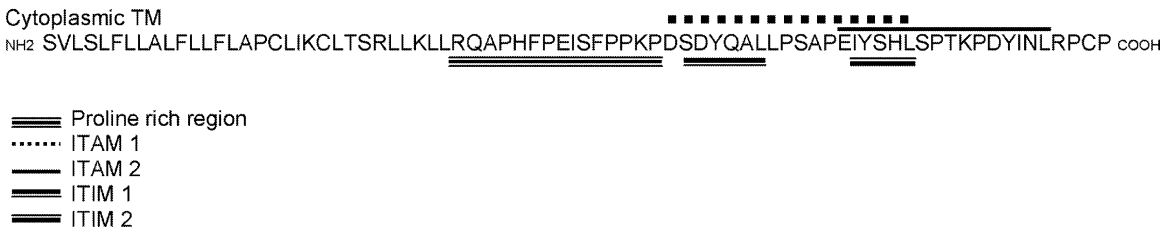

Fig. 1. The amino acid sequence of the cytoplasmic tail of TM. Two overlapping ITAM sequences are found, along with two ITIMs and a proline-rich region upstream of the ITAMs and ITIMs.

molecules contain ITAMs and ITIMs, respectively (Amigorena et al., 1992; Burkhardt et al., 1994; Doody et al., 1995; Sanchez et al., 1993). BCRs cluster upon antigen binding, and there is movement of the BCR into membrane domains enriched in Src-family kinases and Syk kinase (Cheng et al., 2001; Pierce, 2002). Igα and Igβ (CD79a and CD79b, respectively) ITAMs are the main signal transducing components of the BCR and are rapidly phosphorylated on their tyrosine residues, creating binding sites for Src-homology 2 (SH2) domains of non-receptor tyrosine kinase proteins (Gold et al., 1991; Lin and Justement, 1992). The interaction of phosphorylated BCR ITAMs with kinases is responsible for amplifying and propagating the surface signal through the cytoplasm to the nucleus (Hsueh and Scheuermann, 2000). Shortly after ITAM phosphorylation, ITIMs in inhibitory receptors become phosphorylated. Phosphorylated ITIMs bind preferentially to SH2 domains located in phosphatases (Binstadt et al., 1996; Fong et al., 1996). Thus ITIMs function as counterparts to control the activation induced by ITAMs in B cells (Ravetch and Lanier, 2000) and the SH2 domains are the regulatory motifs which coordinate these interactions (Pawson et al., 2001).

ITAMs and ITIMs are found in numerous signaling proteins and play a significant role in coordinating signaling through their interactions with nonreceptor tyrosine kinase proteins and phosphatase proteins (Billadeau and Leibson, 2002; Gergely et al., 1999). In addition to the importance of these motifs as regulators of signaling in immune cells, there are many examples of viral ITAMs that modify host cell responses through interactions with nonreceptor tyrosine kinase proteins (Collette and Olive, 1997; Messerschmitt et al., 1997). These include Epstein–Barr virus latent protein (LMP2A) (Dykstra et al., 2001; Fruehling et al., 1998; Fruehling and Longecker, 1997), HIV and SIV Nef (Collette et al., 1997; Greenway et al., 1999), and Kaposi's sarcoma-associated herpesvirus K1 protein (Lee et al., 1998). These viral–host cell interactions typically rely on phosphorylation of viral ITAM tyrosines (Biesinger et al., 1995; Burkhardt et al., 1992; Du et al., 1995; Dunant et al., 1997; Lagunoff et al., 1999).

Two studies demonstrated the ability of the cytoplasmic tail of BLV TM to activate cells. Chimeric proteins constructed from mouse extracellular and transmembrane CD8α and cytoplasmic BLV TM (CD8/TM) were expressed in B- and T-cell lines. When CD8/TM was crosslinked with antibody against the extracellular portion of CD8α, there was increased tyrosine phosphorylation of cellular proteins, increased intracellular-free Ca^{++}, and in some cell lines, increased interleukin 2 (IL-2) production (Alber et al., 1993; Beaufils et al., 1993). A similar effect was seen with a protein chimera of extracellular CD8α and the cytoplasmic tail of Epstein–Barr LMP2A. Another study showed that two of the BLV TM tyrosine residues were important for viral infectivity in vivo in sheep. Mutation of the two ITAM tyrosine residues closest to the NH_2 terminal end of cytoplasmic TM either reduced viral propagation in vivo or completely abrogated in vivo infectivity (Willems et al., 1995). TM has also been shown to co-immunoprecipitate with the tyrosine phosphatase, SHP-1, and this interaction is dependent on phosphorylation (Cantor et al., 1999). SHP-1 is important in negative regulatory

pathways of the BCR after antigen-mediated stimulation (Adachi et al., 2001; Doody et al., 1995). Recruitment of SHP-1 to the BCR occurs via SH2 binding to tyrosine phosphorylated ITIMs of CD22 (Otipoby et al., 2001) and helps determine the threshold for antigen stimulation through the BCR (Cyster and Goodnow, 1995). Although there is no detectable chronic viremia in BLV infection, several BLV proteins are expressed in vivo, indicating that virus proteins are present and may affect circulating cells. In vivo expression of viral envelope proteins in particular is evident by persistent and high anti-SU (gp51) antibody titers in infected animals (Levy et al., 1980).

Based on (1) the well-conserved sequence of tyrosine-containing motifs in the cytoplasmic tail of BLV TM of different BLV isolates, (2) TM motif mimicry of B-cell signal motifs, (3) the expression of BLV Env protein in infected animals, and (4) the evidence that BLV TM can increase cell activation and associate with important B-cell signal molecules, we hypothesize here that upon activation of the infected host B cell, BLV TM is inductively phosphorylated on ITAM or ITIM tyrosines. This phosphorylation results in modification of normal signaling pathways, altering the normal B-cell response to antigen and resulting in either decreased apoptosis or increased proliferation. To test this hypothesis, we evaluated TM ITAM and ITIM tyrosine phosphorylation by both immunoblotting and by metabolic labeling with ^{32}P in ex vivo lymphocytes from BLV-infected, persistently lymphocytotic cattle, two different cell lines persistently infected with BLV, and a B-cell line expressing the cytoplasmic tail of TM as a dimerized chimeric protein.

Initial experiments were performed on ex vivo bovine lymphocytes and detection of tyrosine phosphorylation was by immunoblotting (Fig. 2A and B). Whole blood was collected from naturally infected cattle exhibiting persistent lymphocytosis, and peripheral blood mononuclear cells (PBMC) were isolated as previously described (Cantor et al., 1999). Since viral proteins are undetectable in uncultured ex vivo PBMCs, but virus, including cell-surface expressed Env protein, is readily demonstrated after short-term culture (Driscoll and Olson, 1977), the cells were cultured in RPMI plus 10% fetal bovine serum (FBS), 100 μM streptomycin and 100 μU penicillin for 10–20 h. Addition of 16 nM phorbol myristate acetate (PMA) and 0.75 μg/ml ionomycin to some cultures was used to stimulate BCR activation pathways and upregulate BLV protein expression (Stone et al., 1996). PMA is a phorbol ester and potent activator of the protein kinase C pathway. Ionomycin is a calcium ionophor. Thus both serve to stimulate pathways involved in B-cell activation through the BCR. In some experiments, the potent phosphatase inhibitor sodium pervanadate (prepared using 1 mM H_2O_2 and 0.1 mM Na_2VO_3 [sodium orthovanadate]) was added to increase tyrosine phosphorylated proteins present by inhibiting phosphatase activity (Cantor et al., 1999). Sodium pervanadate was added to cells for the last 10 min of culture, just prior to washing and lysis of the cells. The cells were lysed in Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris [pH 8], 1% NP-40) with protease inhibitors (0.1 mM *Nα-p*-tosyl-L-lysine chloromethyl ketone, 1.8 mg/ml iodoacetamide, 10 μg/ml aprotinin, 1 μM pepstatin, 100 μM leupeptin, and 1 mM phenyl-methylsulfonyl fluoride) and 0.2 mM Na_2VO_3 to decrease phosphatase activity. Lysates were centrifuged for 10 min at $45\,000 \times g$ to clear cellular debris. Immunoprecipitations were done with equivalent amounts of lysate based on cell counts prior to lysis. To immunoprecipitate TM, a rabbit monospecific, polyclonal antibody generated in our laboratory against the cytoplasmic tail of TM was used (R5169J, Cantor et al., 1999). The immunoprecipitations were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Immunoblots were blocked in Tris-buffered saline blocker (100 mM NaCl and 15 mM Tris, pH 7.4, 0.1% Tween 20 [TBST] and 1% bovine serum albumin [BSA]). The nitrocellulose was probed with mouse monoclonal antibody against phosphorylated tyrosine-containing proteins (RC20, Transduction Laboratories) (Fig. 2A and B).

For a more sensitive assay of phosphorylation, PBMCs were metabolically labeled with ^{32}P (orthophosphate in aqueous solution, Amersham Pharmacia Biotech) (Fig. 2C and D). After 4 h of phosphate starvation in phosphate-free media

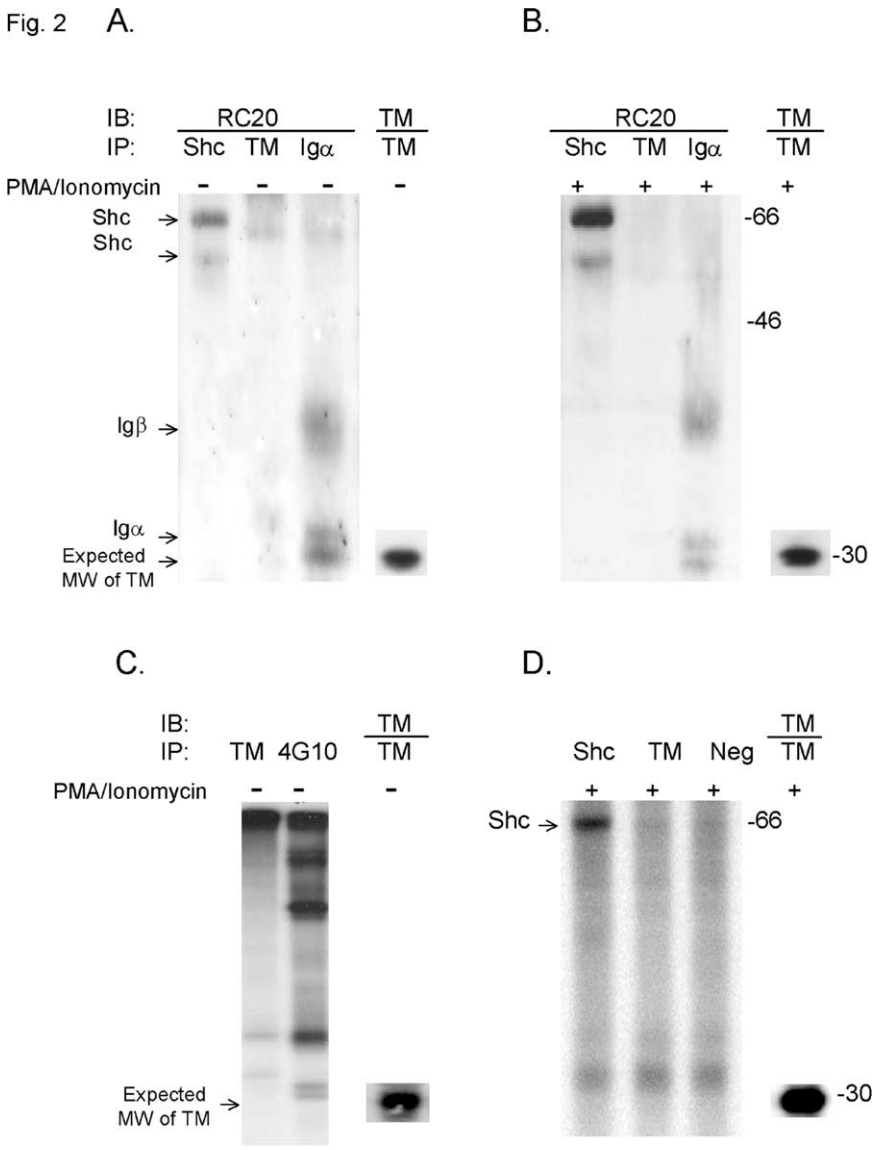

Fig. 2. For immunoblotting experiments, PBMCs from BLV infected cattle were cultured to enhance expression of viral proteins and (A) lysed in 1% NP40 lysis buffer plus protease inhibitors and sodium orthovanadate to inhibit dephosphorylation, or (B) treated with 160 nM PMA and 1.5 ug/ml ionomycin for 6 h, then treated for 10 min with sodium pervanadate to significantly enhance cell phosphorylation, and lysed as in A. Immunoprecipitations were performed with anti-Shc, anti-TM, or anti-Igα antibody as designated (Shc, TM, or Igα). Igα occurs in two forms and co-immunoprecipitates with Igβ (indicated). After SDS-PAGE, proteins were blotted onto nitrocellulose and detected with antibody against phosphorylated tyrosine (RC20) or with antibody against TM, respectively. In all experiments, equivalent cell lysate amounts were utilized based on cell counts prior to lysis. The data shown in all figures are representative of a minimum of three experiments. For metabolic labeling experiments, PBMCs from BLV-infected cattle were cultured to enhance expression of viral proteins, phosphate starved for 3 h and labeled with ^{32}P. Cells were then either (C) lysed as in A or (D) treated with 160 nM PMA and 1.5 ug/ml ionomycin for 6 h, then treated for 10 min with sodium pervanadate and lysed. Immunoprecipitations were performed as indicated based on cpm of incorporated ^{32}P. 4G10, used as a control in one experiment, is an antibody against tyrosine phosphorylated proteins. Neg indicates negative control immunoprecipitations using serum from a rabbit immunized with irrelevant antigen.

(phosphate-free RPMI plus 10% dialyzed FBS, 100 μM streptomycin and 100 μU penicillin), 0.4–0.5 mCi/ml ^{32}P was added and PBMCs (5×10^6 cells per ml) were cultured for 6 h. Phosphorylation was evaluated in both unstimulated and stimulated cells as before. Cells were washed and lysed. After lysis, counts-per-minute (cpm) of incorporated ^{32}P were determined and equivalent cpm were used for immunoprecipitation, averaging 10^5 cpm per immunoprecipitation. Immunoprecipitated proteins were separated by SDS-PAGE and the gels were fixed in 40% methanol, 10% acetic acid and 3% glycerol for 1 h and dried. Phosphorylated proteins were evaluated using a phosphoimaging screen (Molecular Dynamics).

To verify that lack of detection of phosphorylation was not due to technical errors, stringent controls were performed with all experiments. Two tyrosine phosphorylated proteins were immunoprecipitated from the lysates as positive controls in the experiments on PBMCs (except one experiment in which 4G10 (Upstate Biotechnology), an antibody against phosphorylated tyrosine-containing proteins, was used as a positive control [Fig. 2C]). The antibodies used were anti-Igα (Dako Corporation), against one of the ITAM-containing proteins of the BCR (Fig. 2) and anti-Shc (Transduction Laboratories) (Figs. 2, 4 and 5). Igα was chosen as a positive control because it allows verification that phosphorylated ITAM tyrosines were present in the cells and could be immunoprecipitated and detected. Shc is an adaptor protein that occurs as three isoforms of molecular weights 46, 52 and 66 kDa (Pelicci et al., 1992), and was chosen as another positive control in the PBMC experiments because it is tyrosine phosphorylated upon antigen-mediated B-cell upregulation (Baldari and Telford, 1999; Saxton et al., 1994). In addition, tyrosine phosphorylated forms of Shc are widely expressed in many cell types and were present in the non-B-cell lines and the avian B-cell line used in subsequent experiments. As a negative control for TM immunoprecipitation, an irrelevant polyclonal rabbit serum was used (lanes labeled Neg) (Figs. 2D and 5A–C). We demonstrated in each experiment that BLV TM was present in detectable amounts as determined by immunoprecipitation followed by immunoblotting (Figs. 2A–D, 4B and 5B). All results shown are representative of at least three experiments.

Results from the immunoprecipitation-immunoblot experiments with PBMCs indicated that TM was not phosphorylated in cultured, unstimulated PBMCs, although both phosphorylated Shc and phosphorylated Igα could be detected from the same cell lysates (Fig. 2A). This was also true in cultured cells stimulated with PMA and ionomycin (Fig. 2B), and in immunoprecipitated lysates of metabolically labeled, unstimulated or stimulated cells (Fig. 2C and D). A faint 35 kDa phosphorylated band was sporadically detected by immunoblotting of the TM immunoprecipitates of PBMCs from one BLV-infected cow. A band of similar size was also demonstrated in immunoprecipitates from PBMCs of BLV negative animals (not shown) making it unlikely the faint band represents phosphorylated TM.

Since no phosphorylation of BLV TM was detected in PBMCs, TM phosphorylation was evaluated in two different BLV-infected cell lines, fetal lamb kidney cells (FLK) (Deshayes et al., 1977) and bat lung cells (BLV-BLC) (Graves and Ferrer, 1976). Both immunoblotting and metabolic labeling were performed to detect phosphorylated TM. These cell lines were acquired persistently infected and used because they express abundant viral proteins, while viral expression in ex vivo PBMCs may be low. Adherent cell lines were grown for 5-7 days to near confluency (75–85%) in DMEM plus 10% FBS, and then stimulated with PMA (160 nM) and ionomycin (1.5 ug/ml) for 2–4 h, or evaluated without stimulation. Stimulated cells were treated with sodium pervanadate for 10 min and then lifted from the plates with 0.25% trypsin and 1 mM EDTA (Gibco BRL). Metabolic labeling was performed as in the PBMC experiments, by first starving the cells of phosphate for 4 h, then culturing FLK or BLV-BLC cells with ^{32}P (1 mCi/ml phosphorus-32) for 6 h to near-confluency. All cells were washed in phosphate-buffered saline (PBS) after culture. Following cell lysis, immunoprecipitation was performed for both the TM protein and control proteins. In experiments with unlabeled cells, immunoprecipitation was based on cell counts prior to lysis. In metabolically labeled cells,

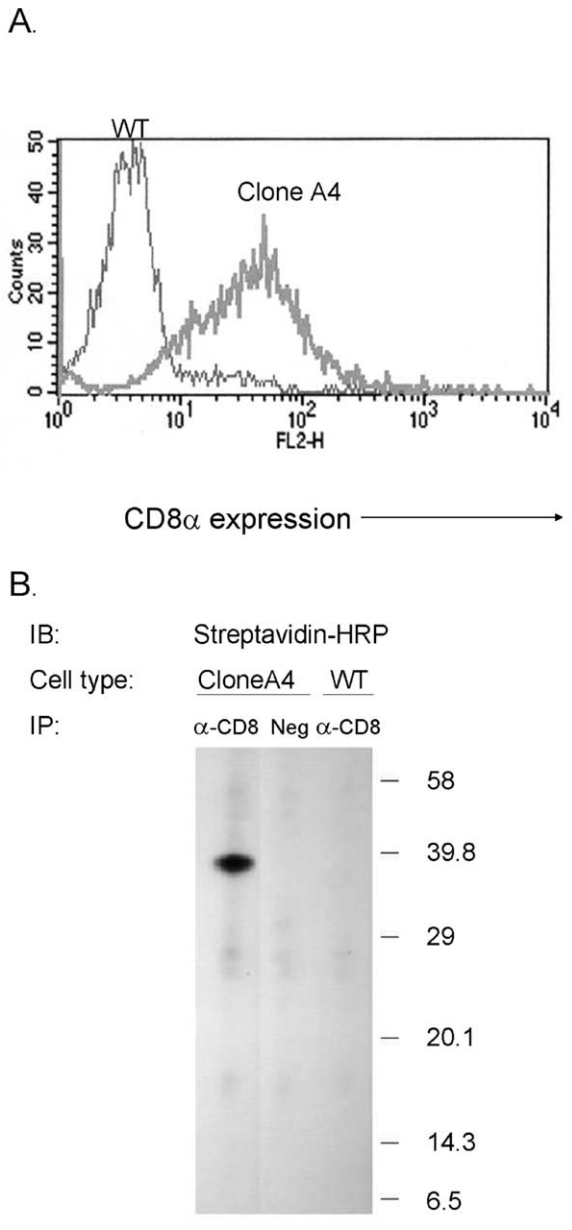

Fig. 3

immunoprecipitations were based on cpm of incorporated ^{32}P and averaged 10^6 cpm per immunoprecipitation. The immunoprecipitates were separated by SDS-PAGE. Gels of immunoprecipitations from unlabeled lysate were transferred to nitrocellulose, the membranes were blocked with TBST + 1% BSA, and probed with RC20 anti-phosphotyrosine antibody. Gels of immunoprecipitations from ^{32}P-labeled cells were dried and exposed to a phosphoimager, as described above. As in the PBMCs, no phosphorylated TM was detected in either FLK or BLV-BLC cell lines by immunoblotting or by metabolic labeling of the cells (data not shown). In each experiment with the cell lines, phosphorylated Shc could be immunoprecipitated and detected, and nonphosphorylated TM was shown to be present.

To confirm the results in a fourth system, we performed similar experiments in the avian DT40 B-cell line. DT40 cells are an easily transfectable B-cell line commonly used in B-cell signaling studies and contain many of the same kinases, phosphatases and signal pathways found in mammalian B cells (Winding and Berchtold, 2001). The plasmid construct, pLVLy2-Hy-BLV-II, encoding CD8/TM (Alber et al., 1993) (kindly provided by M. Reth) was electroporated into DT40 wild-type cells (kindly provided by T. Kurosaki). The DT40 cells were electroporated (550V, 25 μF, BioRad Genepulser II, 0.4 cm cuvette) with the plasmid for 0.62 ms at 0.55 kV. Transfected cells were selected for 4 weeks with 4 mg/ml hygromycin, in RPMI plus 10% FBS, 1% chicken serum, and 1% L-glutamine (designated Clone A4 cells). Expression of CD8/TM was verified by fluorescent antibody labeling with rat anti-mouse CD8α antibody (Biosource International) and goat anti-rat IgG conjugated to Alexifluor 488 (Molecular Probes), followed by flow cytometry (Becton-Dickinson Immunocytometry Systems) (Fig. 3A). As additional verification, cells expressing CD8/TM were surface labeled with biotin by incubating 5×10^7

Fig. 3. DT40 wild type cells electroporated with plasmid pLVLy2-Hy-BLV-II were selected in hygromycin for 4 weeks (designated CloneA4). (A) The cells were stained with rat anti-mouse CD8α antibody (Biosource International) followed by Alexifluor anti-rat 488 (Molecular Probes), and analyzed by flow cytometry. As a control, DT40 wild-type cells (WT) were also labeled and analyzed. (B) Clone A4 cells were biotin labeled and immunoprecipitations were performed with anti-CD8α antibody, followed by separation on SDS-PAGE. Detection was with streptavidin-HRP. As controls, biotin-labeled Clone A4 cells were immunoprecipitated with irrelevant serum (Neg) and biotin-labeled DT40 wild-type cells were also immunoprecipitated with anti-CD8α antibody. CD8/TM is expressed as an approximately 39 kDa protein.

cells per ml in PBS with EZ-link sulfo-NHS-LC-biotin (Pierce) for 30 min, with rotation. The cells were washed two times in PBS and lysed in 1% NP40 lysis buffer. CD8/TM was immunoprecipitated with anti-CD8α antibody and detected with streptavidin-HRP (Fig. 3B).

Phosphorylation of the cytoplasmic tail of TM was tested in both stimulated and unstimulated DT40 cells. Two stimulation methods were used; either stimulation by crosslinking of the BCR or by oligomerization of CD8/TM with biotin-labeled anti-CD8α antibody and avidin. Upregulation of overall tyrosine phosphorylation in the cells was first achieved by crosslinking of the BCR using anti-IgM antibody. Clone A4 cells were stimulated for varying time points from 0 to 5 min using antibody against the BCR (4 μg/ml anti-chicken IgM, M4, Southern Biotechnology Associates). To confirm expression of CD8/TM in every experiment, separate aliquots of the cells were biotin labeled, lysed and immunoprecipitated with anti-CD8α antibody (Fig. 4B). The cells used for stimulation experiments were washed three times in ice-cold PBS and resuspended in cold media prior to stimulation, then added to media containing anti-IgM antibody at 37 °C (or to cold media containing anti-IgM antibody for the 0 time experiment) and incubated at 37 °C for the indicated times. Stimulation was halted by washing the cells in ice-cold PBS followed by immediate lysis in 1% NP40 lysis buffer.

Clone A4 cells were also stimulated through the CD8α extracellular portion of CD8/TM. Based on the sequence of the transmembrane portion of CD8α, CD8/TM is likely expressed as a homodimer on the cell surface (Alber et al., 1993) and use of biotinylated anti-CD8α antibody crosslinked with avidin results in oligomerization of the chimeric protein (Sutherland et al., 1999). Stimulation times were optimized by time trials evaluating overall tyrosine phosphorylation patterns of the cells (data not shown). For these experiments, the cells were washed in cold PBS then resuspended at 1×10^7 cells per ml in cold RPMI plus 10% FBS. Biotinylated rat anti-mouse CD8α antibody and NeutrAvidin (Pierce) were added and the cells were brought to 37 °C. Stimulation was halted by washing the cells in ice-cold PBS. Rat anti-mouse CD8α antibody was obtained from supernate of the hybridoma cell line, 53-6.72 (ATCC) (Sutherland et al., 1999), and purified over a protein G sepharose column. Antibody was then biotinylated using sulfo-NHS biotin and reactivity of the biotinylated antibody with CD8/TM was verified using FACS analysis (data not shown).

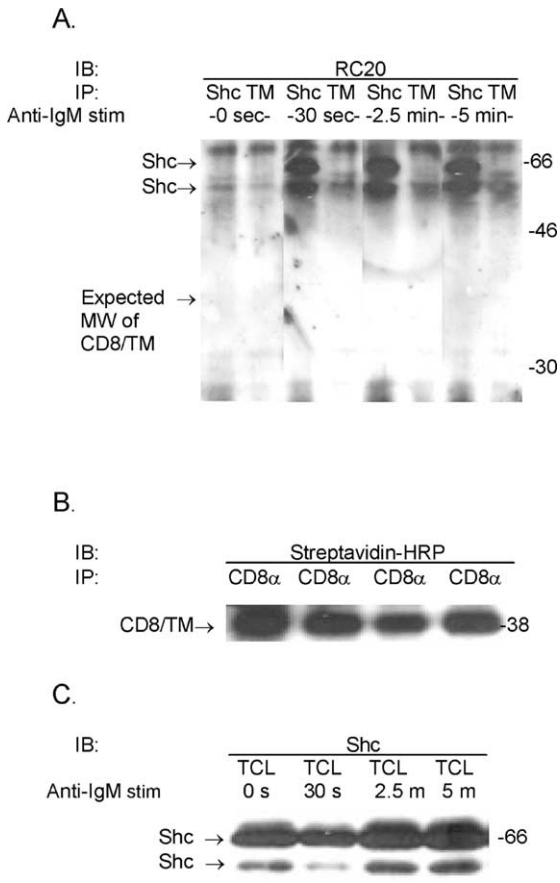

Fig. 4. Clone A4 cells were stimulated for the indicated times by crosslinking with 4 μg/ml anti-chicken IgM antibody. Reactions were stopped by the addition of ice-cold PBS and cells were lysed. (A) Immunoprecipitations were performed with either anti-Shc or anti-TM antibodies, and immunoblots were performed with antibody against phosphorylated tyrosine (RC20). (B) Aliquots of Clone A4 cells were removed prior to the BCR crosslinking, biotin-labeled, lysed and immunoprecipitated with anti-CD8α antibody. CD8/TM was detected with streptavidin-HRP. (C) Total cell lysates of cells stimulated as described above were separated by SDS-PAGE and then transferred to nitrocellulose. After blocking, immunoblotting with anti-Shc antibody was performed.

CD8/TM immunoprecipitates from Clone A4 cells were not tyrosine phosphorylated, as assessed by immunoblotting with RC20. No induced phosphorylation was detected by either BCR (anti-IgM) crosslinking (Fig. 4A) or by oligomerization of the cytoplasmic TM by anti-CD8α antibody (Fig. 5A). Control immunoblots showed that CD8/TM was expressed and could be detected by either biotin labeling of cells or with anti-TM antibody (Figs. 4B and 5B). Phosphorylated and total Shc was detected in the cells by immunoprecipitation followed by immunoblotting (Figs. 4A and C and 5A and C). The phosphorylation of Shc in nonstimulated cells varied slightly in several experiments (Fig. 4A, lane 1 vs. Fig. 5, lane 1), but typically was low as is expected in unstimulated mature B cells and increased with either BCR crosslinking or anti-CD8α oligomerization. Both BCR crosslinking, and oligomerization of CD8/TM did result in a change in the total phosphotyrosine pattern of Clone A4 cells compared with the parent cell line, similar to previous results of Alber et al. (1993) and Beaufils et al. (1993). There was significant increase in tyrosine phosphorylation of a 59 kDa band, and slight increases of 43 and 29 kDa phosphorylated bands from Clone A4 cells as compared with bands from parental or wild-type DT40 cells. There was also decreased tyrosine phosphorylation compared with the wild-type cells of the 88, 65 and 39 kDa phosphorylated bands (Fig. 6). Results were similar in experiments in which the BCR was crosslinked, except for a greater overall increase in tyrosine phosphorylation of proteins from both Clone A4 and wild type cell lines (data not shown).

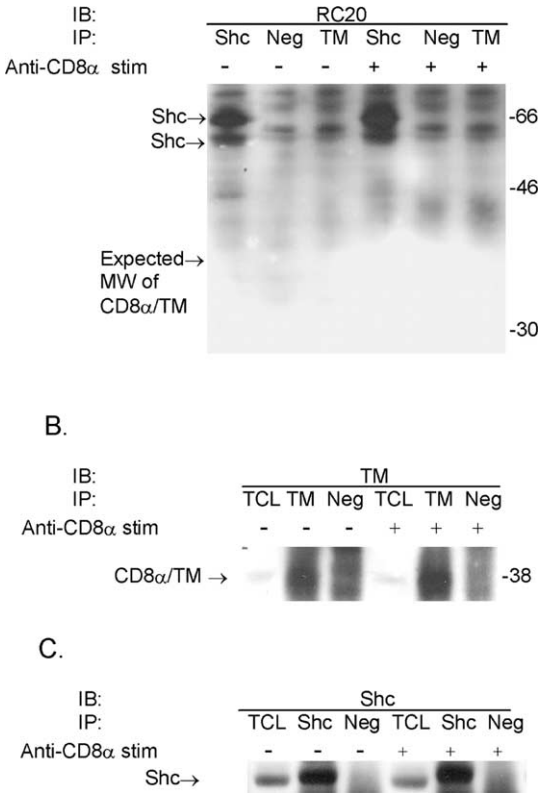

Fig. 5. Clone A4 cells were treated for 15 min at 37 °C with NeutrAvidin® in PBS (−) or with 10 μg/ml biotin-labeled antibody against CD8α and NeutrAvidin®, to oligomerize the CD8/TM protein (+). Reactions were stopped with the addition of ice-cold PBS. (A) Lysates were immunoprecipitated with antibody as indicated and immunoblots were detected with antibody against phosphorylated tyrosine (RC20). (B) Lysates were immunoprecipitated with anti-TM antibody, separated by SDS-PAGE and transferred to nitrocellulose. After blocking, anti-TM antibody was used to detect CD8/TM. (C) Total cell lysates of cells oligomerized through CD8/TM were separated by SDS-PAGE and transferred to nitrocellulose. After blocking, immunoblots were performed with anti-Shc antibody to verify expression of Shc.

We initially hypothesized that the BLV TM tyrosines within the ITAMs or ITIMs were inducibly phosphorylated in host B cells, a process which could alter signal transduction and contribute to the preneoplastic cell expansion seen in BLV-infected animals. To test this hypothesis, we first examined ex vivo BLV-infected PBMCs for induced TM tyrosine phosphorylation using either immunoblotting or metabolic labeling methods. PBMCs from naturally infected cattle represent a natural system of BLV infection in B lymphocytes; however, no phosphorylation of TM was evident in unstimulated, cultured cells or after PMA and ionomycin stimulation. One possibility is that the amount of TM expressed in PBMC culture was inadequate to demonstrate phosphorylation. We have previously shown that only 15–20% of PBMCs express viral p24 upon culture (Dequiedt et al., 1999). Additionally, conditions of culture may not mimic the milieu or the cell stage of virus-expressing cells in vivo, which could alter the degree of phosphorylation seen. One study has

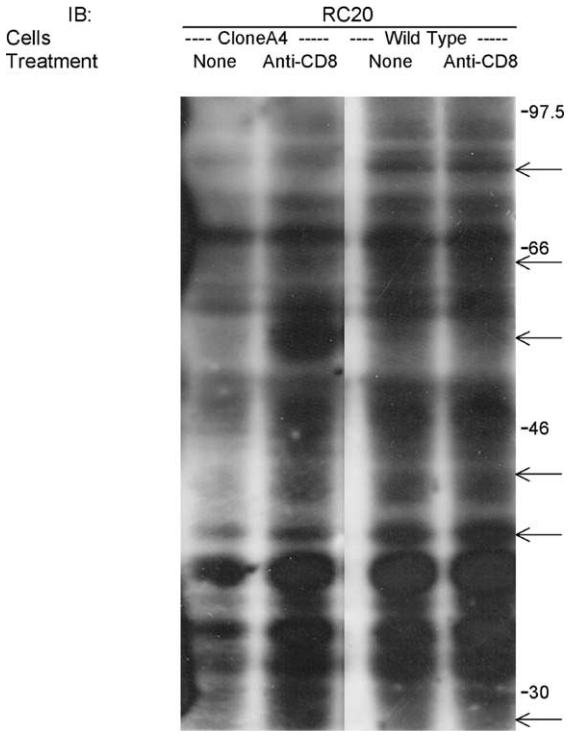

Fig. 6. Clone A4 cells or wild-type DT40 cells were treated for 15 min with PBS alone, or were stimulated with 10 μg/ml of anti-CD8α antibody, then lysed in SDS sample buffer. Total cell lysates were separated by SDS-PAGE and immunoblotted with antibody to detect phosphorylated tyrosines (RC20). Arrows indicate a strong increase in phosphorylation at 59 kDa band, as well as slight increases of 43 and 29 kDa tyrosine phosphorylated bands as compared with bands from the parental or wild-type DT40 cells. There were also bands at 88, 65 and 39 kDa with decreased tyrosine phosphorylation compared with the wild-type cells. Results were similar for cells stimulated through the BCR, but overall phosphorylation was more intense (data not shown).

shown that the infected B cells expressing viral proteins largely remain in the G_0/G_1 portion of the cell cycle during 24–72 h of culture (Stone et al., 2000).

Due to the findings in PBMCs, the experiments were repeated in two different cell lines that stably express abundant viral proteins. The time to near-confluency in these cells (ca. 5–6 days for FLK cells and 6–7 days for BLV-BLC cells) corresponded well to peak viral expression in these cell lines (Llames et al., 2001), yet no tyrosine phosphorylation of TM was detected in either stimulated or unstimulated cells. Finally, experiments were repeated in a B-cell line stably transfected with the cytoplasmic tail of TM. However, neither activation through the BCR nor oligomerization through CD8/TM resulted in tyrosine phosphorylation of TM, despite phosphorylation of the control Shc and modification of the total tyrosine phosphorylation pattern of the cells. Thus even with expression of TM in a variety of cells, at various levels of virus expression, and at different levels of stimulation, phosphorylated BLV TM could not be demonstrated.

Our hypothesis was based on the data supporting a significant role for the tyrosine residues in altering cell signaling by BLV TM. Although most interactions between tyrosine residues in ITAMs and ITIMs and their SH2 binding sites rely on tyrosine phosphorylation (Flaswinkel et al., 1995; Muta et al., 1994), not all do. For example, SH2D1A (or SAP) is an SH2 domain-containing protein that does not rely on recognition of either a tyrosine or phosphotyrosine residue for binding (Hwang et al., 2002). Other binding domains can recognize both phosphorylated and unphosphorylated tyrosine residues, such as with signal proteins known as phosphotyrosine-binding domains (PTBs) (Forman-Kay and Pawson, 1999; Borg et al., 1996; Howell et al., 1999). Thus interactions of tyrosine-containing motifs may not require tyrosine phosphorylation.

Additionally, there are other potential binding domains within BLV TM, such as the proline-rich motifs, that may interact with host signal proteins without requiring tyrosines. Binding of proline-rich motifs to SH3 domains does not require phosphorylation and this binding may be sufficient for interactions between viral and host signal proteins. For example, the binding of Nef to the Src-family kinase Hck is dependent only on binding of a proline-rich motif to the SH3 domain of Hck (Lee et al., 1995; Saksela et al., 1995). Indeed, we demonstrated the importance of proline-rich motifs in vivo in a study in which infection with proviruses with mutations of a TM proline-rich motif resulted in decreased viral loads and lack of progression to tumorigenesis in sheep (Reichert et al., 2001). Simultaneous interactions of both SH2 and SH3 domains with either

an ITAM or ITIM and the proline-rich motif may synergistically cause binding in the absence of tyrosine phosphorylation. Thus the ability of BLV TM to modify B-cell signal pathways by interacting with host signal proteins is still a plausible hypothesis.

Several mutational studies have indicated the importance of specific BLV TM tyrosine residues for signal activity in vitro and for infectivity in vivo. However, interactions of the cytoplasmic tail of BLV TM without tyrosine phosphorylation may still explain these results. Two studies which examined the ability of CD8/TM to activate signal transduction when expressed and oligomerized in cell lines (Alber et al., 1993; Beaufils et al., 1993) demonstrated that specific TM tyrosine residues were necessary for these actions. The mutation of tyrosine to alanine in these studies changes the polarity and charge of the residue, which in turn may change the function of the protein. A study of infectious BLV clones with TM tyrosine to aspartic acid mutations demonstrated decreased viral propagation in vivo when inoculated into sheep (Willems et al., 1995). This mutation avoids the change in charge and maintains the steric organization of the protein, which suggests that tyrosine phosphorylation, rather than the amino acid charge, is important. However, the effect on viral infectivity and propagation could be explained by mechanisms other than interaction with host proteins due to tyrosine phosphorylation, such as alteration of viral assembly of Env protein, or alteration of endocytosis signals, which have not yet been determined to rely on phosphorylation of the tyrosine residue. This may result in increased immune recognition of virus-infected cells (Honing et al., 1996; Hunziker et al., 1994; Matter et al., 1992; Thomas et al., 1993). The tyrosine residues needed for endocytosis are limited to just the four amino acid motif, YXXL, and thus a function in endocytosis alone does not explain the significant conservation of the entire ITAM sequence.

BLV TM in PBMCs from infected PL cattle has previously been shown to co-immunoprecipitate with the phosphatase SHP-1 (Cantor et al., 1999). The specific kind of interaction between TM and SHP-1 and whether it was direct or indirect could not be determined. However, the interaction did rely on tyrosine phosphorylation as demonstrated by enhancement of the association between TM and SHP-1 after treatment with sodium orthovanadate, a phosphatase inhibitor. In light of our current results, one might speculate that the interactions of TM with SHP-1 could result in such a rapid dephosphorylation of TM that TM phosphorylation could not be demonstrated in our system. However, because of the rapid binding kinetics of phosphate groups, this is not likely a cause of our inability to detect a phosphorylated form of TM.

Our current data demonstrated an increase in Shc phosphorylation (albeit somewhat variable) after crosslinking of CD8/TM in DT40 cells. Shc was mildly phosphorylated in unstimulated Clone A4 cells. This was compared with minimal or no Shc phosphorylation in unstimulated DT40 wild type cells. As expected due to its role in antigen-mediated signaling, Shc was phosphorylated upon anti-BCR antibody stimulation of both Clone A4 and wild type cells. Somewhat unexpectedly, Shc phosphorylation increased, although variably, upon crosslinking of CD8/TM. The higher baseline level of phosphorylation of Shc in Clone A4 cells, and the increase seen after crosslinking may be related to TM interaction with inhibitory pathways through SHP-1 (Cantor et al., 1999). Although direct interaction between Shc and SHP-1 is not well described in the literature, the role of Shc in BCR inhibitory pathways has been shown (Saxton et al., 1994; Harmer and DeFranco, 1999). Upon phosphorylation, Shc forms complexes with two important phosphatases, Src homology-2-containing inositol 5′-phosphatase (SHIP) and SHP-2, and two key B-cell downregulatory coreceptors, CD22 (Poe et al., 2000) and FCγRIIb (Koncz et al., 2001). However, since Shc is an adaptor protein seen in a wide range of cells and multiple pathways (Ravichandran, 2001), the increased phosphorylation seen in the Clone A4 cells may be related to culture conditions and unrelated to TM interactions with BCR.

We conclude that while TM may affect host cell signaling pathways, it does not appear to be phosphorylated on tyrosine residues of either the putative ITAMs or ITIMs in our test conditions. ITAMs and ITIMs are characterized by a rela-

tively well-conserved sequence centered on tyrosine residues. Tyrosine phosphorylation is important for binding, but may not be required for binding of SH2 domains to these motifs. However, it is surprising to us that this highly conserved set of ITAM and ITIM sequences with sequence similarity to binding sites for tyrosine kinase family members does not function through phosphorylation. It is still possible there are in vivo conditions that are not repeated in the in vitro cultures and thus the phosphorylation reaction of these motifs are either below detection levels or not recreated in the in vitro cultures. It is likely that protein–protein interactions do occur between BLV TM and host cell proteins, either through unphosphorylated binding of SH2 and SH3 domains to the closely apposed ITAM or ITIM and proline-rich motifs, or via interactions with yet undefined binding domains. The inability of our experimental conditions to demonstrate any phosphorylation of BLV TM does, however, suggest that phosphotyrosine-mediated signal transduction via direct interaction with nonreceptor protein tyrosine kinases or phosphatases is unlikely.

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