Herpesvirus saimiri protein StpB associates with cellular Src

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Herpesvirus saimiri (HVS) induces T cell-proliferative disorders in certain species of New World primates and in rabbits (Jung et al., 1999). HVS isolates can be divided into three subgroups (A, B and C) based on sequence divergence at the left end of the genomic L-DNA (Desrosiers & Falk, 1982; Medveczky et al., 1984). Strains of all subgroups are able to induce T cell lymphomas in cottontop marmosets (Saguinus oedipus), but only members of subgroups A and C cause disease in common marmosets (Callithrix jacchus) (Fleckenstein & Desrosiers, 1982; Duboise et al., 1998). While subgroup A and C strains were able to immortalize primary C. jacchus T lymphocytes in vitro in the absence of exogenous IL-2, subgroup B tested negative under the same conditions (Desrosiers et al., 1986; Szomolanyi et al., 1987). Finally, only subgroup C strains are able to transform human lymphocytes to permanent growth in culture (Biesinger et al., 1992). These biological differences have been assigned to the stp genes encoded at the variable left end of L-DNA. The stp genes of subgroup A and C strains have been shown to be essential for lymphocyte transformation in vitro and lymphoma induction in vivo (Murthy et al., 1989; Duboise et al., 1998). In addition, the saimiri transforming proteins of subgroups A (StpA) and C (StpC) are both oncogenic when expressed in rodent fibroblasts and transgenic mice (Jung et al., 1991; Murphy et al., 1994; Kretschmer et al., 1996). Fibroblast transformation by StpC depends on a cluster of collagen-like repeats in the central part of the protein (Jung & Desrosiers, 1994). Insertion of this repetitive element in the N-terminal part of subgroup B Stp (StpB) converts the non-transforming phenotype of this protein in fibroblasts (Choi et al., 2000). The collagen-like repeats induce self-oligomerization of StpB, suggesting that their function is to induce aggregation of additional cellular binding partners. Potential effector proteins are TNF receptor-associated factors (TRAFs), which associate with StpA and StpC (Lee et al., 1999) as well as with StpB (Choi et al., 2000). This hypothesis is further supported by the finding that an StpC mutant deficient in TRAF interaction no longer transformed rodent fibroblasts in vitro and a virus carrying this mutation was not able to transform primary human T lymphocytes. However, the recombinant virus still immortalized common marmoset lymphocytes and induced lymphoma in this species (Lee et al., 1999), indicating that TRAFs are not the only effectors of StpC. Indeed, cellular Ras has been identified previously as an interaction partner essential for fibroblast transformation (Jung & Desrosiers, 1995) and as a functional substitute for StpC (Guo et al., 1998). While Ras interaction seems to be restricted to StpC, c-Src might be an additional effector for StpA and StpB (Lee et al., 1997; Choi et al., 2000). However, Jung and co-workers recently described significantly reduced Src-binding activity of wild-type StpB compared with StpA (Choi et al., 2000). These observations contrast with data presented here, which suggest similar Src-binding activities of StpA and StpB but differences in the tyrosine kinase substrate properties of both viral proteins.

In order to approach the transforming functions of HVS subgroup B, we first analysed the left-terminal L-DNA of strain SMHL. Virion DNA was cloned into pBluescribe M13+ and plasmids containing left-terminal sequences were identified by colony hybridization with a radioactively labelled fragment of strain C-488 (nt 2683–6652). Four overlapping PstI and NsiI fragments were sequenced as described previously (Albrecht et al., 1992; Ensser et al., 1997) and analysed with the GCG programs using standard parameters (version 9.0; Genetics Computer Group, Madison, WI, USA). The nucleotide se-
Fig. 1. Primary structure of HVS B-SMHI sequences relevant to transformation. (A) Dot plot comparison of the left-terminal nucleotide sequences of HVS B-SMHI and A-11 (GCC programs COMPARE and DOTPLOT; window 32, stringency 24). ORFs and HSUR sequences of HVS B-SMHI are indicated above the dot matrix. DHRF, Dihydrofolate reductase. (B) Amino acid comparison of StpA and StpB. Sequences are aligned manually to produce overlaps for conserved patterns, namely GX,X motifs (open boxes), potential TRAF-binding sites (bold), the SH2-binding consensus YAEV/I (black boxes) and hydrophobic C termini (underlined). Arrowheads indicate the positions of tyrosine residues mutated to phenylalanine (see Fig. 2).

Fig. 2. Binding of StpB to c-Src. AU1 epitope-tagged StpB (StpB), mutants AU–StpB Y62F (Y62F) and AU–StpB Y118F (Y118F) as well as c-Src (Src) were expressed in COS cells either alone or in the combinations indicated. Src complexes were immunoprecipitated from cell lysates with SRC2 antibody (IP SRC2). Precipitation with protein A–Sepharose alone (IP no ab) served as a negative control. AU1-specific immunoblots (IB anti-AU1) were used to detect StpB proteins in precipitates (upper panel) and cell lysates (lower panel). Expression of c-Src was confirmed by an immunoblot with cell lysates and MAb EC10 (IB EC10; middle panel). Sizes of molecular mass markers are given in kDa. *, Background band present in all samples containing protein A–Sepharose.

Fig. 3. (A)–(B) StpB is phosphorylated by c-Src in vivo and in vitro. The vector pcDNA3 (lanes 1, 4, 7) and plasmids encoding AU–StpA (2, 5, 8) or AU–StpB (3, 6, 9) were transfected into COS cells either alone (lanes 1–3) or together with pEF-Src (4–6) or pEF-Src L295 (7–9). (A) Cell lysates (10 µg per lane) were separated by SDS–PAGE and subjected to an immunoblot with anti-phosphotyrosine antibody (upper panel). The membrane was cut horizontally and reprobed with MAb EC10 (IB EC10; middle panel) to detect c-Src complexes were immunoprecipitated from cell lysates with SRC2 antibody (IP SRC2). Precipitation with protein A–Sepharose alone (IP no ab) served as a negative control. AU1-specific immunoblots (IB anti-AU1) were used to detect StpB proteins in precipitates (upper panel) and cell lysates (lower panel). Expression of c-Src was confirmed by an immunoblot with cell lysates and MAb EC10 (IB EC10; middle panel). Sizes of molecular mass markers are given in kDa. *, Background band related to protein A–Sepharose.
Finally, the amino acid sequence YAEV of StpA is conserved in StpB as YAEI (Fig. 1B). This YAEV/I motif resembles the consensus for phosphotyrosine-dependent binding to the SH2 domains of Src family tyrosine kinases (Songyang et al., 1993). Acidic residues N-terminal of this motif that are characteristic for preferred Src kinase substrates (Songyang et al., 1995) are present in StpA but not in StpB. In fact, StpA is phosphorylated on tyrosine residues in vivo in the presence of Src and the tyrosine residue of the YAEV motif has been shown to be essential for the interaction with cellular Src (Lee et al., 1997), suggesting that StpA binds Src via the SH2 domain.
To assess the Src-binding properties of StpB in comparison with StpA, we cloned both reading frames into the expression vector pcDNA3 (Invitrogen) with or without an N-terminal AU1 epitope tag. In addition, two point mutants of StpB were generated by site-directed mutagenesis; codons 62 and 118 were mutated from tyrosine to phenylalanine, resulting in AU–StpB Y\(_{62}\)F and AU–StpB Y\(_{118}\)F. DNA sequences of all plasmids were analysed to confirm the presence of the expected mutations and the absence of unintentional mutations. After \textit{in vitro} translation in the presence of \(^{35}\)S-methionine, StpB, AU–StpB, AU–StpB Y\(_{62}\)F and AU–StpB Y\(_{118}\)F constructs gave rise to 25 kDa bands (data not shown). After transient expression in COS cells, StpB appeared as a triplet of 24–26 kDa proteins on epitope-specific immunoblots (Fig. 3; and data not shown), suggesting post-translational modification of StpB in COS cells.

In order to analyse Src-binding of StpB, COS cells expressing StpB and/or c-Src (Dunant \textit{et al}., 1995) were lysed in TNE buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 2 mM EDTA) with 1% NP-40, protease inhibitors (10 mg/ml aprotinin, 10 mg/ml leupeptin) and phosphatase inhibitors (5 mM NaF, 1 mM Na\(_2\)VO\(_4\)). Immunoblots with antibodies specific to chicken c-Src (EC10 hybridoma supernatant) or to the AU1 epitope (Babco) revealed uniform levels of Src or StpB expression in the respective lysates (Fig. 2, middle and lower panels). A Src-specific rabbit serum (SRC2; Santa Cruz Biotech) and protein A–Sepharose (Pharmacia) were used for immunoprecipitations. Subsequent immunoblots with anti-AU1 antibody (Fig. 2, upper panel) demonstrated the association between StpB and c-Src (lane 4). While Src-binding of StpB was not affected by mutation of \(Y_{62}\) (lane 5), it was disrupted completely by mutation of \(Y_{118}\) (lane 6). A weak signal was also observed after SRC2 immunoprecipitation from lysates of AU–StpB-transfected cells (lane 1), suggesting that StpB associates not only with the co-transfected chicken c-Src but also with the endogenous Src kinase of COS cells. Non-specific precipitation was excluded by controls without antibody (lanes 7–10).

The importance of the YAEI motif for binding suggested that association of StpB with Src is mediated by the SH2 domain. As SH2 binding is dependent on tyrosine phosphorylation, we analysed the phosphorylation state of StpA and StpB that lead to retarded migration appear to be regulated by the activity of Src. As all forms of these proteins may be phosphorylated on tyrosine residues (see below), we suggest that the observed modifications were induced by serine/threonine kinases acting as downstream effectors of Src.

The anti-phosphotyrosine immunoblot (Fig. 3A, upper panel) revealed a strong phosphorylation of all StpB species \textit{in vivo} in the presence of c-Src (lane 6). The amount of phosphorylated StpA was significantly smaller (lane 5) and slower migrating forms were only visible after longer exposure (data not shown). Comparison of the phosphotyrosine-specific and epitope-specific signals (Fig. 3A, lower panel) suggested that StpB is phosphorylated \textit{in vivo} more efficiently than StpA. Endogenous kinases (lanes 2 and 3) and inactive c-Src (lanes 8 and 9) did not lead to detectable levels of StpA or StpB phosphorylation \textit{in vivo}. Aliquots of the same cell lysates were used for AU1-specific immunoprecipitation followed by an \textit{in vitro} kinase reaction (Lang \textit{et al}., 1997) (Fig. 3B). After co-expression with c-Src, AU–StpB and AU–StpA were phosphorylated \textit{in vitro} to a comparable extent (lanes 5 and 6). \textit{In vitro} phosphorylation of StpB by endogenous kinases alone (lane 3) or after co-transfection with inactive c-Src (lane 9) was detectable only after longer exposure and was significantly weaker than for StpA (lanes 2 and 8). These data suggest that StpB is a good substrate for Src or a Src-activated tyrosine kinase \textit{in vivo}, while StpA might be more efficiently phosphorylated \textit{in vitro}.

To prove binding of StpA and StpB to the SH2 domain of Src definitively, we performed co-immunoprecipitation assays with Src deletion mutants (Dunant \textit{et al}., 1996). Both AU–StpA and AU–StpB were co-transfected with wild-type c-Src and with deletion mutants lacking the SH3 or SH2 domain (Fig. 3C, D). Expression of the Src constructs was monitored by an immunoblot with cell lysates and SRC2 antisera (Fig. 3C, D; middle panels). The AU1-specific lysate immunoblot revealed expression of recombinant StpA and StpB in the appropriate lanes (Fig. 3C, D; lower panels). Immunoprecipitation with SRC2 antibodies followed by an AU1-specific immunoblot demonstrated interaction of StpA and StpB with wild-type and SH3-deficient Src (Fig. 3C, D; upper panel). In agreement with published data (Moarefi \textit{et al}., 1997), the SH3-deletion mutant appeared to be more active than wild-type Src, resulting in enhanced mobility shift and binding of StpB (Fig. 3D; lanes 6 and 7). This effect of SH3-deficient Src was less pronounced for StpA, where mainly the slower-migrating form was co-precipitated (Fig. 3C; lanes 6 and 7). In contrast, deletion of the
SH2 domain abolished Src binding of both StpA and StpB (Fig. 3 C, D; upper panel).

Thus, after co-expression with c-Src in COS cells, we did not observe significant differences in the ability of StpA and StpB to interact with the SH2 domain of c-Src via the YAEV/I motif. While acidic residues typical of a Src kinase phosphorylation site are present only in StpA, in vivo tyrosine phosphorylation in this system was higher for StpB. An endogenous tyrosine kinase activated by c-Src overexpression might account for this discrepancy by phosphorylating StpB and generating Src SH2-binding sites. Different expression levels of such a kinase might also explain the contradictory results obtained by Jung and co-workers using 293T cells (Choi et al., 2000).

As our nucleotide sequence indicated, HVS B-SMHI is more closely related to HVS A-11 than to HVS C-488 and, in spite of low similarity, StpA and StpB share all interaction partners identified so far, while StpC shows unique properties. This potent oncoprotein binds Ras and activates MAP kinases (Jung & Desrosiers, 1995), interacts with TRAFs and activates their downstream target, NF-kB (Lee et al., 1999), and, finally, it carries collagen-like repeats capable of inducing protein multimerization (Choi et al., 2000). All these properties are required for transformation of rodent fibroblasts. In addition, lymphocyte transformation and lymphoma induction by HVS C-488 depend on the presence of another viral protein, Tip, which is co-transcribed with StpC and may influence T cell growth regulation by its interaction with the T cell-specific tyrosine kinase Lck (Biesinger et al., 1995; Fickenscher et al., 1996; Duboise et al., 1998; Isakov & Biesinger, 2000).

In contrast, the ability of StpA and StpB to interact with TRAFs does not result in NF-kB activation (Lee et al., 1999; Choi et al., 2000). Despite differences in their tyrosine kinase substrate properties, both proteins appear to bind the SH2 domain of Src with similar efficiency (Fig. 3). However, only StpA is able to transform rodent fibroblasts, although with a moderate phenotype (Jung et al., 1991). In accordance with our data, mutation of the Src-interacting sequences is not required to generate a transforming StpB variant. The component missing in StpB may be self-oligomerization (Choi et al., 2000). As supposed but not proven by Jung and co-workers, this function may also be provided by the collagen-like triplets scattered within the N-terminal half of StpA. While StpA has long been known to be required for the oncogenic phenotype of HVS A-11 (Murthy et al., 1989) and induces lymphoma by itself (Kretschmer et al., 1996), the functions of StpB and of the interaction motifs in lymphocytes remain to be analysed. As demonstrated clearly by the work of Longnecker and co-workers on the Epstein–Barr virus protein LMP2A (Longan & Longnecker, 2000) and references therein, growth-altering effects of viral transformation-associated proteins may depend significantly on the cellular context.

Taken together, the low efficiency of transformation by subgroup B strains seems not to be due to a failure of StpB to bind cellular proteins known to interact with StpA. Comparative analyses of the effects of StpA and StpB in lymphocyte transformation by recombinant HVS might help in future to delineate the role of cellular interaction partners and the influence of additional viral factors on lymphoma induction by subgroup A and B isolates of HVS.

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