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217 BioRad).

218 **Electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared from
219 the BJAB and BCBL1 cell lines. Cy5-labeled double-stranded D1-A (-143 to -125;19
220 nt) oligonucleotide (5'-Cy5- aagtgtattaaggtggact -3'/ 5'-Cy5-agtccaccttaatacactt-3') was
221 prepared (Greiner) and non-labeled D1-A (5'- aagtgtattaaggtggact -3'/
222 5'-agtccaccttaatacactt-3') and mutants (Mut1; 5'-aagtgtattaaggtggaaa-3'/
223 5'-tttccaccttaatacactt-3'), Mut2; 5'-ccccgtattaaggtggact-3'/ 5'-agtccaccttaatacgggg-3',
224 Mut3; 5'-aagtcccctaaggtggact-3'/ 5'-agtccaccttaggggactt-3', Mut4;
225 5'-aagtgtatccccgtggact-3'/ 5'-agtccacggggatacactt-3', Mut5; 5'-aagtgtattaagccccact-3'/
226 5'-agtggggcttaatacactt-3', Mut6; 5'-aagtgtattaaggtggccc-3'/ 5'-gggccaccttaatacactt-3')
227 were prepared. For the gel shift assay, 10 μg of nuclear extract was mixed with 1.25
228 pmol of the Cy5 fluorescent tag probe and incubated for 30 min at 25°C in a binding
229 buffer (100 mM HEPES pH7.9, 5 mM $MgCl_2$, 2.5 mM DTT, 250 □g/ml BSA, 20%
230 Ficoll). Cold D1-A probe (20X) or mutant probes (20X) were used for competition
231 experiments. In the case of supershift analysis, 1μg of each specific antibody against
232 IRF-4 (Sigma, St. Louis, MO) or RFX-5 (Sigma) or OCT-1 (MBL Life Science, Japan)
233 was added to the respective reaction. Normal mouse IgG was used as a control reaction.
234 The reacted mixtures were separated on a 0.25×TBE-4% acrylamide gel. The
235 fluorescent image was obtained with a ChemiDoc™ MP imaging system (BioRad).

236 **Chromatin immunoprecipitation (ChIP).** 1×10^7 BJAB or BCBL-1 cells were
237 crosslinked with 1% formaldehyde at room temperature for 10 min, then glycine was
238 added to a final concentration of 0.125M. After incubation for 5 min, the cells were
239 washed in PBS and harvested by centrifugation. The cells were lysed in 1 ml ice-cold

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240 low salt lysis buffer (10 mM Tris-HCl pH 7.8, 10 mM NaCl, 0.1 mM EDTA) with
241 protease inhibitors. The nuclei were spun down harvested and resuspended in 300 □l
242 high salt buffer (50 mM Tris-HCl, pH 7.8, 500 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$,
243 20% glycerol) with protease inhibitors. After incubation on ice for 15 min the samples
244 were added 700 □l low salt buffer with protease inhibitors. Chromatin was sheared to
245 about 500 bp fragments using a Tomy Ultrasonic Disruptor (UD-201) set to output at 2
246 and duty at 60, 200 times on ice. The chromatin from 10^7 cells was incubated with 2 □g
247 normal mouse IgG and 20 □l Protein G Sepharose™ (GE Healthcare) and incubated for
248 half an hour at room temperature to remove nonspecific binding. The nonspecific
249 binding removed samples were then incubated with 5 □g primary antibody (either normal
250 mouse IgG or a mouse monoclonal anti OCT-1 antibody [MBL]) and 20 □l Protein G
251 Sepharose™ at room temperature for 1 hour on a rotator. The Sepharose™ was washed
252 6 times with a wash buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5%
253 vol/vol of NP-40, 1.0% vol/vol of Triton X-100). The chromatin/immune complexes
254 were incubated in a 200 □l reversal buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 300
255 mM NaCl) at 37°C with 20 □g RNaseA (Roche) for 30 min, then add 10 □l Proteinase K
256 (Roche) at 65°C over night. DNA was extracted once with Monofas DNA purification
257 kit (GL Sciences) according to the manufacturer's direction. Then, the DNA was
258 amplified with primers: ANGPT1-ups Fw8; 5'-ggaagctttgctatattttagtaggtcagc-3',
259 ANGPT1-ups Fw10 RV1; 5'-caatttgtaagacgatcccgcc-3' which included the D1A region.
260 The amplification was quantified by qPCR using Roche Light Cycler® (Roche
261 Diagnostics).
262

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263 **RESULTS**

264

265 **ANGPT-1 expression in KSHV-infected PEL cell lines and secretion into the culture**
266 **medium.** A previous clinical research study revealed the expression of ANGPT-1
267 mRNA in AIDS-associated KS biopsies (29), and we have shown that the expression of
268 ANGPT-1 in PEL cell lines appeared to be much higher than that in the T-Cell
269 Lymphoma (TCL) and Burkitt lymphoma (BL) cell lines (6). To investigate whether
270 ANGPT-1 was really produced in the PEL cell lines, we measured the secretion level of
271 ANGPT-1 in the culture supernatant of PEL cell lines by ELISA, and observed its cellular
272 expression and localization by immunofluorescence assay (IFA). We generated
273 ANGPT-1-expressing stable cell lines, ANGPT-1-VH/BJAB and ANGPT-1-VH/293,
274 were used as positive controls, concomitantly Lac-Z-VH-expressing cell lines, LacZ-VH
275 cell/BJAB, LacZ-VH/293, were used as negative controls. Since the ANGPT-1-VH and
276 the LacZ-VH were tagged by a V5-Histidine hexamer (VH) at each C terminus, we
277 assessed whether a mouse anti-V5 antibody as well as a mouse anti-ANGPT-1 antibody
278 was valuable for IFA. Comparing to the case with no signal by staining with
279 anti-ANGPT-1 antibody in LacZ-VH/BJAB cells, diffused patterns were detected by
280 anti-V5 antibody (Fig. 1A). In ANGPT-1-VH/BJAB cells, both anti-V5 antibody and
281 anti-ANGPT-1 antibody could detect dotted spots. In the case of PEL cell lines,
282 ANGPT-1 was stained diffusely in the cytoplasm in KSHV-infected PEL cells (Fig. 1B).
283 The bigger dot patterns seen in ANGPT-1-VH/BJAB cells may have been due to massive
284 expression of ANGPT-1-VH in the cells.
285 To examine whether ANGPT-1 was really secreted into the culture medium of PEL

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286 cell lines, we measured ANGPT-1 levels in the culture supernatant by ELISA. We could
287 detect the secreted ANGPT-1 in ANGPT-1-VH/293 and ANGPT-1-VH/BJAB cells, which
288 served as positive controls (Fig. 1C). Likewise, we measured secreted ANGPT-1 levels
289 in the culture supernatant of PEL and Burkitt cell lines, and these levels were clearly
290 higher in the culture medium of KSHV-infected cell lines such as BC1, BC3, BCBL1 and
291 TY1 than in KSHV-uninfected cell lines such as BJAB, Raji and Namalwa. On the
292 other hand, ANGPT-2 was detected at the same level among these cell lines, the values
293 were relatively low and did not reach 1ng/ml (Fig. 1E). Collectively, these data
294 confirmed that the KSHV-infected PEL cell lines expressed a high level of ANGPT-1, but
295 the KSHV-uninfected cell lines—including the EBV-infected Burkitt cell lines (Fig.
296 1C)—did not express a high level of ANGPT-1, as shown by the gene expression profile
297 analysis and the data by RT-PCR (6). In addition, we detected ANGPT-1 from the
298 culture supernatant of KSHV-infected cell lines by immunoprecipitation followed by
299 immunoblot analysis, in which ANGPT-1 in the medium was immunoprecipitated with a
300 mouse anti-ANGPT-1 antibody and then identified by Western blotting analysis with the
301 same antibody (Fig. 1D). Together, these results strongly suggested that *ANGPT-1*
302 expression should be up-regulated in KSHV-infected PEL cells.
303
304 **The luciferase activity of the regulatory region of *ANGPT-1* is enhanced in**
305 **KSHV-infected PEL cells.** It has been reported that KSHV regulates host gene
306 expression by encoding several viral genes, including *vIRF3*, *vIRF7*, and a viral protein
307 kinase (ORF36) (12, 33). To understand how *ANGPT-1* was up-regulated in the
308 KSHV-infected PEL cell lines and how KSHV contributed to *ANGPT-1* gene expression,

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309 we constructed *ANGPT-1* promoter-luciferase reporter constructs (-898 ~ +490 Luc,
310 where +1 is the transcription start site and +490 is A in ATG; the ANGPT-1 translation
311 initiation codon) and measured their transcriptional activity. These constructs were
312 tested in KSHV-infected/uninfected cell lines and BJAB-BAC36 cells, which contained a
313 full KSHV genome in the bacterial artificial chromosome (26, 37). The activation of the
314 reporter gene containing the regulatory region of *ANGPT-1* relative to the control was
315 more remarkably observed; i.e., 5.1-fold greater in BC3, 15.1-fold greater in BCBL1 and
316 23.5-fold greater in TY1, and it was activated with 4.1-fold greater in BJAB-BAC36, but
317 not in BJAB cells (Fig. 2). These data suggested that *ANGPT-1* should be specifically
318 up-regulated in KSHV-infected PEL cells.
319
320 **A 19 nt element is responsible for *ANGPT-1* up-regulation.** In the experiments
321 described above, it was shown that *ANGPT-1* expression was enhanced in KSHV
322 infected-PEL cell lines where KSHV could govern the gene expression profile.
323 Therefore, in order to determine the responsible regulatory region for *ANGPT-1*
324 transcription in KSHV-infected PEL cell lines, we made several deletion
325 mutant-luciferase reporters (-898, -579, -178 and +1 Luc as a control) and tested them in
326 the KSHV-infected cell lines BCBL1 and TY1. Two of the deletion reporters (-579 and
327 -178) showed a comparable transcriptional activity to the full deletion reporter (-898)
328 both in BCBL1 and TY1 (Fig. 3A).
329 We next prepared four further deletion mutant-luciferase reporters: -143, -110, -84
330 and -58. Among them, we found a profound decrease in activity between -143 and -110
331 nt (Fig. 3B), and thus we focused on this segment and examined whether it contained a

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332 responsive element that was sufficiently activated in KSHV-infected PEL cells.

333 We divided the 34 nt segment into two regions named D1 (the former half; -143 to

334 -110) and D2 (the latter half; -130 to -102) and put them in front of the -58 reporter,

335 which showed very low activity close to the basal level by only the proximal promoter

336 (Fig. 4A). Compared to the control-luciferase reporter (-58), the transcriptional activity

337 of D1 was clearly higher than that of D2 (Fig. 4A).

338 We further designed two reporter plasmids named D1-A (the former half of the D1;

339 -143 to -125) and D1-B (the latter half of the D1; -128 to -110) by dividing the D1

340 segment, and put them at the front of the -58 reporter (Fig. 4B). Luciferase assay

341 showed that D1-A had a high transcriptional activity similar to that of the positive control

342 (-143) (Fig. 4B). Collectively, these analyses suggested that the 19 nt element (-143 to

343 -125) could be responsible for *ANGPT-1* expression regulation in KSHV-infected PEL

344 cell lines.

345

346 **Nuclear proteins in PEL cells bind to the responsive element (D1-A; -143 to -125) for**

347 ***ANGPT-1* transcriptional regulation.** The above results suggested that the D1-A

348 element (-143 to -125) is necessary and sufficient for *ANGPT-1* expression in

349 KSHV-infected PEL cell lines. To investigate whether any nuclear factor of

350 KSHV-infected PEL cells is involved in the up-regulation of *ANGPT-1*, we performed

351 EMSA to identify the factors binding to the 19 bp element by EMSA. We prepared a

352 Cy5-labeled 19 bp probe, and attempted to react it with nuclear proteins of the

353 KSHV-infected or uninfected cell lines. As shown in Fig. 5A, the specific binding

354 factor contained in the nuclear extracts from KSHV-infected cells (BCBL1) could bind to

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355 the 19 nt element specifically (lane 3). In fact, there appeared to be two specific shifted
356 bands, band1 and band2 (Fig. 5A). These two bands were competed with a non-labeled
357 19 bp probe (D1-A) to some extent, compared to the bands seen in an SP1-binding
358 consensus competitor (lanes 4 &11). The following experiment was done with various
359 kinds of mutated competitors (lanes 5-10). In the initial experiments, we were careful
360 of the overlapped region between D1-A and D1-B and prepared a non-labeled Mut1
361 probe and thereafter a series of mutant probes Mut2 to Mut6. Using these probes, we
362 performed a competition analysis of EMSA (Fig. 5A; lanes 5-10 and 5B). As shown in
363 Fig. 5A, D1-A, Mut1, Mut2, Mut5, and Mut6 competed but not Mut3 and Mut4. These
364 data suggested that a core nuclear factor-binding site in D1-A should be the sequence
365 mutated in Mut3 and Mut4, and thus the sequence should be 5'-GTATTAAG-3'. On the
366 other hand, band2 competed uniformly with these mutated competitors, except the
367 SP1-binding consensus. Therefore, we focused our attention on band1, which exhibited
368 more specific competition.

369 In addition, we prepared reporter constructs with these mutants and checked their
370 luciferase activities (Fig. 5B). The results showed that Mut3 and Mut4 lost the activity,
371 which further confirmed that the mutated region in Mut3 and Mut4 should be important
372 for the nuclear factor binding that promoted *ANGPT-1* expression in the KSHV-infected
373 PEL cell lines. Since Mut5 also lost the activity, some sequences mutated in Mut5 could
374 be involved in the activity without affecting the potential nuclear factor binding.

375

376 **OCT-1 binds to the responsive element of *ANGPT-1*.** We also tried to identify which
377 factor bound with the D1-A element by cutting out the shifted band1 in EMSA followed

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378 by mass-spectrometry (MS). Since Octamer-binding proteins (OCT-1), interferon
379 regulatory factor 4 (IRF-4) and RFX-5 were among the nominated candidates, supershift
380 EMSA analysis using these antibodies was performed. Compared with the anti-IRF-4
381 and the anti-RFX-5 antibody, respectively, the anti-OCT-1 antibody showed a supershift
382 (Fig. 6A), even in a dose-dependent manner (Fig. 6B). Thus it appears that OCT-1
383 could be involved in the up-regulation of *ANGPT-1*. We then checked whether the
384 expression of OCT-1 could up-regulate the luciferase activity of the -58 reporter through
385 the D1-A element. Co-transfection of OCT-1 with the D1-A reporter resulted in higher
386 luciferase activity than did co-transfection with the -58 reporter, in a dose-dependent
387 manner (Fig. 6C).

388 In order to confirm whether OCT-1 was involved in the up-regulation of *ANGPT-1 in*
389 *vivo,* we performed chromatin immunoprecipitation (ChIP) followed by qPCR using
390 mouse normal IgG or an anti-OCT-1 antibody. As shown in Fig. 6D, ChIP enrichment
391 of the region including D1A was about 10-folds higher with the OCT-1 antibody than
392 with normal IgG in case of BCBL1 (Fig. 6D right). This result suggested that OCT-1
393 was involved in in the up-regulation of *ANGPT-1* i*n vivo* by binding with this region,
394 though relatively high background was observed, but no ChIP enrichment was revealed
395 with the OCT-1 antibody in case of BJAB (Fig. 6D left).

396 Taken together, these results show that *ANGPT-1* could be up-regulated in
397 KSHV-infected cells through OCT-1 binding to the D1-A element.

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398 **DISCUSSION**

399

400 Kaposi's sarcoma is recognized as a highly angiogenic and inflammatory tumor (25).
401 It is well understood that KSHV infection could affect the expression and secretion of
402 several cytokines and growth factors, including vascular endothelial growth factor
403 (VEGF) and its receptor (VEGFR), matrix metalloproteinases, and angiopoietins,
404 associated with KS (6, 25-28). The angiopoietin family genes contain four members
405 (ANGPT-1 to 4) and they bind to a tyrosine kinase receptor, Tie1 or Tie2 (34, 35, 38),
406 expressed in endothelial cells. ANGPT-1 is predominantly secreted from mesenchymal
407 cells, and acts as an agonist of Tie2 signaling, whereas Angpt-2 acts as an antagonist (34,
408 35). Activation of Tie2 affects several signal pathways, including PI3K, endothelial
409 nitric oxide synthase (eNOS) and growth factor receptor-bound protein 2 (GRB2).
410 These could regulate the proliferation, migration and survival interaction of endothelial
411 cells (39). Both ANGPT-1 and ANGPT-2 could lead to the development of
412 lymphangiogenesis around islets of Langerhans and pancreaticβ-cell tumors in transgenic
413 mice, although tumor angiogenesis was detected only in ANGPT-2 transgenic mice, and
414 not in ANGPT-1 transgenic ones (40). It is suggested that ANGPT-1 and ANGPT-2
415 collaborate in regulating pathologic lymphangiogenesis and angiogenesis via different
416 pathways (39, 40).

417 In HUVEC cells, KSHV infection could up-regulate the expression of *ANGPT-2,*
418 with activation of the promoter via AP-1 and ETS-1 transcriptional factors, which
419 involves the ERK, JNK, and p38 MAPK pathways (26). KSHV induced rapid release
420 of ANGPT-2 from the Weibel-Palade bodies of endothelial cells (27). In addition, in

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421 KSHV-infected lymphatic endothelial cells, *ANGPT-2* was also up-regulated by two
422 KSHV lytic genes, *v-IL-6* and *v-GPCR*, through the MAPK pathway (28). ANGPT-1
423 could not be detected in KSHV-infected endothelial cells, though it was reported to be
424 expressed at a low level in KS tumor (29). In KSHV-infected PEL cell lines, *ANGPT-1*
425 expression was clearly higher than in the other lymphatic cell lines at the RNA level (6),
426 and thus KSHV and/or the KSHV-infected PEL cell environment could evoke some
427 advantageous effects for the *ANGPT-1* expression. On the other hand, an ANGPT-1
428 receptor, Tie-2, was not expressed in KSHV-infected PEL cell lines, and thus the
429 over-produced ANGPT-1 would not cause an autocrine effect on these cells but
430 probably on endothelial cells, a putative origin of KS.

431 Here we provided quantitative evidence that in KSHV-infected PEL cells, ANGPT-1
432 was localized in the cytoplasm and secreted into the culture medium (Fig. 1), as we
433 showed in our previous DNA array analysis (6). ANGPT-1 overexpression was unique
434 in KSHV infected PEL cell lines, since KSHV uninfected Burkitt cell lines originated
435 from B cell lineage as PEL did not show high expression of ANGPT-1. As for
436 ANGPT-2 that was highly expressed in KSHV infected endothelial cells (26), there was
437 no difference in production among these cell lines. In order to understand how
438 *ANGPT-1* was up-regulated, reporter constructs were prepared with the regulatory
439 sequence. The regulatory region up to -898 of *ANGPT-1* showed high activity in all
440 KSHV-infected PEL cell lines, but not in BJAB cells. No significant enhancement in
441 BJAB cells, while containing a full KSHV genome in the bacterial artificial chromosome
442 (BJAB-BAC36), could indeed enhance the transcriptional activity. Consistently, a -898
443 Luc reporter showed high activity in BJAB cells containing the full length KSHV

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444 genome in the BAC (37, 41).

445 A series of deletion mutant analysis of the *ANGPT-1* regulatory region showed that

446 the D1-A region (-143 to -125; AAGTGTATTAAGGTGGACT) should be necessary and

447 sufficient for up-regulation of *ANGPT-1* transcriptional activity in KSHV-PEL cell lines

448 (Fig. 4). In addition, we demonstrated that some nuclear proteins of KSHV-infected

449 cells could bind with the D1-A element but not uninfected cells (Fig. 5A). Mutation

450 analysis of the D1-A sequence further revealed that the core element should be

451 5'-GTATTAAG-3' and some sequences 5'-GTGG-3' that were mutated in Mut5 might be

452 involved, since the Mut5 reporter plasmid did not show up-regulated activity (Fig. 5B),

453 although the fragment competed with a factor binding to the D1-A probe in EMSA (Fig.

454 5A).

455 Among the factors nominated (IRF-4, RFX-5, and OCT-1) based on the results of

456 mass-spectrometry, *IRF-4* and *RFX-5* are well expressed in the KSHV-infected PEL cell

457 lines (6, 42). While overexpression studies of IRF-4 and RFX-5 and supershift analyses

458 using specific antibodies against IRF4 and RFX-5, respectively, did not show that these

459 factors were involved in *ANGPT-1* up-regulation in the KSHV-infected PEL cell lines, an

460 anti-OCT-1 antibody caused supershift (Fig. 6A and 6B). The 5'-GTATTAAGG-3'

461 sequence is partially contained in 5'-GyATGnTAATGArATTCy-3', which is bound with

462 OCT-1 interacting with the HSV VP16 transactivator (the underlined nucleotides are

463 completely matching). Furthermore, ChIP with an OCT-1 antibody followed by qPCR

464 enriched the D1A region compared with the same experiment using normal mouse IgG.

465 Collectively, these data suggested that OCT-1 should be involved in *ANGPT-1*

466 up-regulation in the KSHV-infected PEL cell lines *in vitro* and *in vivo*.

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467 As noted, KSHV-infection and/or the PEL cell environment could affect *ANGPT-1*
468 up-regulation. We tested whether several well-known latent genes of KSHV, such as
469 *LANA*, *v-CYC*, *v-FLIP* and *v-IRF3,* were involved in the up-regulation. However, none
470 of the genes tested showed any activation on the D1-A reporter construct (data not
471 shown). Thus, currently we do not know how KSHV is involved in this up-regulation,
472 though KAPOSIN has not been tested due to cloning failure. Such environment
473 governing PEL might be achieved epigenetically in the long course of PEL establishment.
474 Namely, in the PEL cells, *ANGPT-1* regulatory region could be more accessible for
475 OCT-1 compared to that of KSHV uninfected Burkitt cells or others.
476 Since the ANGPT-1 receptor, Tie2 is not expressed on the PEL cell lines (data not
477 shown), ANGPT-1 overproduction from PEL cells should not promote PEL cell growth
478 itself but affect pathophysiology such as exudation/effusion of body fluid and
479 angiogenesis in AIDS patients. In order to know what ANGPT-1 does in the AIDS
480 patient, it will be interesting to measure ANGPT-1 in PEL harboring AIDS patients with
481 or without KS and to evaluate their prognosis.
482 Taking these results together, we showed that *ANGPT-1* was activated through an
483 element termed D1-A in which the core element was 5'-GTATTAAG-3' and this core
484 element was bound with OCT-1 in the KSHV-infected PEL cell lines. Though further
485 investigation must be done to understand the mechanism, ANGPT-1 overproduction may
486 profoundly affect the pathophysiology of patients with PEL under AIDS setting.
487

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652 **FIGURE LEGENDS**

653

654 **Fig. 1.** ANGPT-1 expression in KSHV-infected PEL cells and ANGPT-1 and ANGPT-2
655 secretion into the medium. (A and B) ANGPT-1-VH/BJAB and LacZ-VH/BJAB cells
656 were stained with an anti-V5 antibody (A left; green staining) and an anti-ANGPT-1
657 antibody (A right; green staining) followed by anti-mouse IgG conjugated with
658 Alexa488™. KSHV-infected BC1, BC3, BCBL1, and TY1 cells were stained with an
659 anti-ANGPT-1 antibody followed by anti-mouse IgG conjugated with Alexa488™. (C)
660 ELISA of ANGPT-1 secreted into the medium. The KSHV-infected BC1, BC3, BCBL1,
661 and TY1 cell lines, and the KSHV-negative BJAB, Raji, and Namalwa cell lines were
662 cultured until reaching a density of 10^6/ml, and then the supernatant was harvested and
663 ANGPT-1 was measured by ELISA (28). ANGPT-1-VH/BJAB and ANGPT-1-VH/293
664 were used as positive controls, while LacZ-VH/BJAB and LacZ-VH/293 were used as
665 negative controls. (D) Detection of secreted ANGPT-1 by Western blot. The
666 supernatant from ANGPT-1 was immunoprecipitated with an anti-ANGPT-1 antibody and
667 protein-G Sepharose™ and subjected to Western blotting analysis. IN, input; IP,
668 immunoprecipitated. 1. LacZ-VH/BJAB; 2. ANGPT-1-VH/BJAB; 3. BC1; and 4. TY1.
669 (E) Detection of secreted ANGPT-2 with an ELISA kit. KSHV infected PEL cell lines
670 and EBV infected or uninfected Burkitt cell lines were tested as ANGPT-1.

671

672 **Fig. 2.** Transcriptional activity of the *ANGPT-1* regulatory region in the KSHV-infected
673 PEL cell lines. PEL cell lines, KSHV-negative BJAB cells, and BAC36/BJAB cells
674 were transfected with a luciferase reporter plasmid that contained -898 to +490 bp of the

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675 *ANGPT-1*, and a luciferase reporter plasmid that contained +1 to +490 bp was used as a
676 control. Forty-eight hours after transfection, the lysate was prepared and the luciferase
677 activity was measured as described in the MATERIALS AND METHODS. Data are
678 shown as the fold activities compared to the activity of a basal reporter; the activity of +1
679 construct was set at 1 fold.
680

681 **Fig. 3.** Deletion analyses of the transcriptional activity of the *ANGPT-1* regulatory
682 region in KSHV-infected PEL cells. (A) BCBL1, TY1 cells were transfected with
683 luciferase reporter plasmids containing a deleted upstream regulatory region of *ANGPT-1*
684 as indicated (from -898, -579, or -178 to +490), and a control plasmid (from +1 to +490).
685 The luciferase activity was measured at 48 h after transfection as described in the text.
686 (B) BCBL1 and TY1 cells were transfected with luciferase reporter plasmids containing
687 the further deleted upstream *ANGPT-1* regulatory region as shown (from -178, -143, -109,
688 -84, or -58 to +490), and a control reporter plasmid (from +1 to +490), and the luciferase
689 activity was again measured at 48 h after transfection. Data were shown as the fold
690 activities compared to the activity of a basal reporter; the activity of +1 construct was set
691 at 1 fold.
692

693 **Fig. 4.** Identification of the minimal enhancing element of the *ANGPT-1* in the
694 KSHV-infected PEL cell lines. (A) Schematic illustration of two DNA fragments, D1
695 (from -143 to -110, oblique shaded rectangle) and D2 (from -130 to -102, white
696 rectangle) and the reporter analysis of the D1 and D2 fragments. D1 and D2 fragments
697 were inserted into the region upstream of the -58 to +490 reporter as the sense orientation

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698 and the luciferase activity was measured. (B) Schematic drawing of the deletion
699 constructs with D1, D2, D1-A (from -143 to -125) and D1-B (from -128 to -110) regions.
700 D1-A and D1-B fragments were inserted into the region upstream of the -58 to +490
701 reporter, and transfected into BCBL1 cells. Luciferase activity was measured. Data
702 are shown as the fold activities compared to the activity of a basal reporter; the activity of
703 -58 construct was set at 1 fold.
704
705 **Fig. 5.** Electrophoretic mobility shift assay (EMSA) of the D1-A element. (A) Left;
706 Schematic illustration of the D1-A region and mutants of the D1-A region. Right;
707 Nuclear extracts of KSHV-infected PEL cells (BCBL1) were mixed with D1-A labeled
708 with Cy5. 20 times of each cold mutant probe was used for the competition experiment.
709 Minus (–) shows no NE or no competitor, respectively. In lane 2, 10 μg BJAB NE was
710 input and in lane 3 to 11, 10 μg BCBL1 NE was used. (B) Mutant plasmids were
711 constructed by inserting each mutant element into the region upstream of the -58 to +490
712 reporter which contains the *ANGPT-1* promoter. Data are shown as the fold activities
713 compared to the activity of a basal reporter; the activity of -58 construct was set at 1 fold.
714
715 **Fig. 6.** A supershift EMSA using antibodies. (A) Nuclear extracts of KSHV-infected
716 PEL cells (BCBL1) were mixed with D1-A labeled with Cy5. 1000 ng of each
717 antibodies was used for the supershift experiment. Anti-IgG (1000 ng) was used as a
718 negative control. (B) 10 ng, 100 ng, and 1000 ng of anti-OCT-1 were prepared for the
719 dose-dependent experiment. (C) pCGN OCT-1 (none, 0.3, 0.9, 1.5 □g) was transfected
720 with the D1A reporter or -58 nt reporter (0.3 □g) into BCBL1 cells; the ratio of luciferase

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721 activity of D1A/-58 of each combination was calculated and the ratio of D1A/-58 without
722 pCGN OCT-1 was set at 1 fold. (D) Quantitative PCR (qPCR) assays were performed
723 with ChIP DNA to test enrichments of the D1A region in the *ANGPT-1* promoter. DNA
724 by ChIP either with normal mouse IgG or an anti-OCT-1 antibody was amplified with
725 primers around the D1A region. Amount of the amplified product was quantified with
726 qPCR. Quantified ratio was calculated to the input DNA of the ChIP samples. Thus,
727 values under the graph shows ratio of amplified amount with each IgG to that of the input
728 values (ChIP enrichment) and fold enrichment values was calculated by setting normal
729 IgG values at 1 (Fold enrichment).