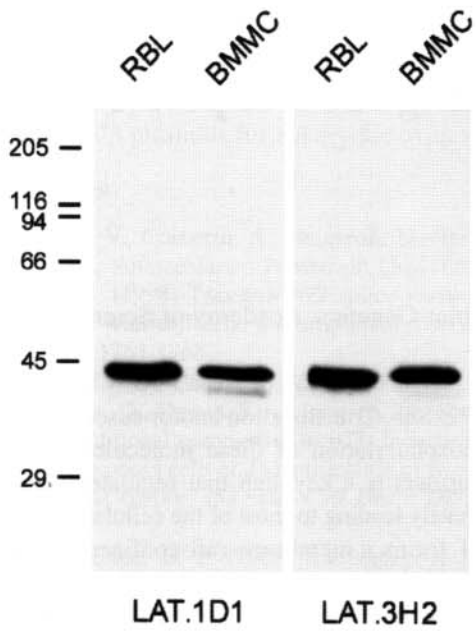

Fig. 1. RBL-2H3 cells (2×10^5) or mouse bone marrow-derived mast cells (BMMC; 3×10^5) were lysed in a lysis buffer (10 mM NaH_2PO_4, pH 7.2, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonylfluoride), resolved by SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane. The membrane was developed with anti-LAT antibodies as indicated, followed by anti-IgG conjugated to horseradish peroxidase. The positions of molecular mass standards are indicated on the left in kDa.

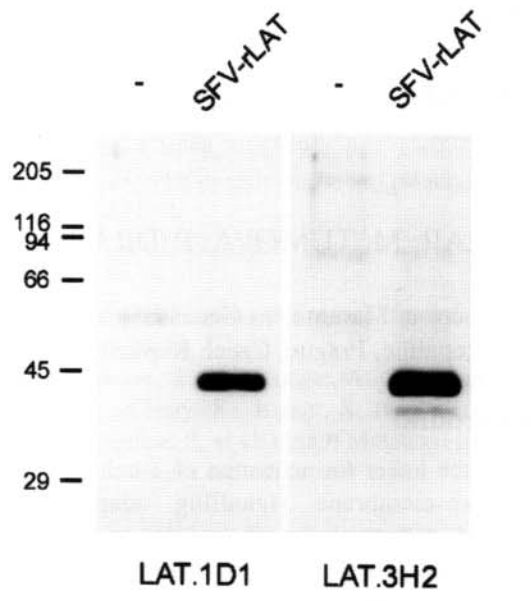

Fig. 2. COS cells were mock-infected (-) or infected with recombinant Semliki Forest Virus containing cDNA of rat *LAT* (SFV-*rLAT*), lysed and analysed by immunoblotting as in Fig. 1. The positions of molecular mass standards are indicated on the left in kDa.

cell lines previously reported to express LAT (data not shown). The reactivity was similar on gels under reducing conditions. The same band was recognized in the light density fractions of the RBL-2H3 mast cell line after sucrose gradient ultracentrifugation (not shown). Both antibodies detected a ~40 kDa protein in lysates of COS cells transiently expressing rat LAT (Fig. 2).

In immunofluorescence assays, the antibodies gave a uniform membrane staining pattern on detergent-permeabilized and paraformaldehyde-fixed RBL cells, but not on non-permeabilized cells as revealed by confocal microscopy (data not shown). The antibodies could also be used for LAT immunoprecipitation under non-denaturing conditions. Since LAT undergoes tyrosine phosphorylation upon MIRR engagement, we tested whether the phosphorylation would affect the reactivity of the antibodies. Fig. 3 shows that the antibodies immunoprecipitated and detected the same amount of LAT from resting as well as antigen-stimulated mast cells, although the phosphorylation of LAT increased dramatically in antigen-stimulated cells. These data indicate that the antibodies can be used for analysis of LAT under various physiological conditions.

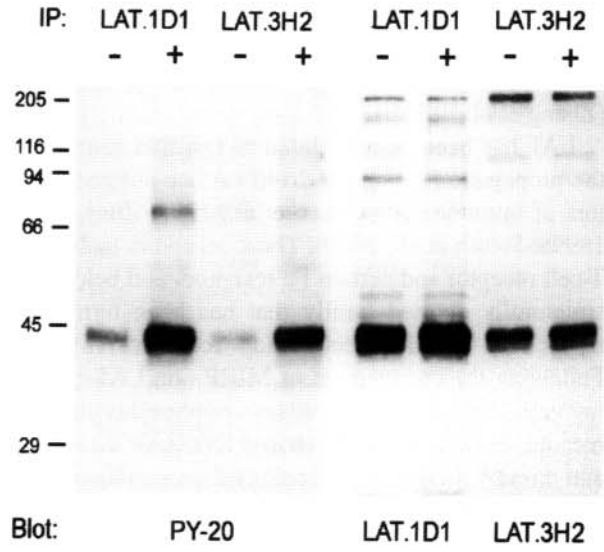

Fig. 3. RBL-2H3 cells (10^7) were sensitized with trinitrophenyl (TNP)-specific IgE and then stimulated (+) or not (–) with 1 μg/ml TNP-bovine serum albumin conjugate. After 5 min at 37°C the cells were lysed as described in Fig. 1 and subjected to immunoprecipitation with anti-LAT antibodies covalently bound to Ultralink Biosupport Medium (Pierce, Rockford, IL). The immunoprecipitates were eluted and analysed by immunoblotting either with anti-phosphotyrosine antibodies (PY-20, Transduction Laboratories, Lexington, KY), or with anti-LAT antibodies (LAT.1D1, LAT.3H2). The positions of molecular mass standards are indicated on the left in kDa.

Properties

Antibodies LAT.1D1 (IgG_{2a}, pI 7.8–8.3) and LAT.3H2 (IgG_1, pI 6.1–6.2) can react with mouse and rat LAT under denaturing as well as non-denaturing conditions.

Acknowledgement

We thank Hana Mrázová and Romana Budovičová for technical support.

References

Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E., Weiss, A. (1998) LAT is required for TCR-mediated activation of PLCγ1 and the Ras pathway. *Immunity* **9,** 617-626.

Gringhuis, S. I., Leow, A., Papendrecht-Van Der Voort, E. A., Remans, P. H., Breedveld, F. C., Verweij, C. L. (2000) Displacement of linker for activation of T cells from the plasma membrane due to redox balance alterations results in hyporesponsiveness of synovial fluid T lymphocytes in rheumatoid arthritis. *J. Immunol.* **164,** 2170-2179.

Saitoh, S., Arudchandran, R., Manetz, T. S., Zhang, W., Sommers, C. L., Love, P. E., Rivera, J., Samelson, L. E. (2000) LAT is essential for FcεRI-mediated mast cell activation. *Immunity* **12,** 525-535.

Wange, R. L. (2000) LAT, the linker for activation of T cells: a bridge between T cell-specific and general signaling pathways. *Science's Signal Transduction Knowledge Environment.* www.stke.org/cgi/content/full/OC_sigtrans;2000/63/re1.

Weber, J. R., Orstavik, S., Torgersen, K. M., Danbolt, N. C., Berg, S. F., Ryan, J. C., Tasken, K., Imboden, J. B., Vaage, J. T. (1998) Molecular cloning of the cDNA encoding pp36, a tyrosine-phosphorylated adaptor protein selectively expressed by T cells and natural killer cells. *J. Exp. Med.* **187,** 1157-1161.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., Samelson, L. E. (1998a) LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* **92,** 83-92.

Zhang, W., Trible, R. P., Samelson, L. E. (1998b) LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity* **9,** 239-246.

Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., Long, E. O., Love, P. E., Samelson, L. E. (1999a) Essential role of LAT in T cell development. *Immunity* **10,** 323-332.

Zhang, W., Irvin, B. J., Trible, R. P., Abraham, R. T., Samelson, L. E. (1999b) Functional analysis of LAT in TCR-mediated signaling pathways using a LAT-deficient Jurkat cell line. *Int. Immunol.* **11,** 943-950.