Distinct Isoforms of the CD45 Protein-tyrosine Phosphatase Differentially Regulate Interleukin 2 Secretion and Activation Signal Pathways Involving Vav in T Cells*

(Received for publication, March 16, 1995, and in revised form, August 25, 1995)

Daniel W. McKenney, Hideo Onodera, Linda Gorman, Toshihide Mimura, and David M. Rothstein§

From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06529-8029 and the Third Department of Internal Medicine, Faculty of Medicine, Tokyo University, Tokyo 113, Japan

The CD45 family of transmembrane protein-tyrosine phosphatases plays a crucial role in the regulation of lymphocyte activation by coupling activation signals from antigen receptors to the signal transduction apparatus. Multiple CD45 isoforms, generated through regulated alternative mRNA splicing, differ only in the length and glycosylation of their extracellular domains. Differential distribution of these isoforms defines subsets of T cells having distinct functions and activation requirements. While the requirement for the intracellular protein-tyrosine phosphatase domains has been documented, the physiological role of the extracellular domains remains elusive. Here we report the generation of CD45-antisense transfected J urkat T cell clones that lack CD45 or have been reconstituted to uniquely express either the smallest, CD45(0), or the largest, CD45(ABC), isoform. These cells exhibited marked isoform-dependent differences in IL-2 production and tyrosine phosphorylation of cellular proteins, including Vav after anti-CD3 stimulation. These results demonstrate that the distinct CD45 extracellular domains differentially regulate T cell receptor-mediated signaling pathways. Furthermore, these findings suggest that alterations in CD45 isoform expression by individual T cells during thymic ontogeny and after antigen exposure in the periphery directly affects the signaling pathways utilized.

Activation of resting T lymphocytes through the T cell receptor (TCR) requires expression of the CD45 family of transmembrane protein-tyrosine phosphatases (PTPases) (1, 2). CD45 has been shown to regulate the basal activity of the Fyn and Lck protein-tyrosine kinases (PTKs) by dephosphorylation of their respective regulatory carboxyl-terminal tyrosine residues (3–7). However, it is not clear that these are CD45's sole functions. For example, new evidence suggests that CD45 can also dephosphorylate certain PTK substrates, such as the TCR "in a 270-base pair segment (from the P1 transcription initiation site to the initiation codon) was amplified by polymerase chain reaction from genomic CD45 DNA (clone LCA.512, from Dr. H. Saito, Dana-Farber Cancer Institute, Boston, MA (16)). Polymerase chain reaction primers incorporating unique restriction sites allowed ligation into the RCSR plasmid vector (27) in an antisense orientation, generating the AS-CD45 plasmid vector. To generate CD45 cDNA con-

*This work was supported by Donaghue Foundation Fellowship Award DF93–010 (to D. W. M.) and National Institutes of Health Grant DK02011 (to D. M. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§To whom correspondence should be addressed: Dept. of Internal Medicine, Section of Nephrology, LCI 208, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06529-8029. Tel.: 203-785-6738; Fax: 203-785-7068.
1 The abbreviations used are: TCR, T cell receptor; PTPase, protein-tyrosine phosphatase; PTK, protein-tyrosine kinase; mAb, monoclonal antibody; GAM, goat anti-mouse; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.

24949
Differential Regulation of Signaling by CD45 Isoforms

Structures in the sense orientation, the pSP5R8s LCA.1 and LCA.6 constructs (28), encoding the smallest, CD45R(0), and largest, CD45R(ABC), isoforms, respectively, (from Dr. M. Streuli, Dana-Farber Cancer Institute), were modified by removing a 5′ SacI-SphiI segment, to reduce overlap between AS-CD45 and these CD45 cDNAs to 40 base pairs.

Cell Lines and Transfections—The Jurkat human leukemic CD4+ T cell line was maintained in RPMI 1640 media containing 10% fetal calf serum, 4 mM l-glutamine, and 50 μg/ml gentamycin at 37°C in humidified atmosphere with 5% CO2. Cells were transfected by electroporation, and G418-resistant colonies were screened for loss of CD45 by immunofluorescence. Two of the CD4+ CD45+ clones (J-AS-1 and J-AS-2) were selected for further study. J-AS-1 was co-transfected at a 10:1 ratio with DNA constructs for either the CD45R(0) or CD45R(ABC) isoform plus the pPGK-hyg plasmid (29) encoding Hygromycin B resistance. Resistant colonies (G418 and Hygromycin) were screened by immunofluorescence for CD45 expression as well as for expression of appropriate cell surface markers described below. Clones were sorted (fluorescence activated cell sorting) as necessary to obtain similar CD45 and CD4 expression, as described below.

Antibodies, Immunofluorescence, Phenyotyping, and Cell Sorting—Immunofluorescence analysis was performed as described previously (26). Mouse anti-human mAbs reactive with CD2, CD3, CD4, CD28, CD45, and CD45RA were obtained as ascendents (generously provided by Dr. Chikao Morimoto, Dana-Farber Cancer Institute) or as phycocyanin conjugates (from Molecular Immunology, Hialeah, FL), anti-CD45RO (obtained with the kind permission of Dr. Peter Beverly, University College Hospital, London), and goat anti-mouse IgG-FITC (from Southern Biotechnology, Birmingham, AL). Cell phenotype was routinely monitored for these markers using a BD FACSTAR IV (10,000 cells/sample). Cell sorting was performed using a BD FACSTAR IV, after staining by direct or indirect immunofluorescence, as described (26). Fluorochrome conjugates were dialyzed to remove unbound dyes prior to use.

Rabbit polyclonal Ab to Vav was developed by immunization of rabbits with a synthetic peptide containing residues 575–594 of (mouse) Vav. Immunoprecipitation of a 95-kDa band with the antiserum was specifically blocked by addition of the immunizing peptide to the lysate before immunoprecipitation (data not shown).

IL-2 Secretion—10⁶ cells/well in triplicate flat bottom 96-well tissue culture plates were stimulated with anti-CD3 (purified OKT3) at the concentrations indicated, essentially as described (27). Cross-linking was performed with goat anti-mouse (GAM) (at a 1:1 ratio with anti-CD3). For anti-CD3 × anti-CD28 cross-linking, anti-CD3 (1 μg/ml) and anti-CD28 (1:400 dilution of ascites) were cross-linked with GAM (2 μg/ml). PMA (1 ng/ml) was added to all wells as described (30). 24-h supernatants were assayed for IL-2 concentration using CTLL-2 cells.

IL-2 units were determined in each assay by comparison to a standard curve using human rIL-2 (generously supplied by Chiron Corp., Emeryville, CA). The data were normalized to the response of J urkat cells transfected with plasmid CD45R0 (1 μg/ml). A 1:1 AS-CD45 antisense (RNA and encoding either the smallest isoform, denoted CD45(0), lacking alternative exons, or the largest isoform, denoted CD45(ABC),isoforms,respectively(from Dr. M. Streuli, Dana-Farber Cancer Institute, La Jolla Research Institute, La Jolla, CA, or from UBI, Lake Placid, NY). All immunoblots were developed using horseradish peroxidase-conjugated secondary reagents and developed using ECL (Amersham).

RESULTS AND DISCUSSION

Like peripheral T cells, the Jurkat human T cell leukemia line normally expresses CD45 at high levels, and individual cells express multiple isoforms simultaneously (17–19). To examine the role of CD45 and its individual isoforms, free from the potential confounding influences of unknown mutations, we directly targeted endogenous CD45 expression by stable transfection of a plasmid construct (AS-CD45) expressing an antisense RNA directed at a 270-base pair region of genomic CD45 just upstream from the coding region. (Fig. 1A). Of the several independent CD45+ colonies selected and subcloned, two, denoted J-AS-1 and J-AS-2, were selected on the basis of CD4 expression comparable to that of parental Jurkat. (Fig. 1B). J urkat expresses high levels of total CD45 and lower levels of both the smallest CD45 isoform (CD45R0) and the largest (two) isoforms which contain exon A (CD45RA). J-AS-1 and -2 lack detectable CD45 expression either on the surface or in the cytoplasm (Fig. 1, B and C).

J-AS-1 was then stably transfected with CD45 cDNA constructs modified to minimize overlap with AS-CD45 antisense RNA and encoding either the smallest isoform, denoted CD45(0), lacking alternative exons, or the largest isoform, denoted CD45(ABC), which includes all three alternative exons. These isoforms best exemplify differential distribution on T cell subsets having distinct functions and activation preferences (19, 21, 23). Each of the CD4+ clones arising expressed solely the transfected CD45 isoform by both immunofluorescence and by immunoblotting (Fig. 1, and C). The independent CD45(ABC) transfectants isolates (J[ABC]-1, -2, and -3) and two CD45(0) transfected isolates (J[0]-1 and -2) were selected for further study, based on CD45 expression and wild-type levels of CD3. The clones were then sorted to obtain stable populations expressing similar levels of CD4 and CD45. When matched for their surface expression, J[0] and J[ABC] clones expressed identical levels of CD45 by immunoblotting, indicating no inherent differences in the relative distribution of intra- and extracellular CD45 (data not shown). Although total CD45 expression was lower in the transfectants, the expression of individual CD45(0) and CD45(ABC) isoforms by J[0] and J[ABC] cells, respectively, was similar to their expression in wild-type cells. The expression of CD3, CD2, and CD28 was nearly identical in each of the cell lines (Fig. 1B and data not shown).

Current evidence indicates that CD45 regulates the activity of proximal components of the signaling apparatus such as the Src family PTKs, Lck and Fyn, and, presumably, their substrates (3–6). First, TCR/CD3-induced IL-2 secretion, which depends on the coordinated activation of multiple transcription factors (34), was examined as an integrated measure of such signaling events. The dose-response curve to anti-CD3 (Fig. 2A)
CD45 transfectants at both 0.05 and 1.0 lines produced significantly less IL-2 than either Jurkat or the CD45(0) isoform (see Fig. 3). As before, clones expressing CD45(0) secrete wild-type levels of IL-2. As shown, increased CD45 expression by J[ABC] transfectants did not augment IL-2 secretion. Both sorted and unsorted J[ABC] populations averaged just 24% of the wild-type levels of IL-2.

Given differential anti-CD3-induced IL-2 secretion by these cell lines, more proximal signaling events were next examined. Comparison of Lck and Fyn activities by immune complex kinase assays failed to reveal isoform-dependent differences (data not shown). T cell activation is associated with alterations in the tyrosine phosphorylation of a number of cellular proteins. Therefore, we compared the tyrosine phosphorylation of cellular proteins in each cell line before and at various time points after, anti-CD3 stimulation (Fig. 4). Under basal conditions, J-AS-1 consistently revealed hyperphosphorylation of a limited set of bands at ~70–76 kDa and decreased tyrosine phosphorylation of several other bands (~105, ~95, and ~50–52 kDa) when compared to J urkat (Fig. 4B).

After anti-CD3 stimulation of J urkat, there was rapid phosphorylation (peaking at 30 s to 1 min) and subsequent dephosphorylation of a number of bands. Although many of the same bands were ultimately phosphorylated (within 5–10 min) after stimulation of J-AS-1 cells, the kinetics were significantly slowed. Furthermore, once phosphorylated, these bands did not undergo dephosphorylation, consistent with decreased action of the CD45 PTPase and perhaps of other cellular PTPases whose activities depend on regulated tyrosine phosphorylation (35, 36).

Re-expression of either the CD45(0) or the CD45(ABC) isoforms generally restored basal and activation-related tyrosine phosphorylation, although the kinetics were somewhat prolonged compared to wild-type J urkat (Fig. 4B). This may reflect the lower overall levels of CD45 expression in these cells. More importantly, direct comparison reveals clear isoform-dependent differences in the relative phosphorylation of several bands. For example, J[ABC] cells consistently exhibited relative hyperphosphorylation of a band at ~95 kDa when compared to J[0] cells.

This prompted a comparison in our cells of the tyrosine phosphorylation of p95 Vav (Vav) which is rapidly and transiently phosphorylated on tyrosine after ligation of the TCR (33, 37), CD28 (38), or upon the binding of IL-2 to its receptor (39). While the exact function of this proto-oncogene product in signal transduction is unclear, gene ablation studies document the important role of Vav in the activation and proliferation of mature lymphocytes as well as in the normal developmental

Fig. 1. A, schematic representation of DNA inserts encoding antisense CD45 RNA (AS-CD45), CD45(0), and CD45(ABC) isoforms. The arrow under each insert indicates the direction of transcription once inserted into the respective expression vectors. Sense and antisense constructs have minimal overlap. B, representative immunofluorescence analysis of cell surface expression of various markers on J urkat, CD45 (J-AS-1), and single CD45 isoform transfectants expressing either the CD45(0) isoform (J[0]-1) or the CD45(ABC) isoform (J[ABC]-1). Isotype-matched negative controls are depicted as dotted lines. The x and y axes represent log fluorescence and cell number, respectively. C, anti-CD45 immunoblotting of whole cell lysates from: Raji (human B cell line), J urkat, CD45 (J-AS-1), CD45(0) transfectants (J[0]-1 and -2), and CD45(ABC) transfectants (J[ABC]-1 and -2). 300–19 (mouse pre-B cell) is shown as a negative control. Arrows on left indicate previously established human isoforms at 220, 205, 190, and 180 kDa (17). Lower M, bands represent immature forms, not yet glycosylated at O-linked sites (17). Arrows at right indicate nonspecific bands present in control 300–19 cells.

Reveals that, in contrast to J urkat, the CD45 (J-AS) cell lines secreted minimal IL-2 in response to all doses of anti-CD3 tested. Furthermore, no enhancement was seen after co-stimulation by cross-linking anti-CD3 and anti-CD4 (data not shown). Reconstitution with the CD45(0) isoform resulted in wild-type levels of IL-2 secretion after stimulation with anti-CD3 (0.005 μg/ml). In contrast, the CD45(ABC) transfected cell lines produced significantly less IL-2 than either J urkat or the CD45(0) transfectants at both 0.05 and 1.0 μg/ml anti-CD3, secreting at maximum, 30% of wild-type levels. Increasing the anti-CD3 dose to 5 μg/ml had no additional effect on IL-2 secretion by any of the cell lines (data not shown). However, at lower doses of anti-CD3 (0.005 μg/ml), transfectants expressing either individual isoform secrete much less IL-2 than J urkat, possibly owing to the lower levels of overall CD45 expression. Stimulation with anti-CD2 gave overall results similar to those observed above (not shown).

Similar responses by J-AS-1 and each of its single isoform-constituted derivatives after stimulation by Ab-mediated cross-linking of CD3 and CD28 (Fig. 2B), or with PMA plus ionomycin (Fig. 2C), documents similar inherent capacity of each cell line to secrete IL-2 when the proximal signaling machinery, or the requirement for CD45 (30), are bypassed, respectively.

Thus, after stimulation with anti-CD3 (at 0.05 to 1 μg/ml), IL-2 secretion by J[0] transfectants is not significantly different from wild-type cells, despite 6–7-fold lower CD45 expression. Nonetheless, it is possible that decreased IL-2 secretion by J[ABC] cells compared to J[0] cells is due to their somewhat lower levels of CD45 expression. To rule out this possibility, we sorted J[ABC] clones to obtain CD45 expression equal to that of J[0] cells and then compared IL-2 secretion by these cell populations after anti-CD3 stimulation (see Fig. 3). As before, clones expressing CD45(0) secrete wild-type levels of IL-2. As shown, increased CD45 expression by J[ABC] transfectants did not augment IL-2 secretion. Both sorted and unsorted J[ABC] populations averaged just 24% of the wild-type levels of IL-2.

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
expansion of lymphocyte precursors in the marrow and thymus (40, 41).

Basal Vav tyrosine phosphorylation was minimal but detectable in each of our cell lines (Fig. 5A). Anti-CD3 stimulation consistently induced significantly greater tyrosine phosphorylation of Vav within 1 min, in Jurkat and particularly in all three J[ABC] transfectants compared to either of the two J[0] transfectants or the CD45- AS-1 cells. Reprobing the same membrane with anti-Vav antisera confirmed similar loading of Vav protein in each lane (Fig. 5B). These differences are not secondary to altered kinetics, since the same pattern is observed 4 min after anti-CD3 stimulation, at which time phosphorylation of Vav in Jurkat and single-isoform transfectants is decreasing (data not shown and Ref. 33).

Our results are the first to demonstrate that signaling pathways utilized by the TCR are differentially regulated by the extracellular domain of distinct CD45 isoforms. Stimulation of the TCR leads to the phosphorylation of a number of cellular proteins including Vav. Although the signaling pathways involving Vav have not yet been clarified, Vav contains an array of signaling and DNA-binding motifs, including SH2 and SH3 domains, a Dbl domain, and a helix-loop-helix, which all appear to be involved in the generation of downstream signals (33, 42-45). Activation-related tyrosine phosphorylation directs SH2-mediated interactions between Vav and several other signaling molecules. Thus, Vav has been shown to associate with Shc, Grb2, ZAP-70, phosphatidylinositol 3-kinase (p85), CD19, VAP-1, and several other uncharacterized bands through SH2 and/or SH3-mediated interactions after activation of B or T lymphocytes (37, 46-48).

How different CD45 isoforms might regulate this pathway remains speculative. Particular CD45 isoforms might directly dephosphorylate Vav or could differentially regulate the activ-
Differential Regulation of Signaling by CD45 Isoforms

In conclusion, our results indicate that the regulated expression of distinct CD45 isoforms in different developmental and functional subsets of T cells may impose preferential utilization of particular TCR-mediated signaling pathways. Alterations in CD45 isoform expression by individual T cells in response to thymic selection or peripheral antigen exposure, may consequently allow that cell to respond to TCR ligation using a different subset of signals. We speculate that the delivery of these different signals to the cell nucleus might have a significant influence on cell differentiation, the expression of functional repertoire, or in allowing T cells to “fine-tune” their responsiveness. A more complete understanding of these differences is likely to have important implications for signal transduction and for the interpretation of the highly regulated expression of CD45 isoforms in lymphocytes.

Acknowledgments—We are grateful to Dr. Tomas Mustelin and Dr. Alfred Bothwell for their critical reading of this manuscript, Drs. Yoshihiko Nogima, Chris Rice, and Toshiaki Tanaka for reagents and helpful advice, and to Drs. Amnon Altman, Brian Drucker, Chikao Morimoto, Haruo Saito, and Michel Streuli for generously providing various mAbs and CD45 genomic segments and cDNAs.

REFERENCES
1. Pingel, J. T. & Thomas, M. L. (1989) Cell 58, 1055-1065
2. Koretzky, G. A., Picus, J., Thomas, M. L. & Weiss, A. (1990) Nature 346, 66-68
3. Mustelin, T., Pessa-Morikawa, T., Auto, M., Gassmann, M., Andersson, L., Sahlen, C. G. & Burn, P. (1992) Eur. J. Immunol. 22, 1173-1178
4. Mustelin, T., Coggeshall, K. M. & Altman, A. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 6302-6306
5. Cahir-McFarland, E. D., Hurley, R. T., Pingel, J. T., Setton, B. M., Shaw, A. & Thomas, M. L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1602-1606
6. Hurley, T. R., Hyman, R. & Setton, B. (1993) Mol. Cell Biol. 13, 1651-1656
7. Ostergaard, H. L., Shackelford, D. A., Hurley, R. T., Johnson, P., Hyman, R., Setton, B. M. & Trowbridge, I. S. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 8959-8963
8. Furakawa, T., Itoh, M., Krueger, N. X., Streuli, M. & Saito, H. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10328-10332
9. Schirren, B., Schoenhaut, D., Bruyens, E., Koretzky, G., Eckerskorn, C., Wallich, R., Kirchgesнер, H., Sakorafas, P., Labkovsky, B., Ratnoffo, S. & Meuer, S. (1994) J. Biol. Chem. 269, 29102-29111
10. Koretzky, G. A., Kehnert, M. A., Keddie, T. & Weiss, A. (1992) J. Immunol. 149, 1138-1142
11. Volarevic, S., Pessa-Morikawa, T., Auto, M., Weissman, A. M. & Thomas, M. L. (1993) Science 260, 544-547
12. Hovis, R. R., Donovan, J. A., Musci, M. A., Moto, D. G., Goldman, F. D., Ross, S. E. & Koretzky, G. A. (1993) Science 260, 544-546
13. Doria, D., Sap, J., Silverman, O., Schiessinger, J. & Weiss, A. (1994) EMBO J. 13, 4002-4010
14. Streuli, M., Hall, R. L., Saga, Y., Schlossman, S. F. & Saito, H. (1987) J. Exp. Med. 166, 1548-1566
15. Ralph, S. J., Thomas, M. L., Morton, C. C. & Trowbridge, I. S. (1987) EMBO J. 6, 1251-1257
16. Hall, L. R., Streuli, M., Schlossman, S. F. & Saito, H. (1988) J. Immunol. 141, 3778-3787
17. Rudd, C. E., Morimoto, C., Wong, L. L. & Schlossman, S. F. (1987) J. Exp. Med. 166, 1758-1773
18. Rothstein, D. M., Sohen, S., Daley, J. F., Schlossman, S. F. & Morimoto, C. (1990) Cell Immunol. 129, 449-467
19. Rothstein, D. M., Saito, H., Streuli, M., Schlossman, S. F. & Morimoto, C. (1992) J. Biol. Chem. 267, 7139-7147
20. Tedder, T. F., Clement, L. T. & Cooper, M. D. (1985) J. Immunol. 134, 2983-2988
21. Smith, S. H., Brown, M. H., Rowe, D., Callard, R. E. & Beverley, P. C. L. (1986) Immunology 58, 63-70
22. Tedder, T. F., Cooper, M. D. & Clement, L. T. (1985) J. Immunol. 134, 2989-2994
23. Morimoto, C., Letvin, N. L., Distaso, J. A., Aldrich, W. R. & Schlossman, S. F. (1985) J. Immunol. 134, 1508-1515
24. Akbar, A. N., Terry, L., Timms, A., Beverley, P. C. L. & Janossy, G. (1988) J. Immunol. 140, 2171
25. Wallace, V. A., Fang-Leung, W. P., Timms, E., Gray, D., Kishihara, K., Loh, D. Y., Penninger, J. & Mak, T. W. (1992) J. Exp. Med. 176, 1657-1663
26. Rothstein, D. M., Yamaoka, A., Schlossman, S. F. & Morimoto, C. (1991) J. Immunol. 146, 1175-1183
27. Tanaka, K., Kamoeoka, J., Yaron, A., Schlossmann, S. F. & Morimoto, C. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4586-4590
28. Streuli, M., Morimoto, C., Schriever, M., Schlossman, S. F. & Saito, H. (1988) J. Immunol. 141, 3910-3914
29. te Riele, H., Maandag, E. R., Clarke, A., Hooper, M. & Berns, A. (1990) Nature 348, 649-651
30. Koretzky, G. A., Picus, J., Schultz, T. & Weiss, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2037-2041
31. Rothstein, D. M., da Silva, A., Sugita, K., Yamamoto, M., Prasad, K. V., Morimoto, C., Schlossman, S. F. & Rudd, C. E. (1993) Int. Immunol. 5, 409-418
Differential Regulation of Signaling by CD45 Isoforms
Distinct Isoforms of the CD45 Protein-tyrosine Phosphatase Differentially Regulate Interleukin 2 Secretion and Activation Signal Pathways Involving Vav in T Cells

Daniel W. McKenney, Hideo Onodera, Linda Gorman, Toshihide Mimura and David M. Rothstein

J. Biol. Chem. 1995, 270:24949-24954.
doi: 10.1074/jbc.270.42.24949

Access the most updated version of this article at http://www.jbc.org/content/270/42/24949

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 33 of which can be accessed free at http://www.jbc.org/content/270/42/24949.full.html#ref-list-1