A Cell Surface ADP-ribosyltransferase Modulates T Cell Receptor Association and Signaling*

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ART-1, a cell surface ADP-ribosyltransferase, is imbedded in the membrane by a glycosylphosphatidylinositol anchor. Function of this enzyme in mouse T lymphocytes is to transfer ADP-ribose groups from NAD to arginine residues, exposed on the extracellular domain of cell surface molecules. As a consequence, T cell responses are modulated. To explore the precise action of the enzyme, the T cell lymphoma EL-4 was transfected with the ART-1 gene, and its effects were examined. It is shown that ART-1 ADP-ribosylates distinct cell surface molecules, causing inhibition of T cell receptor signaling, concomitant to suppression of p56lck kinase activation. These effects are explained by failure of T cell receptors and co-receptors to associate into a contiguous and functional receptor cluster.

Post-translational protein modifications constitute a central mechanism in cellular signal transmission. Although this has been long known in case of intracellular signaling pathways, it has not yet been documented for signal transmission on the outer cell membrane. That such regulatory mechanisms might exist is suggested by the presence of cell surface enzymes with specificity for peptides in the extracellular milieu. However, proof that these enzymes regulate cell surface receptor function by modifying them is still lacking. We recently described an ecto-enzyme on mouse cytotoxic T cells, named ART-1, that functions as an ADP-ribosyltransferase, attaching ADP-ribose groups to arginine residues of cell surface molecules (1). As a consequence, T cell functions such as cytolytic activity and cell proliferation are inhibited (1, 2). Here we explore the function of this enzyme in a stable transfectant generated from lym-

MATERIALS AND METHODS

Treatment of Cells with NAD, Assays for IL-2 Secretion, and Inositol Phosphate Generation—EL-4 cells were incubated in 1 mM NAD for 3 h at 37 °C, seeded on 104 cells/well into 96-well flat bottom plates in complete DMEM, and then stimulated with 125 ng/ml phorbol ester (PMA) and 100 μM calcium ionophore (CIO). Receptor ligation was induced with 2 μg/ml anti-CD3 (500A2) and 10 μg/ml anti-CD28 (37.51) (PharMingen, San Diego, CA). Culture supernatants were assayed 20 h later for IL-2 by enzyme-linked immunosorbent assay (PharMingen, San Diego, CA). To induce inositol phosphates, cells were labeled in complete DMEM medium with 30 μCi/ml [3H]inositol (ICN, Costa Mesa, CA) for 4 h (107 cells/ml). During the last 2 h, 1 mM NAD was added. After washing, cells were resuspended in complete DMEM medium containing 1 mM NAD and incubated for 1 h at 37 °C. Cells (107 cells/ml) were activated by adding anti-CD3 (500A2, 2 μg/ml) and anti-CD28 (37.51, 10 μg/ml), followed by incubation at 37 °C for 10 min. Cell pellets (107 cells) were lysed and analyzed for inositol phosphates as described (2).

Cell Labeling with 32P-NAD, Immunoprecipitation, Immunoblotting, and Kinase Assays—32P-NAD-labeled cells were assayed for labeled proteins by polyacrylamide gel electrophoresis as described (1). Immunoprecipitations of crude cell lysates prepared in 1% Nonidet P-40 were performed as described (4) using the following antibodies: anti-LFA-1 (3G8), anti-CD2 (L293), anti-CD3ε (500A2), anti-CD4 (7G12), anti-CD43 (S7), anti-CD44 (IM7), anti-CD45 mAb (30F11.1) (PharMingen, San Diego, CA), anti-CD3ζ (6B10, Zymed Laboratories Inc., South San Francisco, CA) (3), anti-CD3γ, anti-CD3ζ, anti-TCRα, and anti-TCRγ (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots were performed as described (4), using the following antibodies: anti-CD3ε, anti-CD28 (PharMingen, San Diego CA), anti-CD3ζ, anti-p56lck mAb, anti-p59fyn mAb, and rabbit anti-ZAP70 (Santa Cruz Biotechnology, Inc.). Blots were developed by the ECL method (Amersham Pharmacia Biotech). Assays for autophosphorylation kinase activities were performed as described (4, 5) on either nonactivated cells or cells activated by both anti-CD3ε and anti-CD28 mAb for 10 min at 37 °C. The following antibodies were used to precipitate kinases: anti-p56lck mAb (lck-3A5, Zymed Laboratories Inc., San Francisco, CA), anti-p59fyn mAb, anti-ZAP70 (Santa Cruz Biotechnology, Inc.).

Fluorescent Confocal Microscopy—Cells were treated with 1 μM NAD for 3 h at 37 °C and then incubated with 10 μg/ml anti-CD3emAb (500A2) on ice for 30 min. After washing, the cells were resuspended in PBS containing 10 μg/ml rhodamine-labeled cross-linking secondary goat anti-hamster antibodies and incubated at 37 °C for 15 min. After washing with ice-cold PBS containing 0.1% NaN3, cells were either stained with 10 μg/ml FITC-labeled anti-CD45 mAb (50F11.1, PharMingen, San Diego, CA) or PBS containing 0.1% NaN3 for 30 min on ice or permeabilized with 1% saponine and stained with rabbit anti-human lck antibody (Upstate Biotechnology, Inc., Lake Placid, NY) diluted in PBS containing 0.1% NaN3 for 30 min on ice, followed by incubation with FITC-labeled goat anti-rabbit antibody (Jackson, West Grove, PA). After fixation with 4% paraformaldehyde, cells were spread onto glass slides, mounted, and examined by confocal microscopy.

RESULTS AND DISCUSSION

To examine the function of ART-1, its gene was expressed in a mouse T cell lymphoma lacking this enzyme (6). EL-4 cells were chosen because they possess TCRs whose ligation induces IL-2 secretion. Fig. 1A shows that PMA and CIO stimulate secretion of large quantities of IL-2 in EL-4 as well as its ART-1 transfectant C17. Addition of ART-1 substrate, NAD, at concentrations as high as 1000 μM exerts little effect on IL-2 secretion in either cell line. The high NAD concentration is physiologically relevant, because cell lysis caused by cytotoxic lymphocytes could result in local concentrations of this magnitude. The failure then of high NAD concentrations to inhibit cytokine secretion demonstrates that NAD is not toxic.

To examine whether ART-1 and its substrate have effects on...
CIO (C17 cells were treated with NAD and then stimulated with PMA and EL-4 and phosphates in C17 cells induced by CD3/CD28 ligation. Anti-CD3 was precipitated, which precipitates both CD3 complexes and therefore the intact CD3 complex, among those CD4, CD8, LFA-1, CD43, CD45, and CD44 (16–18). The demonstration therefore (Fig. 2A) that several of these molecules, i.e.

receptor-mediated cell activation, cells were incubated with anti-CD3 and anti-CD28 to induce receptor ligation and IL-2 secretion. It is seen in Fig. 1B that NAD has no effect in EL-4 cells, but there is a significant suppression of IL-2 secretion in C17 cells. To show that this effect depends on cell surface ADP-ribosyltransferase activity, C15, a transfectant expressing the ART-2/R66.1 gene, and therefore only one-fifteenth of cell surface protein complexes of CD28 activity of CD17 was used: In C15 cells no suppressive effect of NAD on receptor-induced IL-2 secretion was demonstrable (data not shown). Therefore, ART-1 transfectants expressing high enzyme activity are sensitive to NAD-mediated regulation. The ART-1 enzyme effect likely occurs on the level of receptor-mediated cell activation, raising the question of whether the enzyme modifies CD3 and/or CD28 and thereby inhibits signal transmission. To find out, cells were incubated with 32P-NAD, and cell lysates were analyzed for kinase activities, phosphorylation of p59 and p56 (Fig. 2, B, C–E). A similar result is seen with ZAP70 but not with phospholipase C-γ1, generation of inositol phosphates, and mobilization of calcium (5–10). To examine whether these early events in TCR signaling are regulated by ART-1, the induction of inositol phosphates and activation of TCR-associated protein phosphokinases was assayed. It is seen in Fig. 1C that NAD has no effect on induced levels of inositol phosphates in EL-4 cells, but there is a very strong inhibition in C17 cells. To examine the activation of TCR-associated protein phosphokinases, auto-phosphorylation of p56 and p59, and ZAP70 was assayed (5, 11, 12). It is seen in Fig. 2 (C, D, and E) that in exponentially growing EL-4 and C17 cells not stimulated by receptor ligation, NAD has no effect on kinase activities, although there is some suppression of p56 autophosphorylation in C17 cells. When cells are activated by receptor ligation a significant increase in p56 and somewhat smaller increase in p59 and ZAP70 autophosphorylation is seen (Fig. 2, C, D, and E). These increases are not affected by NAD in EL-4 cells. In C17 cells, however, NAD suppresses p56 autophosphorylation activity, induced by receptor ligation, to the level of nonstimulated controls (Fig. 2C). A similar result is seen with ZAP70 but not with p59 (Fig. 2, D and E), hence ART-1 action involves an early step in receptor signaling, i.e. a reaction preceding the generation of inositol phosphates and p56 autophosphorylation.

Circumstantial evidence suggests that activation of p56 in is the consequence of cell surface receptor rearrangements in the contact zone between T cell and antigen presenting cell. This reorganization culminates in formation of a receptor cluster between the two cells, which serves to stabilize cell contact and facilitates contact between p56 and CD45, which activates p56 by dephosphorylation (13–15). Antibody-mediated co-capping and precipitation experiments have shown that many co-receptors are part of the TCR cluster, among those CD4, CD8, LFA-1, CD43, CD45, and CD44 (16–18). The demonstration therefore (Fig. 2A) that several of these molecules, i.e.
CD45, LFA-1, CD43, and CD44 are ADP-ribosylated, raises the intriguing possibility that inhibition of TCR signaling is caused by interference of ADP-ribosylated co-receptors with formation of a functional signal transmitting TCR cluster.

This possibility was investigated by examining antibody-induced cap formation on a single cell level as a model for T cell APC interaction. Cells were incubated with anti-CD3, followed by a rhodamine-conjugated second antibody and examined by fluorescent confocal microscopy. Results in Fig. 2A show that CD3 ligation in the absence of NAD induces very large caps in EL4 and C17 cells. To visualize the association of TCRs with \( p56^{\text{ck}} \), permeabilized cells were reacted with anti-\( p56^{\text{ck}} \) and a fluorescein-conjugated secondary antibody. It is seen that \( p56^{\text{ck}} \) co-localizes with CD3 in the cap. Virtually identical results are seen in EL4 cells, incubated with NAD. In C17 cells, however, the presence of NAD inhibits formation of the cap, and only small aggregates of CD3 and \( p56^{\text{ck}} \) molecules are discernable. Therefore, in NAD-treated C17 cells there is inhibition of formation of a cap, containing CD3 and \( p56^{\text{ck}} \) molecules. These effects are also demonstrable, albeit less dramatically, under conditions in which receptor clustering is induced with one antibody only (Fig. 1), mediating receptor cross-linking via engagement with Fc receptors on adjacent cells.

Results in Fig. 2C had shown that NAD inhibits CD3/CD28-mediated activation of the \( p56^{\text{ck}} \) kinase in C17 cells; therefore we predict that the interaction between \( p56^{\text{ck}} \) and CD45 is likely disturbed. The possibility that ADP-ribosylation of CD45 inhibits its phosphatase activity and is therefore responsible for the effect was found not to be the case (data not shown). An alternative explanation is that association of CD45 with the kinase is inhibited. It is seen in Fig. 3B that a CD3 cap that contains CD45 is formed in both EL-4 and C17 cells in the absence of NAD. However, in the presence of NAD cap formation is inhibited in C17 cells, and only small aggregates of CD3 and CD45 are discernable. Therefore, formation of a signal transmitting cap containing CD3, CD45, and \( p56^{\text{ck}} \) does not take place in NAD-treated cells expressing ART-1.

These results provide conclusive evidence that the inhibitory action of NAD in TCR-mediated activation of cells expressing ART-1 occurs at the level of transmembrane signaling. The inability of the TCR complex to transmit signals can now be explained by the failure of ADP-ribosylated co-receptors to aggregate with the TCR to form a functional signaling structure. Our demonstration that ADP-ribosylation of cell surface molecules causes inhibition of transmembrane signaling uncovers an entirely novel mechanism of cell regulation in which cell surface receptors rather than their ligands are modified by an ecto-enzyme. The modification alters the ability of receptors to associate and to form a functional cell surface cluster. Hence, ADP-ribosyltransferases represent a new family of cell surface enzymes able to regulate T lymphocyte function by modulating receptor association and transmembrane signaling.

REFERENCES

1. Wang, J., Nemoto, E., Kets, Y., Kaslow, H. R., and Dennert, G. (1994) J. Immunol. 153, 4048–4058.
2. Nemoto, E., Stuhlman, S., and Dennert, G. (1996) J. Immunol. 156, 85–92.
3. Okamoto, S., Oiga, A., Yu, Y., Russo, E., and Dennert, G. (1998) J. Immunol. 160, 4180–4189.
4. Wang, J., Nemoto, E., and Dennert, G. (1996) J. Immunol. 156, 2819–2827.
5. Samelson, L. E., Phillips, A. F., Luone, E. T., and Klausner, R. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4358–4362.
6. Yu, Y., Okamoto, S., Nemoto, E., and Dennert, G. (1997) DNA Cell Biol. 16, 235–244.
7. Weiss, A. (1995) Cell 73, 209–212.
8. Perlmuter, R. M., Levin, S. D., Appleby, M. W., Anderson, S. J., and Alberola-Iilana, J. (1995) Annu. Rev. Immunol. 11, 481–499.
9. Bolon, J. B., Thompson, P. A., Eisenman, E., and Horak, I. D. (1991) Adv. Cancer Res. 57, 103–149.
10. Sercr, J. P., Karnitz, L., and Abraham, R. T. (1991) J. Biol. Chem. 266, 12135–12139.
11. Weber, J. R., Bell, G. M., Han, M. Y., Pawson, T., and Imboden, J. B. (1992) J. Exp. Med. 176, 373–379.
12. August, A., and Dupont, B. (1994) Biochem. Biophys. Res. Commun. 199, 1446–1473.
13. Koretsky, G. A., Piers, J., Thomas, M. L., and Weiss, A. (1990) Nature 345, 66–68.
14. Volaravi, S., Niklinska, B. B., Burns, C. M., June, C. H., Weissman, A. M., and Ashwell, J. D. (1993) Science 260, 541–544.
15. Oosterhout, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Selton, B. M., and Trowbridge, I. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8959–8963.
16. Shaw, A. S., and Dustin, M. L. (1997) Immunity 6, 361–369.
17. Pardi, R., Inverardi, L., Ragari, C., and Bender, J. R. (1992) J. Cell Biol. 116, 1211–1220.
18. Poggi, A., Pella, N., Cantoni, C., Zocchi, M. R., and Moretta, L. (1996) Int. Immunol. 8, 1947–1953.