Post-translational Modification of CD38 Protein into a High Molecular Weight Form Alters Its Catalytic Properties*

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Human CD38 is a 45-kDa transmembrane protein that acts as a bifunctional ectoenzyme, catalyzing the synthesis of cyclic ADP-ribose (cADPR) from NAD$^+$ and the hydrolysis of cADPR to ADP-ribose. All-trans-retinoic acid (RA) is a potent and specific inducer of CD38 in myeloid cells. In this report, we demonstrate that RA-induced CD38 protein from human myeloid (HL-60) leukemia cells coimmunoprecipitates with another protein of molecular mass ~190 kDa (p190). The p190 protein is localized exclusively in the membranes and is a consequence of post-translational cross-linking of CD38 protein. This conclusion was based on the observations that purified CD38 effectively competes with p190, its accumulation is preceded by the accumulation of CD38, it immunoreacted with three different monoclonal anti-CD38 antibodies on immunoblots, and its peptide map revealed several peptides in common with CD38. Furthermore, CD38 could serve as a suitable substrate for transglutaminase (TGase)-catalyzed cross-linking reactions in vitro, and the accumulation of p190 in RA-treated HL-60 cells is effectively blocked by the presence of TGase-specific inhibitor. The purified p190 showed at least three times more cyclase activity than CD38. Conversely, p190 was at least 2.5-fold less active than CD38 in hydrolyzing cADPR to ADPR. These results suggest that post-translational modification of CD38 may represent an important mechanism for regulating the two catalytic activities of this bifunctional enzyme.

Human CD38 is a 45-kDa type II transmembrane glycoprotein with a short N-terminal cytoplasmic domain and a long C-terminal extracellular domain (1). Its expression is widely used as a phenotypic marker of differentiation and activation in T and B lymphocytes (2). Sequence analysis of the cDNAs encoding murine CD38 (3), human CD38 (1), and ADP-ribosyl cyclase revealed a 24% sequence identity among these proteins (4). ADP-ribosyl cyclase is a 29-kDa cytosolic enzyme, previously identified cyclic nucleotide with potent Ca$^{2+}$ mobilizing activity from intracellular stores and has been suggested to be a regulator of calcium-dependent calcium release (8–12).

Human CD38 antigen and its murine homologues were recently reported to catalyze the conversion of NAD$^+$ into cADPR and the hydrolysis of cADPR to ADP-ribose (13–16). Both of these activities are associated with the extracellular C-terminal region of CD38 (13). The ability of CD38 protein to function as a bifunctional enzyme is likely to contribute to some of its cell-regulatory functions.

Ligation of CD38 with agonistic monoclonal antibodies (mAb) has been shown to stimulate the proliferation of T and B lymphocytes, suggesting that CD38 may be involved in transduction of signals that lead to activation and proliferation of these cells (2). Perturbation in intracellular Ca$^{2+}$ concentrations is known to affect the signals involved in cell growth and proliferation. The observation that CD38 can effectively catalyze the conversion of NAD$^+$ into cADPR, which functions independently from inositol trisphosphate, suggests that CD38 may participate in signal transduction for cell growth and proliferation. However, an extracellular enzymatic role for CD38 poses an interesting topological dilemma. How does the extracellularly produced cADPR help mobilize Ca$^{2+}$ from intracellular pools? One possibility is that CD38 is internalized following its interaction with an as yet unknown ligand. An alternative is that CD38 undergoes post-translational changes in response to some external stimuli and then acts intracellularly. Indeed, ligation of receptors on interaction with specific ligands is a well-known mechanism for subsequent internalization.

In this paper we demonstrate that all-trans-retinoic acid (RA)-induced CD38 protein in HL-60 cells undergoes post-translational modification into a high molecular weight form (p190) and that this post-translational modification of CD38 results in altered cyclase to hydrolase activities.

**EXPERIMENTAL PROCEDURES**

Cdl Culture and Radiolabeling—HL-60, a human myeloblast leukemia cell line, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum, 2 mML-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cells (2 x 10⁷) were cultured overnight in the presence or absence of 0.1 μM RA (supplied by Aroneex Pharmaceuticals, Inc, The Woodlands, TX). Prior to metabolic labeling, the cells were incubated in cysteine-free medium for 30 min and pulsed with [35S]cysteine (50–100 μCi/ml, specific activity >1000 Ci/mmol; Amersham Corp.) for 2 h in the same medium with RA or in fresh medium without RA.

Immunoprecipitation and Electrophoretic Analysis—The metabolized-saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propane sulfonate; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; TGase, transglutaminase; MOPS, 3-(N-morpholinio)propanesulfonic acid; FPLC, fast protein liquid chromatography; NGD, nicotinamide guanine dinucleotide; cADPR, cyclic GDP-ribose.
cally labeled cells (1 × 10⁷) were washed twice with ice-cold phosphate-buffered saline (PBS) containing 10 mM unlabeled cysteine and lysed in lysis buffer (PBS containing 3% CHAPS and 1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4 °C for 20 min. The nuclei were removed by centrifugation (10,000 × g, 20 min), and the cleared lysates were preabsorbed with an irrelevant mAb, followed by the addition of 20 µg of protein A-Sepharose (Bio-Rad). The cell lysates were incubated at 4 °C for 2 h with 5–15 µg of anti-CD38 antibody (IB4 or IB6 mAbs) and then for 15 min with a goat anti-mouse immunoglobulin G (IgG). The immune complexes thus formed were recovered by incubating the mixture with protein A-Sepharose at 4 °C for 2 h. Immunoprecipitates were washed twice, extracted by boiling for 4 min in SDS sample buffer, and subjected to electrophoresis on 10% polyacrylamide gel according to a procedure described elsewhere (18). Purified CD38 protein (10 µg) was included in the labeled cell lysate during immunoprecipitation with anti-CD38 antibody.

To study the kinetics of p190 accumulation, HL-60 cells (2 × 10⁷) were incubated in the presence or absence of RA for 2, 4, 6, 10, or 16 h. Two hours prior to harvesting, the cells were metabolically labeled with [35S]cysteine. The labeled cells were washed twice with ice-cold PBS, lysed, and immunoprecipitated by using anti-CD38 mAb, as already described. The immunoprecipitates were analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) after electrophoresis in SDS-10% polyacrylamide gel.

Isolation of Cell Membranes—The cell pellets were resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 1 mM PMSF. The cells were lysed in this buffer by three freeze-thaw cycles and separated from lysate by centrifugation (3,000 × g, 10 min). The supernatant was recovered and centrifuged at 100,000 × g for 90 min to sediment the membranes (19). The pellets were solubilized in lysis buffer containing 1% CHAPS, 1 mM EDTA, and 1 mM PMSF. Two-hour supernatants were recovered and centrifuged at 100,000 × g. The pellets were solubilized in lysis buffer containing 1% CHAPS, 1 mM EDTA, and 1 mM PMSF.

Isolation of the p190 band—The cell lysates (5–10 mg of protein) from RA-treated HL-60 cells were used for the immunoprecipitation. The cell lysates were probed with anti-CD38 mAb (IB4), as already described. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and the membrane was probed with anti-CD38 mAb (IB4), as already described. Separation of p190 from CD38—The p190 was separated from CD38 in a two-step procedure consisting of anti-CD38 immunoprecipitation followed by gel filtration chromatography. Five milliliters of desalted IgG fraction containing anti-CD38 mAb (4.6 mg of protein/ml) were conjugated to Affi-Gel 10 (Bio-Rad) according to the manufacturer’s instructions. The gel was packed in a column (12 × 1.5 cm) and washed extensively with 0.1 mM MOPS buffer to remove unbound IgG.

The cell lysates were subjected to electrophoresis, transferred onto nitrocellulose membrane, and the membrane was probed with anti-CD38 mAb (IB4), as already described.

RESULTS AND DISCUSSION

Overnight incubation of HL-60 cells with RA (0.1 µM) resulted in a 30-fold higher NADase activity than in untreated control cells (Fig. 1A). A similar increase in NADase activity had been observed earlier in response to RA treatment in HL-60 and other myeloid cells (24–27). The RA-induced increase in NADase activity was associated with a parallel increase in the synthesis and accumulation of CD38 protein. Immunoprecipitation of metabolically labeled HL-60 cells, using a monospecific anti-CD38 mAb (IB4), revealed no significant levels of CD38 protein (45 kDa) in untreated cells (Fig. 1B, lane 1), but treatment of cells with RA (0.1 µM for 18 h) resulted in a marked increase in accumulation of CD38 protein (Fig. 1B, lane 2).

In addition to the expected 45-kDa band, we detected a high level of CD38 protein (45 kDa) in untreated cells (Fig. 1B, lane 3). A similar increase in NADase activity was observed earlier in response to RA treatment in HL-60 and other myeloid cells (24–27). The RA-induced increase in NADase activity was associated with a parallel increase in the synthesis and accumulation of CD38 protein. Immunoprecipitation of metabolically labeled HL-60 cells, using a monospecific anti-CD38 mAb (IB4), revealed no significant levels of CD38 protein (45 kDa) in untreated cells (Fig. 1B, lane 1), but treatment of cells with RA (0.1 µM for 18 h) resulted in a marked increase in accumulation of CD38 protein (Fig. 1B, lane 2).
molecular weight protein (p190) that communoprecipitated with anti-CD38 mAb IB4 from RA-treated HL-60 cell lysates (Fig. 1B, lane 2). Similar bands of 45 and 190 kDa were obtained when two other anti-CD38 antibodies, IB6 (anti-CD38 mAb) and rabbit anti-CD38 peptide, were used for immunoprecipitation (data not shown). These results suggested that CD38 may be an integral part of p190. To determine the relationship between p190 and CD38, we next studied the ability of purified CD38 protein to inhibit immunoprecipitation of radio-labeled bands by anti-CD38 mAb IB4. The inclusion of purified CD38 protein during the immunoprecipitation step almost completely blocked the appearance of 45- as well as 190-kDa radiolabeled bands (Fig. 1B, lane 3). These results suggested that p190 represents a high molecular weight precursor of CD38, a post-translationally modified cross-linked product of CD38, or a protein that is closely associated with CD38 and communoprecipitates along with CD38.

To test this further, we determined the kinetics of CD38 and p190 accumulation. The cell lysates from HL-60 cells after 2, 4, 6, 10, or 16 h of RA treatment were immunoprecipitated with IB4. Results shown in Fig. 2 suggest that accumulation of CD38 protein (45 kDa) precedes the accumulation of p190. A significant accumulation of CD38 protein was evident as early as 4 h after RA treatment (Fig. 2, lane 3). However, a comparable level of p190 accumulation was seen only after 6 h of treatment with RA, and thereafter both the proteins showed a comparable increase and accumulation with time for at least up to 10 h of treatment (Fig. 2, lanes 5 and 6). Since CD38 appeared before p190, the latter cannot serve as a precursor for CD38 protein. On the contrary, p190 may represent a post-translational modified product of CD38 protein.

To test further whether p190 is a post-translational modified product of CD38, we determined the immunoreactivity of p190 to three different anti-CD38 antibodies that recognize different epitopes on the CD38 molecule. The immunoprecipitates (obtained by using the anti-CD38 mAb IB4) from untreated or RA-treated HL-60 cells were fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were then probed with anti-CD38 mAbs (IB4 or IB6) or rabbit anti-CD38 peptide antibody. The results shown in Fig. 3A revealed the presence of two strong immunoreactive bands at 45 and 190 kDa in the immunoprecipitates from RA-treated HL-60 cells when the blots were probed with mAb IB4 (Fig. 3A, lane 2). The other two antibodies, IB6 and anti-CD38 peptide antibody, showed equally strong immunoreactivity with these proteins (45 and 190 kDa) in a Western blot assay (data not shown). The immunoprecipitate from untreated HL-60 cells showed no or very poor immunoreactivity with IB4 (Fig. 3A, lane 1), IB6, or anti-CD38 peptide antibodies. These results strongly suggested that p190 is not a CD38-associated protein but may represent a post-translational modified product of CD38.

To define further the relationship between CD38 and p190, we compared their peptide maps generated by limited proteolysis with V8 Staphylococcus aureus protease. Both CD38 and p190 were digested with V8 protease for various times in SDS-containing buffer as discussed under "Experimental Procedures." Fig. 3B shows an autoradiogram of peptide maps obtained from CD38 and p190 after 0, 2, 4, or 8 h of V8 treatment at a 20:1 substrate:enzyme ratio (w:w). The comparison of CD38 and p190 peptide maps revealed the presence of several common peptide fragments ranging from 7.2 to 39 kDa (Fig. 3B; labeled ▲). Except for the 66-kDa peptide (Fig. 3B; labeled ▼), p190 yielded a peptide pattern identical to that of CD38 following its digestion with the V8 enzyme. It is interesting that the qualitative pattern of peptide fragments did not change significantly even after 8 h of digestion. Similarly, the complete digestion of CD38 and p190 could not be achieved even after prolonged digestion (8 h) with increasing concentrations of V8 protease (data not shown). These results suggest the high specificity of V8 protease for cleaving the peptide bond at a specific site (C-terminal of glutamic acid) under the experimental condition employed in our assay system. Furthermore, the strikingly similar peptide profiles strongly supported the contention that CD38 is an integral part of the p190 protein and may represent a covalently modified product of CD38.

The induction of CD38 is a specific response of HL-60 cells to RA rather than a general response to differentiation (25, 26). A similar specificity has been reported for induction of another enzyme, tissue TGase (28). Thus, treatment of HL-60 cells with RA has been shown to induce a rapid and large accumulation of TGase that was not seen with other agents that induce HL-60 differentiation (28). TGase is a Ca\(^{2+}\)-dependent enzyme that...
catalyzes irreversible cross-linking of proteins by promoting the formation of isopeptide bonds between protein-bound glutamine and lysine residues (29). Since there are 14 glutamine and 19 lysine residues in human CD38 protein, providing a total of 33 potential sites for cross-linking by TGase, it was tempting to speculate that CD38 could be a substrate for TGase.

To test this notion, we next determined the ability of purified CD38 protein to serve as an acyl donor substrate in TGase-catalyzed reactions. Results shown in Fig. 4A suggested that CD38 could indeed serve as a suitable acyl donor substrate for TGase-catalyzed reactions. Incubation of purified CD38 protein with Ca2+ and purified TGase (from guinea pig liver) resulted in an appreciable incorporation of [3H]putrescine. TGase-catalyzed incorporation of putrescine into CD38 was linear for at least up to 10 min of reaction (Fig. 4A) and was completely dependent on the presence of Ca2+. Replacing Ca2+ with EGTA in the reaction mixture fully inhibited the reaction (Fig. 4A), suggesting that the incorporation of putrescine into CD38 protein is mediated by TGase. To our knowledge there is no other Ca2+-dependent process by which putrescine could be incorporated into proteins. The ability of CD38 to serve as substrate for TGase was further tested by studying the post-translational changes, if any, induced by purified TGase. Results shown in
Fig. 5. Isolation of p190 from CD38. Cells (5 x 10^8) were treated overnight with 0.1 μM RA and lysed in CHAPS buffer. The cell extracts were passed through an affinity column of anti-CD38 mAb coupled to Affi-Gel 10. The resulting eluate was extensively dialyzed against 0.1 M Tris-HCl buffer (pH 7.4) and checked for the enzyme activity. The active fractions were pooled, concentrated, and placed in a FPLC Superose 12 HR 10/30 gel filtration column (A). One-milliliter fractions, at 0.5 ml/min flow rate, were collected. The p190 (peak a) and CD38 (peak b) were recovered in elution volumes of 5 and 15 ml, respectively. The NADase activity associated with each fraction was determined (dashed line). B, silver-stained SDS-polyacrylamide gel containing fraction a (lane 2) and fraction b (lane 1) from the Superose 12 HR 10/30 column shown in A.

| Table I | Determination of cyclase and NADase activity in crude cell extracts and purified fractions |
|---------|-------------------------------------------------------------------------------------|
|         | GDP-ribosyl | NAD | GDP-ribosyl |
|         | cyclase | glycohydrolase | cyclase/NADase |
| Untreated | 0.16 ± 0.02 | 0.22 ± 0.02 | 0.72 |
| HL-60 cell lysates | 4.4 ± 0.08 | 6.2 ± 0.16 | 0.71 |
| RA-treated | 14.4 ± 1.44 | 43.8 ± 4.0 | 0.52 |
| CD38 (45 kDa) | 696.0 ± 14.4 | 174.8 ± 2.4 | 4.04 |

*The enzyme activities are expressed as a net change in fluorescence (excitation 300 nm, emission 410 nm) produced by 1.0 μg of protein during 1-h reaction.

The data shown represent the average ± S.D. of three independent observations.

Fig. 4B demonstrate that CD38, in the presence of TGase and Ca^{2+} (Fig. 4B, lane 1), undergoes heavy cross-linking into a high molecular weight form (~190 kDa). TGase-mediated cross-linking of CD38 was completely inhibited when Ca^{2+} was replaced with EGTA in the reaction mixture (Fig. 4B, lane 2). These results suggested that p190 that communoprecipitates with CD38 from RA-treated HL-60 cells (Fig. 1) may be the product of TGase-catalyzed cross-linking of CD38 protein. Indeed, the presence of monodansylcadaverine, a competitive inhibitor of TGase, in culture medium at 50 or 100 μM concentrations (Fig. 4C, lanes 2 and 3, respectively) almost completely prevented the RA-induced accumulation of p190 in HL-60 cells. The inhibitory effect of monodansylcadaverine on p190 accumulation was a specific rather than a trivial toxic effect, since the accumulation of CD38 protein (45 kDa) in RA-treated cells was not significantly affected by the presence or absence of the inhibitor (Fig. 4C).

TGase has been shown to induce post-translational cross-linking of several proteins in tissues and body fluids (30). Of particular interest is the post-translational modification of phospholipase A_2 and interleukin-2. TGase-mediated dimerization of phospholipase A_2 has been shown to result in a 10-fold increase in its catalytic activity with respect to monomeric enzyme (31). Similarly, TGase-catalyzed dimerization of interleukin-2 has been shown to render this protein cytotoxic to oligodendrocytes (32). In view of these observations, it was tempting to determine whether p190 has any altered catalytic functions when compared with CD38 protein.

To test this, we purified p190 from CD38 protein by taking advantage of the fact that the two proteins differ substantially in their molecular masses. p190 and CD38 were isolated from RA-induced HL-60 cells in a two-step procedure, the anti-CD38 immunoaffinity column and size-exclusion chromatography, as described under “Experimental Procedures.” The bound fraction obtained from the immunoaffinity column, when subjected to FPLC gel filtration, yielded two fractions containing NADase activity with elution volumes of 5 ml (Fig. 5A, fraction a) and 15 ml (Fig. 5A, fraction b), respectively. Electrophoresis of the first active fraction (fraction a) on SDS-polyacrylamide gel under reducing conditions revealed the presence of a major band at the 190-kDa position (Fig. 5B, lane 2), whereas the second active fraction (fraction b) showed a single band of 45-kDa size, as determined by silver staining (Fig. 5B, lane 1).

Enzymatic assay of fractions obtained from the size-exclusion chromatography revealed the presence of a low NAD-glycohydrolase activity in the p190 fraction (Fig. 5A). Since CD38 also displays ADP-ribosyl cyclase activity (13–16), it was necessary to determine the cyclase activity in the p190 fraction. For this purpose, we took advantage of a recently described fluorometric assay. This assay is based on the fluorescence properties of cGDPR, produced from a nonfluorescent NGD substrate by Aplysia cyclase and human CD38-catalyzed reaction (22, 26). Moreover, cGDPR, unlike cADPR, is a poor substrate for the hydrodase activity of CD38 (22), and GDP-ribose is not fluorescent. To determine the presence of cyclase activity in the p190 fraction, both p190- and CD38-containing fractions were incubated with 0.1 mM NGD^+ at 37 °C to induce cyclization reaction, and the fluorescence emission was then determined at 410 nm. It was interesting that the p190 fraction (Fig. 5A, fraction a) showed markedly different catalytic properties from the fraction containing CD38 (Fig. 5A, fraction b). The former showed three times more GDP-ribosyl cyclase activity than CD38; the difference in the ratio of cyclase to hydrodase activity was even more striking for the two proteins (Table I). These results further supported the premise that CD38 may become intimately associated with some critical stimulatory subunits under physiological conditions, accounting for not only the enzymatic activity associated with p190 but also the altered cyclase to hydrodase activity. Recent site-directed mutagenesis studies by Tohgo et al. (33) suggested that the two cysteines present in the human CD38 molecule at positions 119 and 201 play a critical role in the synthesis and hydrolysis of cADPR (33). Thus, C_{119K} and/or C_{201E}-mutated CD38 exhibited only ribosyl cyclase activity (33). From these observations, it is tempting to speculate that the preferential cyclase activity associated with p190 may result from the masking of these two cysteine residues in the CD38 molecule. It is also apparent that a region distinct from the active site is involved in post-trans-
FIG. 6. Localization of p190 in the membrane fraction. A, membrane and cytosolic fractions from metabolically labeled RA-treated HL-60 cells were separated by ultracentrifugation (100,000 × g). The fractions were individually immunoprecipitated and analyzed as described in the legend to Fig. 1. Immunoprecipitates from membrane (lanes 1 and 2) and cytosolic (lanes 3 and 4) fractions of untreated (lanes 1 and 3) and RA-treated (lanes 2 and 4) HL-60 cells are shown. B, the Coomassie Blue-stained polyacrylamide gel containing total proteins from the membrane (lanes 1 and 2) and cytosolic (lanes 3 and 4) fractions of untreated (lanes 1 and 3) and RA-treated (lanes 2 and 4) HL-60 cells.

Post-translational Modification of CD38

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