Brief Definitive Report

Involvement of the PPPGHR Motif in T Cell Activation Via CD2

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Summary

Prior studies identified a segment of the CD2 cytoplasmic domain between amino acid (aa) residues 253 and 287 as important in T lymphocyte signal transduction. This region contains two repeats of the sequence motif PPPGHR, thought to form a "cage" structure involved in CD2-mediated signaling. To evaluate this segment, a series of mutant human CD2 molecules were produced by oligonucleotide-directed mutagenesis and inserted into the ovalbumin-specific, I-A^d-restricted murine T-T hybridoma 3DO54.8 using the DOL retroviral system. CD2 M1 (271–272), CD2 M2 (278–279), and CD2 M4 (264–265) mutants replaced the positively charged adjacent aa histidine and arginine (HR) in the wild-type CD2 sequence with aspartic and glutamic acid (DE) at positions 271–272, 278–279, and 264–265, respectively. In addition, a truncation mutant, CD2 M3 (268), containing only 57 of the 117 cytoplasmic aa and terminating before the second PPPGHR sequence, was generated. Stimulation of transfectants CD2 FL, CD2 M1 (271–272), and CD2 M2 (278–279) with anti-$T11_2$ + anti-$T11_3$ antibodies resulted in a rise in cytosolic-free calcium ($[Ca^{2+}]_i$) and subsequent interleukin 2 (IL-2) secretion. In contrast, CD2 M4 (264–265) transfectants could not be activated in either assay. Thus, alteration of histidine 264 and/or arginine 265 within the first PPPGHR motif affects the process of signal transduction via CD2, whereas identical mutations in residues at 271–272 or 278–279 were individually without effect. Consistent with these data, CD2 M3 (268) transfectants were able to generate a detectable amount of IL-2 via CD2 triggering. These data support the notion that the PPPGHR motif at aa 260–265 is important for activation of T lymphocytes via the CD2 molecule.

The CD2 (T11) molecule is a 50–55-kD transmembrane glycoprotein expressed on a vast majority of thymocytes and virtually all peripheral T lymphocytes (reviewed in reference 1). Functional characterization of the human molecule has clearly established that CD2 serves a dual role in mediating T cell adhesion and activation (2–6). Based on microchemical analysis of human CD2, as well as cDNA and/or genomic cloning of mouse, rat, and human molecules, the structure of the CD2 molecule has been well defined (reviewed in reference 1). Human CD2 consists of a 185-amino acid (aa) extracellular domain, a 25-aa transmembrane segment, and a 117-aa cytoplasmic domain rich in prolines and basic residues. By analysis of murine T cell transfectants expressing the human wild-type CD2 cDNA as well as deletion mutants, we and others have previously established that the CD2 cytoplasmic region is necessary for activation via CD2 (7–9). More importantly, we were able to identify a segment of the cytoplasmic domain between aa 253 and 287 necessary for CD2-mediated activation (7). This region contains four histidines at aa positions 264, 271, 278, and 282, and includes two tandemly repeated segments (PPPGHR, aa 260–265 and 274–279). A highly homologous region is found in murine CD2 in a corresponding position as well. To further evaluate the role of this segment for activation via CD2, a series of substitution mutants of human CD2 have been generated by oligonucleotide-directed mutagenesis and expressed in the 3DO54.8 murine T cell line for functional characterization.

Materials and Methods

Construction of Truncated and Mutated Human CD2 Molecules and Transfection into a Murine T Cell Hybridoma. Constructs were generated by oligonucleotide-directed in vitro mutagenesis, as previously described (7). The full-length human CD2 cDNA, PB2 insert (10), was subcloned into the BamHI site of M13mp18. The synthetic oligonucleotides used for mutation are 5′-CAGGCACCTAGTGATGAGCCCCCGCCTCCT-3′ for CD2 M1 (271–272), which changes the wild-type sequence CATCGT (His-Arg) into GATGAG (Asp-Glu) at aa 271–272; 5′-GCCTCCTGGAGATGAGGTTCAGCACCAG-3′ for CD2 M2 (278–279), which changes the wild-type sequence CACCGT (His-Arg) into GATGAG (Asp-Glu) at

aa 278–279); 5′-CATCGTTCCCAGTAACCTAGTCATCGT-3′ for CD2 M3 (268), which changes the wild-type sequence GCA (Ala) into TAA, a stop codon resulting in a CD2 molecule truncated at aa position 268; and 5′-CCACCACCTGGTGATGAGTCCC-AGGCACCT-3′ for CD2 M4 (264–265), which changes the wild-type sequence CATCGT (His-Arg) into GATGAG (Asp-Glu) at aa 264–265. The mutant DNAs were subcloned into the retroviral expression vector DOL and transfected by Ca^{2+} precipitation into $\psi 2$ cell to generate the individual virus stock, as described in detail previously (7). Each virus was then used to infect the murine T cell hybridoma 3DO54.8. The G418-resistant clones were further selected by indirect immunofluorescence for surface CD2 expression and maintained as described (7). A single colony arising from one well in selection media was considered clonal.

Sequence Analysis of Human CD2 cDNAs in the Mutant. DNA fragments corresponding to bp 739–1117 of the human CD2 cDNA (10) were generated by amplifying single-stranded cDNA generated with the 3′ primer of CD2 and reverse transcriptase (Life Sciences, St. Petersburg, FL) from the total cytosolic RNA of each T cell clone by PCR with CD2-specific primers (11). The conditions used to perform PCR were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, for a total of 40 cycles of amplification. The 5′ primer 5′-AGGATCCAACAGAGGAGTCGGAGAAAT-3′ corresponds to bp 739–758 of CD2 cDNA and contains a BamHI restriction enzyme recognition sequence. The 3′ primer 5′-GGGATCCACA-GTGCTTTTTATTGAA-3′ corresponds to bp 1100–1117 of CD2 cDNA and includes a BamHI recognition sequence. The fragments generated were subcloned into the BamHI site of pGEM-4 blue (Promega Biotec, Madison, WI) and subjected to double-stranded DNA sequencing to confirm the presence of the correct mutation for each clone.

Measurement of Cytosolic Ca^{2+} and IL-2 Production. Measurement of cytosolic Ca^{2+} concentrations was determined according to a previously described method (7). For quantification of IL-2 production, 10^5 cells/well were incubated in 96-well round-bottomed plates for 24 h in the presence of either OVA (1 mg/ml final concentration) plus 10^5 A20-11 B lymphoma cells or anti-$T11_2$ + anti-$T11_3$ (ascites 1:200) or culture medium. A 5-ng/ml final concentration of PMA was added to all experimental samples including media control. Subsequently, supernatants were harvested and titrated in triplicate for their ability to support the growth of 10,000 CTLL-20 cells by [^{3}H]thymidine incorporation, as previously described (7), in parallel to known concentrations of rIL-2 (Biogen, Cambridge, MA).

Results and Discussion

A series of mutants was generated within the cytoplasmic region of human CD2 including the two tandem repeats of the sequence PPPGHR (Fig. 1). In three mutants, two negatively charged aa residues (D and E) were substituted for adjacent positively charged H and R residues in the CD2 sequence at aa positions 264–265, 271–272, or 278–279. In another mutation, a termination codon was introduced at aa 268, thereby resulting in the loss of the second PPPGHR repeat as well as the other COOH-terminal components of the CD2 cytoplasmic tail. These four modified cDNAs were individually transfected into the 3DO54.8 cell line, a murine T cell hybridoma specific for OVA in the context of the H-2 molecule (I-A^d), using a previously described retroviral system (7).

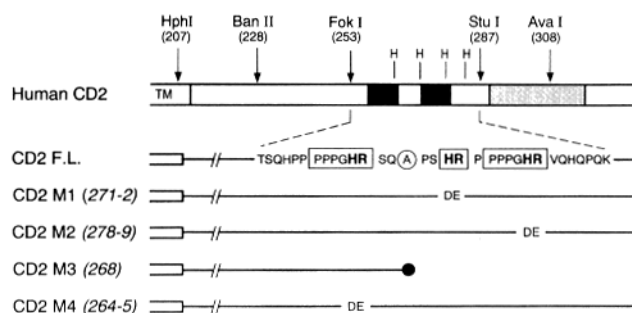

Figure 1. Schematic structure of transmembrane and cytoplasmic regions of human CD2 and mutated CD2 molecules. The region with the highest degree of homology (59%) between human and mouse CD2 proteins is stippled, and the two repeated PPPGHR segments are marked in black. Restriction sites used to generate the truncated CD2 molecules previously used in retroviral gene transfer experiments (9) are indicated by arrows. Numbers in parentheses correspond to aa residues. The CD2 sequence between aa 253 and 287, previously shown to be essential for signal transduction, is given in one-letter code. CD2 substitution and truncation mutants are diagrammed below the CD2 schematic with mutated aa shown in corresponding positions in one-letter code. HR, histidine-arginine; DE, aspartic acid–glutamic acid; TM, transmembrane region.

Clones expressing each of these forms of CD2 were obtained and compared with CD2FL clone 1, a clone derived from 3DO54.8 transfected with wild-type human CD2 expressing a similar level of surface CD2 (7). Nomenclature of clones used in the subsequent functional analyses is as follows: CD2 M1 (271–272) clones express CD2 cDNA with a DE for HR substitution at aa positions 271–272; CD2 M2 (278–279) clones express the DE for HR substitution at aa positions 278–279; CD2 M4 (264–265) clones express the DE for HR substitution at aa 264–265; and CD2 M3 (268) clones express CD2 molecules truncated after 57 cytoplasmic residues. The sequence of each mutation was confirmed by both double-stranded sequencing of the plasmid used for transfection as well as by sequencing the PCR products corresponding to nucleotides 739–1117, as amplified from cellular RNA derived from individual transfected clones.

Because perturbation of the CD2 extracellular segment with anti-$T11_2$ + anti-$T11_3$ antibodies induces human T cell activation and initiates a rise in intracellular-free calcium ($[Ca^{2+}]_i$) (1, 5), we tested the CD2 mutants for their ability to alter $[Ca^{2+}]_i$ in the transfected 3DO54.8 cell lines in which they were expressed. In Fig. 2, a clear rise in $[Ca^{2+}]_i$ (~200-nM increment) equivalent to the maximal level observed after stimulation of the wild-type human CD2-containing cells CD2 FL (clone 1) was reproducibly observed after stimulation of CD2 M1 (clone 232) and CD2 M2 (clone 38.4) cells. In contrast, we repeatedly failed to observe any calcium increase upon stimulation of CD2 M3 (clone 176) or CD2 M4 (clone 41). Given that an immediate $[Ca^{2+}]_i$ rise was observed after addition of the calcium ionophore A23187 to each of these cells, the absence of a response was not due to failure to load the cells with indo-1. These results imply that M3 and M4 mutations interfere with the function of CD2 signal transduction that modulates $[Ca^{2+}]_i$. These

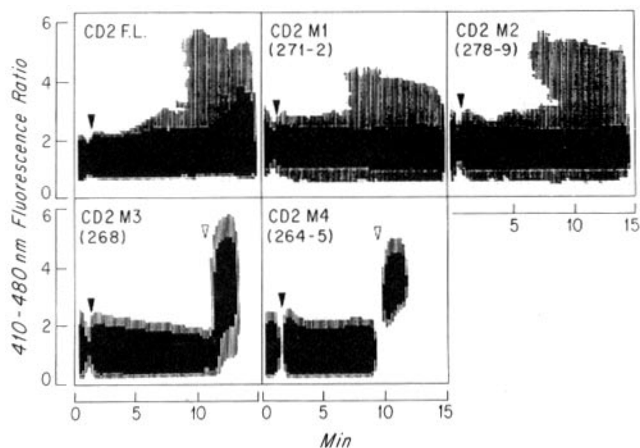

Figure 2. Increase of $[Ca^{2+}]_i$ mediated upon CD2 triggering. Changes in cytosolic Ca^{2+} concentration were monitored in cells loaded with the calcium-sensitive dye indo-1. Cells were stimulated with either anti-$T11_2$ (1old24C1) plus anti-$T11_3$ (1mono2A6) mAbs (ascites 1:200 dilution) (*filled arrowhead*) or calcium ionophore A23187 (*open arrowhead*). Results are expressed as ratio of 410:480–nm indo-1 fluorescence in arbitrary units (*y*-axis) vs. time in minutes (*x*-axis). One arbitrary unit represents ~200 nM $[Ca^{2+}]_i$. The surface expression of the CD2 molecule in these clones is ~3,000–8,000 molecules/cell. Results are representative of five experiments.

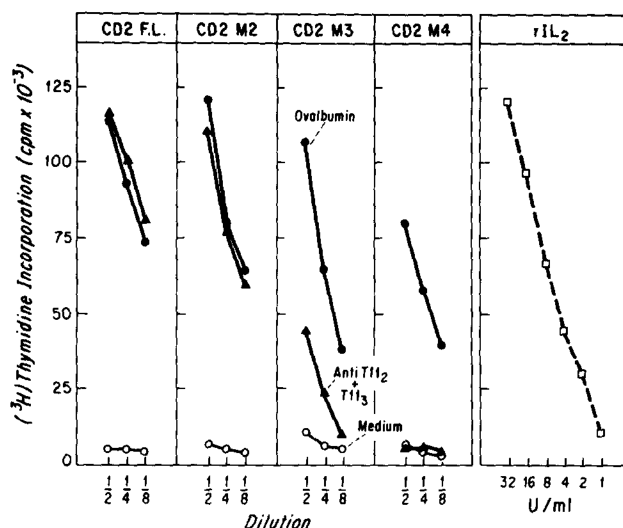

Figure 3. Stimulation of IL-2 production through wild-type and mutant CD2 molecules expressed on murine T cells. Serial twofold dilutions of culture supernatants from unactivated and stimulated transfectants have been analyzed for their capacity to support the growth of the CTLL-20 IL-2-dependent cell line, as evaluated by $[^3H]$thymidine incorporation. Each point is the mean of a triplicate determination (with SD ≤5%, in general). The proliferative effect of known amounts of rIL-2 (Biogen) is shown in the right panel of the figure. Specific clones used in this experiment were CD2 FL clone 1, CD2 M2 clone 38.1, CD2 M3 clone 176, and CD2 M4 clone 41. The CD2 expression of these clones present in copy number per cell is 5,000, 30,000, 8,000, and 4,000, respectively. This experiment is representative of five independent experiments using in addition clones CD2 FL clone 2, CD2 M2 clone 38.4, CD2 M3 clone 95, CD2 M4 clone 36, and CD2 M4 clone 122, with 4,500, 3,500, 4,000, 2,500, and 3,500 CD2 molecules expressed per cell, respectively, as evaluated by indirect immunofluorescence and FACS analysis.

findings were confirmed with two additional M3 clones (clones 17 and 95) and two additional M4 clones (clones 36 and 122).

Perturbation of the CD2 molecule with a combination of anti-$T11_2$ + anti-$T11_3$ mAbs was previously shown to result in IL-2 production (2). We, therefore, next determined whether this mitogenic combination of anti-CD2 mAbs stimulated any of the CD2 mutants, and compared the level of IL-2 produced with that obtained by stimulating each cell type with OVA in the context of $I\text{-}A^d$ APC. As shown in Fig. 3, substantial levels of IL-2 were produced by CD2 FL (clone 1), CD2 M2 (clone 38.1), CD2 M3 (clone 176), and CD2 M4 (clone 41) upon antigenic stimulation, as assayed by the ability of supernatants from transfected cells to induce proliferation of CTLL-20. The amount of IL-2 generated from the CD2 FL clone after CD2 triggering was comparable with that after antigen stimulation. A similar result was obtained for CD2 M2 cells (Fig. 3). As we previously reported, this was also the case for CD2 M1 (clone 232; reference 7). Although cells of the CD2 M2 clone 38.1 express higher levels of surface CD2 than CD2 FL, no defect in CD2 M2 function was observed upon triggering cells of the CD2 M2 clone 38.4, which express levels of CD2 comparable with that on CD2 FL cells (see Fig. 3 legend). Thus, the IL-2 induction function of the CD2 M2 molecule is not altered. In contrast, anti-CD2 stimulated no detectable IL-2 production from CD2 M4 and induced significantly less IL-2 from CD2 M3 relative to stimulation by antigen and MHC. These results indicate that IL-2 production from TCR stimulation of CD2 M4 and CD2 M3 clones is intact but that mutations of the CD2 molecule expressed in these clones has altered their signaling capacity. As indicated in the Fig. 3 legend, each of three CD2 M4 clones (M4 clone 36, M4 clone 41, and M4 clone 122) failed to produce IL-2 upon CD2 stimulation. Furthermore, the lack of IL-2 production was not a consequence of reduced CD2 expression, because the level of CD2 surface expression on the nonfunctional M4 series can be compared with the level of CD2 found on the functional M2 series (3,000–4,000 copies/cell, respectively).

Collectively, the above results show that alteration of the positively charged histidine 264 and arginine 265 residues within the first PPPGHR motif of the human CD2 cytoplasmic domain markedly affects the process of signal transduction via this molecule. In contrast, alteration of histidine and arginine residues at positions 271–272 or 278–279 were without detectable effect, implying that the effect of the nonconservative substitutions is strictly sequence position dependent. Such a finding might not have been anticipated if the histidine residues were each involved in forming a portion of a cage for an ion or small, charged regulatory molecule. While the position of residue substitutions in the mutant CD2 molecules may define the precise site(s) involved in CD2 signal transduction, we cannot exclude the possibility that the nonconservative substitutions structurally altered the CD2 cytoplasmic domain elsewhere. Furthermore, the ability of the M3 truncation to function in signaling IL-2 production,

although at reduced levels relative to CD2 FL, supports the importance of the histidine and/or arginine residues in the first PPPGHR motif. These data are also consistent with our earlier observations from analysis of a panel of CD2 cytoplasmic truncation mutants (7), and extend the findings by suggesting that some component between aa 268–287, aside from the histidine residues, contributes to signal transduction.

A number of important issues are raised by the data herein. First, what is the structural nature of the PPPGHR motif? We suspect that a core sequence consisting of PPGH may be the critical structural element, in view of the fact that the PPGH repeat, but not the entire PPPGHR motif, is present in a corresponding position of the murine CD2 sequence (1). Second, it is yet to be proven whether this CD2 segment is required for function or, rather, facilitates function. For example, we cannot rule out the possibility that CD2 mutant molecules of the M4 type might facilitate signal transduction if overexpressed by one to two orders of magnitude relative to wild-type CD2 expression. Third, the apparent dissociation between triggering an increase in $[Ca^{2+}]_i$ vs. IL-2 production noted with the M3 mutant needs to be investigated further. While it is possible that such a dissociation in signal transduction functions exists, the greater sensitivity of the IL-2 bioassay relative to the Ca^{2+} assay might make this difference more apparent than real. In any event, the characterization of the putative ligand for the segment addressed in the present mutational analysis needs to be considered. The use of recombinant DNA techniques to mass produce the cytoplasmic domain of CD2 protein could be helpful in answering this question.

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