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# Mutational Analysis of the Human 2B4 (CD244)/CD48 Interaction: Lys<sup>68</sup> and Glu<sup>70</sup> in the V Domain of 2B4 Are Critical for CD48 Binding and Functional Activation of NK Cells<sup>1</sup>

Stephen O. Mathew,<sup>2\*</sup> Pappanaicken R. Kumaresan,<sup>2\*†</sup> Jae Kyung Lee,\* Van T. Huynh,\* and Porunelloor A. Mathew<sup>3\*</sup>

Interaction between receptors and ligands plays a critical role in the generation of immune responses. The 2B4 (CD244), a member of the CD2 subset of the Ig superfamily, is the high affinity ligand for CD48. It is expressed on NK cells, T cells, monocytes, and basophils. Recent data indicate that 2B4/CD48 interactions regulate NK and T lymphocyte functions. In human NK cells, 2B4/CD48 interaction induces activation signals, whereas in murine NK cells it sends inhibitory signals. To determine the structural basis for 2B4/CD48 interaction, selected amino acid residues in the V domain of the human 2B4 (h2B4) were mutated to alanine by site-directed mutagenesis. Following transient expression of these mutants in B16F10 melanoma cells, their interaction with soluble CD48-Fc fusion protein was assessed by flow cytometry. We identified amino acid residues in the extracellular domain of h2B4 that are involved in interacting with CD48. Binding of CD48-Fc fusion protein to RNK-16 cells stably transfected with wild-type and a double-mutant Lys<sup>68</sup>Ala-Glu<sup>70</sup>Ala h2B4 further demonstrated that Lys<sup>68</sup> and Glu<sup>70</sup> in the V domain of h2B4 are essential for 2B4/CD48 interaction. Functional analysis indicated that Lys<sup>68</sup> and Glu<sup>70</sup> in the extracellular domain of h2B4 play a key role in the activation of human NK cells through 2B4/CD48 interaction. *The Journal of Immunology*, 2005, 175: 1005–1013.

Natural killer cells are bone marrow-derived lymphocytes that function as key players in innate immunity by recognizing viral, bacterial, and parasitic infections and neoplastic target cells (1–3). NK cell recognition is regulated by specific receptors that, upon interaction with their respective ligands, may send stimulatory or inhibitory signals (4–7). These receptors belong to two structurally distinct superfamilies: the Ig superfamily and the lectin superfamily. In the Ig superfamily, the CD2 family plays a major role in lymphocyte function and regulation (8, 9). The CD2 family includes the CD2, CD58 (LFA-3), CS1 (CD2-like receptor activating cytotoxic cells), CD84, B lymphocyte activator macrophage expressed, CD229 (Ly-9), SF-2001, NTB-A, CD150 (signaling lymphocytic activation molecule), 2B4 (CD244), and CD48 (10). Members of the CD2 family have been shown to interact with other members of the same family or with themselves, and these interactions play an important role regulating a variety of immune responses. The 2B4 is expressed on all NK cells, a subset of CD8<sup>+</sup> T cells, TCR $\gamma\delta$ <sup>+</sup> T cells, monocytes, and basophils (11–13). Ligation of surface 2B4 with a specific mAb or

its ligand CD48 sends activation signals to NK cells, as evident from increased cytolytic function, induction of IFN- $\gamma$  production, and NK cell invasiveness (13–17). Recently, we have generated and characterized 2B4-deficient mice that revealed an *in vivo* role for 2B4 in immune response against tumor (18). Further studies on these 2B4-deficient mice revealed that murine 2B4 acts as a non-MHC-binding inhibitory receptor both *in vitro* and *in vivo*, and this inhibition is independent of signaling lymphocytic activation molecule-associated protein expression (19, 20). The 2B4 is expressed early in NK cell development, before the expression of other NK cell-specific markers such as NK1.1 and Ly-49 family members, suggesting a vital role in NK cell development and function (21, 22). Defective signaling via 2B4 due to a genetic defect in signaling lymphocytic activation molecule-associated protein/Src homology 2 domain protein 1A has been implicated in the pathogenesis of X-linked lymphoproliferative disease (23–26).

Unlike 2B4, its ligand CD48 is widely expressed on cells of hemopoietic origin. Anti-CD48 mAb can inhibit the proliferation of T cells *in vitro* and suppress cell-mediated immunity *in vivo*, suggesting an important role for T cell activation (27). CD48-deficient mice have severe defect in CD4<sup>+</sup> T cell activation (28). Earlier studies have shown that CD48 also binds to CD2, strongly in rodents and weakly in humans (27, 29–31). However, the affinity of the interaction between human CD2 (hCD2)<sup>4</sup> and hCD48 is ~100-fold lower than hCD2 and hCD58 (31). The 2B4/CD48 interaction has ~10-fold higher affinity than CD2/CD48 interaction (32). Moreover, the strength of the 2B4/CD48 interactions is conserved across the species, with similar affinities being observed for mouse and human 2B4 (h2B4)/CD48 interactions (32). Because 2B4/CD48 interaction has a much higher affinity than CD2/CD48 interaction, 2B4 is the physiological ligand for CD48. The

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<sup>4</sup> Abbreviations used in this paper: hCD, human CD; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; 3-D, three-dimensional; h2B4, human 2B4.

Table I. PCR primer sequences and their position relative to h2B4 cDNA

Name	Sequence	Relative nucleotide position
h2B4K47A FP	5'-AAA CAG CAT ACA GAC GGC GGT TGA CAG CAT TGC ATG GAA G-3'	231-270
h2B4K47A RP	5'-CTT CCA TGC AAT GCT GTC AAC CGC CGT CTG TAT GCT GTT T-3'	270-231
h2B4D49A FP	5'-AAC AGC ATA CAG ACG AAG GTT GCC AGC ATT GCA TGG AAG AAG-3'	232-273
h2B4D49A RP	5'-CTT CTT CCA TGC AAT GCT GGC AAC CTT CGT CTG TAT GCT GTT-3'	273-232
h2B4S50A FP	5'-AAC AGC ATA CAG ACG AAG GTT GAC GCC ATT GCA TGG AAG AAG-3'	232-273
h2B4S50A RP	5'-CTT CTT CCA TGC AAT GGC GTC AAC CTT CGT CTG TAT GCT GTT-3'	273-232
h2B4K54A FP	5'-TTG ACA GCA TTG CAT GGG CGA AGT TGC TGC CCT CAC-3'	251-286
h2B4K54A RP	5'-GTG AGG GCA GCA ACT TCG CCC ATG CAA TGC TGT CAA-3'	286-251
h2B4K55A FP	5'-TTG ACA GCA TTG CAT GGA AGG CGT TGC TGC CCT CAC-3'	251-286
h2B4K55A RP	5'-GTG AGG GCA GCA ACG CCT TCC ATG CAA TGC TGT CAA-3'	286-251
h2B4N61A FP	5'-TGC TGC CCT CAC AAG CTG GAT TTC ATA ACA TAT TGA AGT GGG-3'	275-316
h2B4N61A RP	5'-CCC ACT TCA ATA TGT GAT GAA ATC CAG CTT GTG AGG GCA GCA-3'	316-275
h2B4K68A FP	5'-TTT CAT CAC ATA TTG GCG TGG GAG AAT GGC TCT TTG CCT TCC-3'	295-336
h2B4K68A RP	5'-GGA AGG CAA AGA GCC ATT CTC CCA CGC CAA TAT GTG ATG AAA-3'	336-295
h2B4E70A FP	5'-TTT CAT CAC ATA TTG AAG TGG GCG AAT GGC TCT TTG CCT TCC-3'	295-336
h2B4E70A RP	5'-GGA AGG CAA AGA GCC ATT CGC CCA CTT CAA TAT GTG ATG AAA-3'	336-295
h2B4T110A FP	5'-GGC CTC TAC TGC CTG GAG GTC GCC AGT ATA TCT GG-3'	415-449
h2B4T110A RP	5'-CCA GAT ATA CTG GCG ACC TCC AGG CAG TAG AGG CC-3'	449-415
h2B4G114A FP	5'-TAC CAG TAT ATC TGC AAA AGT TCA GAC AGC CAC GTT CC-3'	435-472
h2B4G114A RP	5'-GGA ACG TGG CTG TCT GAA CTT TTG CAG ATA TAC TGG TG-3'	472-435
h2B4K115A FP	5'-CAC CAG TAT ATC TGG AGC AGT TCA GAC AGC CAC G-3'	435-468
h2B4K115A RP	5'-GGA ACG TGG CTG TCT GAA CTG CTC CAG ATA TAC TGG TG-3'	468-435
h2B4K68AE70A FP	5'-TTT CAT CAC ATA TTG GCG TGG GCG AAT GGC TCT TTG CCT TCC-3'	295-336
h2B4K68AE70A RP	5'-GGA AGG CAA AGA GCC ATT CGC CCA CGC CAA TAT GTG ATG AAA-3'	336-295

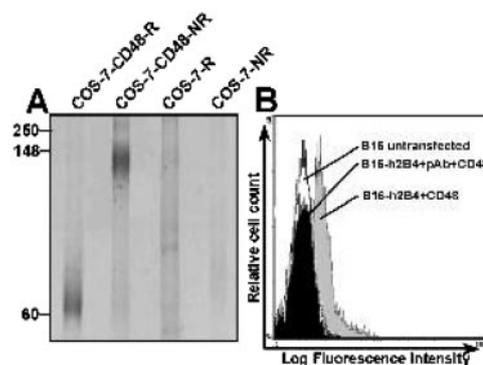
2B4 and CD48 are structurally related, and the genes that encode 2B4 and CD48 are located in chromosome 1 at band 1q21-24 (33, 34).

The 2B4/CD48 interaction not only regulates NK cell functions, but also induces B cell and T cell proliferation (14, 35). Recently, it has been shown that 2B4 molecule acts as a ligand for enhancing activation and proliferation of neighboring T cells through CD48 (36). The 2B4/CD48 interaction among T cells themselves augments CTL lysis of specific targets (37). In the CD2 family, CD2 and CD58, receptor-ligand pair, was the first identified heterophilic cell adhesion interaction (38). Studies on the interaction of CD2 with its ligands have been informative with respect to the mechanisms of protein-protein interaction at the cell surface (9). Because 2B4/CD48 is structurally similar to CD2/CD58, we exploited this information to select potential amino acids that might be involved in 2B4/CD48 interactions. X-ray crystallography and nuclear magnetic resonance have provided important details on the structure of CD2 and CD58. The crystal structure of soluble forms of rat and hCD2 provided the initial views on the charged residues clustered at the ligand binding site of CD2 and CD58 (39, 40). To assess the relative functional importance of individual charged amino acid residues in 2B4/CD48 interaction, we have produced single amino acid variants of the 2B4 in the V domain by mutating charged amino acid to neutral amino acid. These 2B4 mutants were expressed in B16F10 cells, and their binding to soluble CD48-Fc fusion protein was analyzed by flow cytometry. NK92 cells were able to mediate cell cytotoxicity of h2B4-transfected K562 cells, but it was significantly impaired with the double-mutant K68AE70A. Similarly, double-mutant K68AE70A-transfected RNK-16 cells also showed reduced cytolytic activity against P815 cells transfected with hCD48 as compared with the wild-type h2B4-RNK-16 transfectants. This was further confirmed by the calcium flux experiments. Our study showed that several charged amino acids in the V domain of h2B4 make relative contributions to its interaction with CD48 and the amino acid residues Lys<sup>68</sup> and Glu<sup>70</sup> are essential for 2B4/CD48 interaction.

## Materials and Methods

### Cell lines and reagents

B16F10 (mouse melanoma cell line), COS-7, and YT (human NK cell line) cells were cultured in medium RPMI 1640 supplemented with 10% FBS (HyClone), 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin,



**FIGURE 1.** Characterization of soluble CD48-Fc fusion protein. *A*, The pCD48-Fc was transiently transfected into COS-7 cells using Fugene-6 transfection reagent. Two days after transfection, cells were <sup>35</sup>S labeled with Trans label (ICN Biochemicals; 150 mCi/ml) for 4 h, and the supernatants were assayed for the presence of CD48-Fc by precipitation with protein A-agarose (Bio-Rad). The precipitated protein was separated on SDS-PAGE under reduced (COS-7-CD48-R) and nonreduced (COS-7-CD48-NR) conditions, and the gel was exposed for autoradiography. COS-7-R and COS-7-NR are the negative controls. Molecular masses are indicated in kDa. *B*, Soluble CD48-Fc binding to h2B4 is specific and blocked by anti-h2B4. B16F10 cells transfected with h2B4 (filled black) were incubated with polyclonal antisera against 2B4 to specifically block binding to CD48 and then incubated with CD48-Fc fusion protein. B16F10-untransfected cells (open) and B16F10 cells transfected with h2B4 (filled gray) were incubated with CD48-Fc fusion protein, which served as the negative and positive control, respectively. FITC-conjugated anti-human IgG was then used to detect the Fc portion of fusion protein.

10 mM HEPES, and 10 mM nonessential amino acids. Cells were maintained at 37°C in a humidified 5% CO<sub>2</sub>/95% air incubator. All of the cell culture reagents were obtained from Invitrogen Life Technologies, unless otherwise noted. The mAb that specifically recognizes h2B4 (mAb C1.7), and all the secondary Abs were purchased from Corixa. All enzymes were purchased from New England Biolabs, unless otherwise stated. All custom synthesized oligonucleotides used in this study were supplied by Integrated DNA Technologies.

*Construction and expression of soluble CD48-Fc fusion protein*

Soluble CD48-Fc fusion protein was produced by fusing the extracellular domain of the CD48 with Fc portion of the human IgG. The extracellular domain of the hCD48 (GenBank Accession M59904) was amplified by PCR using forward primer, CD48FP, 5'-TGG TGG CTA GCG ATT CAA GGT CAC TTG-3' and reverse primer, CD48RP, 5'-AAG AAT GGA TCC ACC GTG ACC ACT AGC C-3'. The amplified product was subcloned in front of human Fc gene at *NheI* and *BamHI* cloning sites in pCD5lneg1 vector (kindly provided by B. Seed, Harvard Medical School, Boston, MA), which contain the C<sub>H</sub>2 and C<sub>H</sub>3 regions of the human IgG1. To verify the orientation and folding of the fusion protein, 2 µg of pure pDNA was transfected into 50% confluent COS-7 cells using Fugene-6 transfection reagent (Roche Diagnostic Systems). After 48 h, the cells were washed three times with PBS and changed to cysteine- and methionine-depleted RPMI 1640 medium (Mediatech). A total of 150 mCi/ml <sup>35</sup>S-labeled cysteine and methionine (ICN Radiochemicals) was added to medium, and after 48 h supernatant was collected. A total of 5 g of protein A-agarose beads (Bio-Rad) was added in the supernatant to precipitate the CD48-Fc fusion protein. The beads were washed well with PBS to remove the nonspecific binding proteins. Then beads were split into two halves and eluted with SDS-loading buffer with and without DTT. The proteins were separated by 10% SDS-PAGE under reducing and nonreducing conditions, and the gel was dried to expose for autoradiography. For checking the CD48 portion of fusion protein, immunoprecipitated proteins were electrophoretically transferred to polyvinylidene difluoride (Pierce) membrane. Membranes were incubated with anti-CD48 mAb (1/100 dilution) in blocking buffer overnight. Peroxidase-conjugated secondary Abs were used at 1/2000 dilution, and immunoreactive bands were visualized using ECL (Kirkegaard & Perry Laboratories), as per manufacturer's description. For isolation of large quantities of the CD48-Fc fusion protein, COS-7 cells were transfected in large quantities; supernatants were collected at days 3, 6, and 9; and protein was isolated by protein A affinity column chromatography.

*Production of polyclonal Ab against h2B4*

The 2B4-Fc fusion protein was produced, as described above, for CD48-Fc fusion protein. The extracellular domain of the h2B4 was PCR amplified using forward primer, h2B4FP, 5'-TCA AGG TGC TAG CCG GCA AAG GAT GCC AGG GAT CAG-3' and h2B4RP, 5'-CAA AAA CGG CGG ATC CCT GAA TTC CTG ATG GGC ATT CTG-3'. A total of 100 µg of pure h2B4-Fc protein was separated on 8% SDS-PAGE, and the protein with gel was excised and used for immunizing rabbits. The protein with the gel was minced very well to form a paste and immunized rabbit s.c. Once the rabbit showed high titer against the h2B4 Ag after repeated immunization, blood was collected for antisera. The antisera were tested for binding to h2B4-transfected B16F10 cells.

*Site-directed mutagenesis of h2B4*

The h2B4 (GenBank Accession AF107761) cloned in pCI-neo vector was used as template DNA for site-directed mutagenesis. The mutants were constructed using Quik Change Site-directed mutagenesis kit (Stratagene). The primer sequences used to construct the mutant clones are given in Table I. PCR conditions were 94°C for 30 s, primer annealing at 55°C for 1 min, and an extension at 68°C for 6 min for 12 cycles. The PCR product was digested with *DpnI* and transformed into XL1-blue super competent cells (Stratagene). The amino acids in extracellular domain of h2B4 were mutated to noncharged alanine residues at positions K47, D49, S50, K54, K55, N61, K68, E70, T110, G114, and K115. The mutations were confirmed by sequencing the mutated pCIneo-h2B4 clones. For the double mutation, a single PCR primer contained both mutations because the two mutations were close to each other.

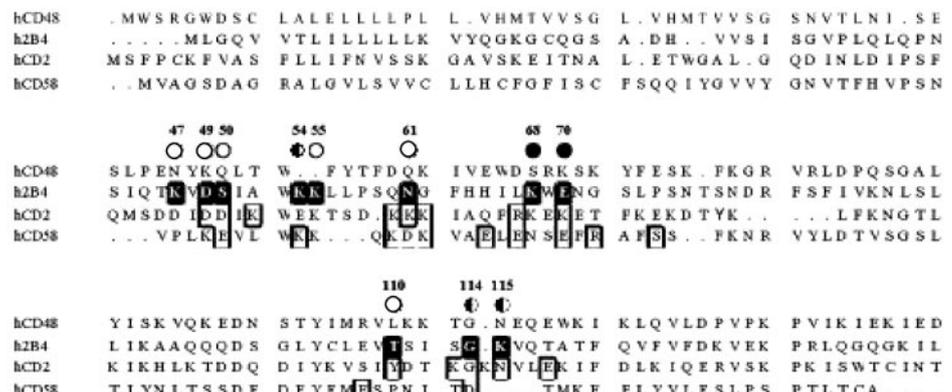
*Transfection and flow cytometry analysis for h2B4-mutated clones*

B16F10 cells were transiently transfected with h2B4-mutated pDNA using Fugene-6 transfection reagent (Roche Diagnostic Systems). After 48 h, cells were incubated with specific mAbs (C1.7), polyclonal Ab against 2B4, and CD48-Fc fusion protein to detect the expression of the molecules. They were then washed with cold PBS at low speed and incubated with FITC-conjugated goat anti-mouse IgG for C1.7, FITC-conjugated goat anti-rabbit IgG for polyclonal 2B4 Ab, and FITC-conjugated goat anti-human IgG for CD48-Fc fusion protein for 40 min at 4°C. Samples were washed twice and were analyzed by flow cytometry (Corixa EPICS XL-MCL). The mean fluorescence intensity in the binding assay represents the average of three independent trials with similar results. Wild-type and double-mutant K68AE70A h2B4 were also stably transfected into RNK-16 and K562 cells. cDNA of each strain was subcloned into *SalI* and *XbaI* sites of expression vector pEMCV.SRα (a kind gift from J. Schatzle, University of Texas Southwestern Medical Center, Dallas, TX). Constructs were confirmed by sequencing before transfections. Electroporations were performed using 50 µg of *ClaI*-linearized plasmids that had been purified by two rounds of CsCl centrifugation. Cells were selected in medium containing 500 µg/ml G418 (Sigma-Aldrich). Positive clones were analyzed for wild-type and double-mutant h2B4 expression by flow cytometry using polyclonal anti-2B4 as well as C1.7 mAb.

*Cytotoxicity assay*

Target cells were labeled by incubating 1 × 10<sup>6</sup> cells with 2 MBq of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (NEN Research Products) for 90 min at 37°C under 5% CO<sub>2</sub> in air. The target cells (100 µl) were incubated with effector cell suspension (100 µl) under various conditions. After incubation for 4 h at 37°C under 5% CO<sub>2</sub> in air, the cells were pelleted at 250 × g for 5 min, 100 µl of the supernatants were removed, and their radioactivity was measured. The percentage of specific lysis was calculated by the following equation:  $(a - b/c - b) \times 100$ , where *a* is the radioactivity of the supernatant of target cells mixed with effector cells, *b* is that in the supernatant of target cells incubated alone, and *c* is that in the supernatant after lysis of target cells with 1% Nonidet P-40.

**FIGURE 2.** Amino acid sequence alignment of extracellular domain of hCD48, h2B4, hCD2, and hCD58. Shaded residues show the amino acids that were mutated. Amino acid residues involved in CD2-CD58 interaction are boxed. h2B4 residues that are involved in binding soluble CD48-Fc are shown in either ● (strong effects) or half ● (partial effects). Analyzed residues not significantly involved in binding to CD48-Fc are shown in ○ (no significant effect). The alignment data were generated by GCG software.



### Calcium flux assay

We used a protocol similar to that used by Prasanna et al. (41). Briefly, to measure intracellular calcium mobilization, RNK-16 cells stably expressing wild-type and double-mutant K68AE70A h2B4 were seeded on coverslips and loaded in 3  $\mu$ M fura 2 dye (Molecular Probes) in a modified Krebs-Ringer buffer solution (in millimoles: 115 NaCl, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 24 NaHCO<sub>3</sub>, 5 KCl, 5 glucose, and 25 HEPES, pH 7.4) for 30 min at 37°C. Then, sCD48-IgG (Fc) fusion protein or anti-NKR-P1A (10/78) mAb was used to induce receptor cross-linking, and cross-linking goat anti-human or goat anti-mouse IgG (20  $\mu$ g/ml) (BD Pharmingen) was added. The fura 2 fluorescence from these cells was monitored at 37°C by the ratio technique (excitation at 340 and 380 nm, emission at 500 nm) under a Nikon Diaphot microscope using Metafluor software (Universal Imaging). Calibrations were performed *in vivo*, and conditions of high intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) were achieved by adding the Ca<sup>2+</sup> ionophore 4-Bromo-A23187 (1–3  $\mu$ M; Calbiochem), whereas conditions of low [Ca<sup>2+</sup>]<sub>i</sub> were obtained by adding EGTA (4–5 mM). Statistical significance of [Ca<sup>2+</sup>]<sub>i</sub> between control and treatments was determined parametrically by Student's *t* test at *p* < 0.05.

## Results

### Recombinant CD48-Fc fusion protein is dimeric and binds h2B4

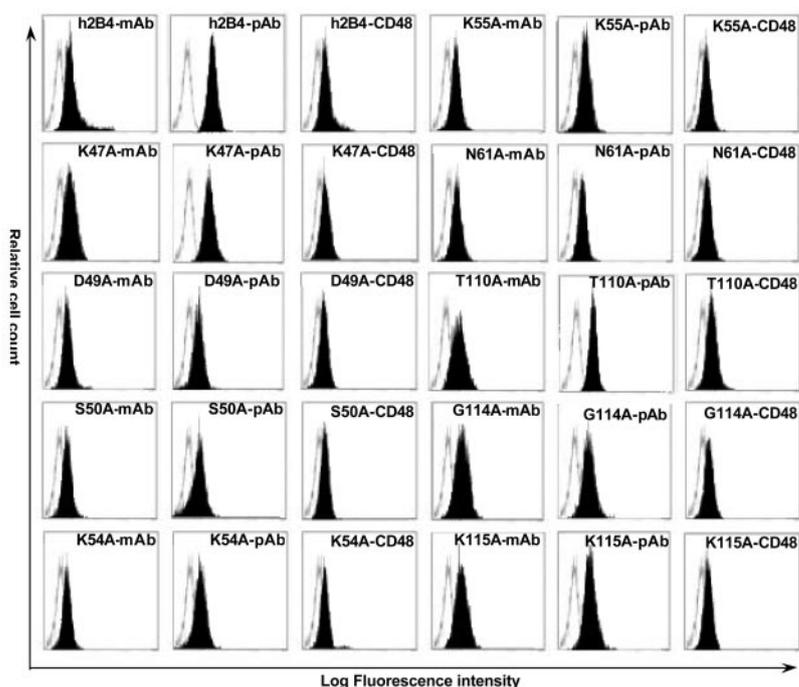
To study 2B4/CD48 interactions, we generated soluble CD48-Fc fusion protein by ligating the extracellular domain of CD48 with Fc portion of human IgG at the C terminus. <sup>35</sup>S-labeled CD48-Fc protein was immunoprecipitated by protein A-agarose and separated on SDS-PAGE to check purity and biochemical structure. Autoradiogram of the gel showed bands of 135 and 65 kDa under nonreducing and reducing conditions, respectively, indicating CD48-Fc fusion protein as a dimer and in proper folding (Fig. 1A). Affinity-purified fusion protein was used to detect the binding of CD48 to h2B4 by flow cytometry (Fig. 1B). The CD48-Fc fusion protein binds specifically to B16F10 cells transfected with h2B4 and not to untransfected cells, suggesting that it is functionally active. The binding was inhibited after blocking with h2B4 Abs, suggesting that the interaction is specific for h2B4 (Fig. 1B).

### Amino acid residues in the V domain of 2B4 contribute to 2B4/CD48 interaction

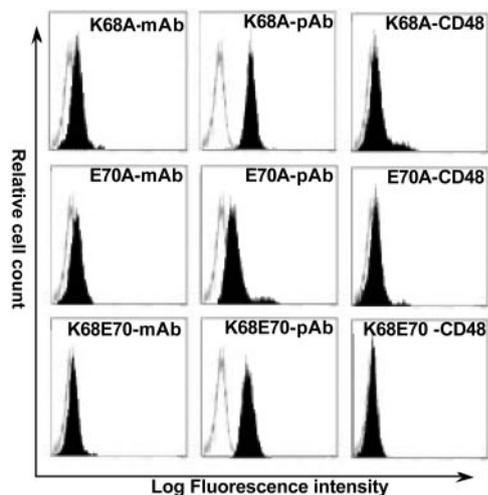
The CD2/CD58 pair was selected to compare 2B4/CD48 interaction studies because both pairs are structurally and functionally

related. CD2 also binds to CD48 weakly as compared with 2B4 (29). Early studies suggested involvement of the charged residues in protein recognition and cell adhesion (42, 43). Recently, CD2-CD58 receptor-ligand interaction is well studied using x-ray crystallography, nuclear magnetic resonance, and site-directed mutation analysis (44–46). To identify the charged amino acids that may be involved in 2B4/CD48 interaction, the extracellular domains of 2B4, CD48, CD2, and CD58 were aligned using the GCG software (Fig. 2). Based on the earlier point mutational and the crystallographic studies, the key amino acids involved in the CD2/CD58 interaction were selected. In CD2, mutations K34, K41, K43, K51, D31, D32, R48, and K91 and in CD58, mutations E25, K32, E39, E42, R44, D33, K34, E37, E78, E84, and E95 showed to interrupt the CD2-CD58 interaction modest to completely (42, 43, 45). The amino acid residues in 2B4 selected for mutagenesis are given in bold. Table I gives the list of amino acids selected for point mutation along with PCR primer sequences used for site-directed mutagenesis.

Preliminary experiments were conducted by expressing all of the 11 single mutants and wild-type 2B4 in B16F10 melanoma cells by transient transfection. The expression of the h2B4 mutants was verified by FACS analysis with C1.7 (mAb against h2B4) as well as polyclonal antisera against h2B4. Figs. 3 and 4 show the expression pattern of wild-type h2B4 and mutants in B16F10 cells. All of the mutants are expressed on the cell surface, like wild-type h2B4. Binding of C1.7 indicated that some of the point mutations affect the mAb binding; in particular, K68A and E70A severely disrupted the C1.7 binding, indicating that these two residues may be involved in recognition of h2B4 by mAb C1.7. The role of these two residues in C1.7 binding is further evident, as seen in Fig. 5C. This indicates that some residues involved in ligand binding and mAb C1.7 binding may overlap. This is not surprising because mAb C1.7 activates human NK cells. The expression of the mutated clones was also checked by Western blot (data not shown). Both the FACS and Western blot analysis depict the same results that the anti-h2B4 Abs bind to all h2B4 mutants like wild-type h2B4. This suggests that by mutating to alanine, the three-dimensional structure of h2B4 is not altered and can be used to identify



**FIGURE 3.** CD48-Fc binding to h2B4 expressed in B16F10 cells. Expression of h2B4 wild-type and single mutated clones and binding with soluble CD48-Fc fusion protein. B16F10 cells transfected with pCI-neo empty vector (open) and B16F10 cells transfected with h2B4 wild-type and mutants (filled) were incubated with mAb (C1.7) denoted by mAb, 2B4 polyclonal Ab denoted by pAb, and soluble CD48-Fc fusion protein denoted by CD48. FITC-conjugated anti-mouse IgG, anti-rabbit IgG, and anti-human IgG were used to detect C1.7, polyclonal anti-2B4, and CD48-Fc fusion protein, respectively, by flow cytometry. Results are representative of three independent experiments.



**FIGURE 4.** The double mutation K68AE70A abolishes CD48 binding to 2B4. The double mutation K68AE70A abolishes CD48 binding to 2B4. B16F10 cells transfected with pCI-neo empty vector (open) and B16F10 cells transfected with mutants K68A, E70A, and double-mutant K68AE70A (filled) were incubated with mAb (C1.7) denoted by mAb, 2B4 polyclonal Ab denoted by pAb, and soluble CD48-Fc fusion protein denoted by CD48. FITC-conjugated anti-mouse IgG, anti-rabbit IgG, and anti-human IgG were used to detect C1.7, polyclonal anti-2B4, and CD48-Fc fusion protein, respectively, by flow cytometry. Results are representative of three independent experiments.

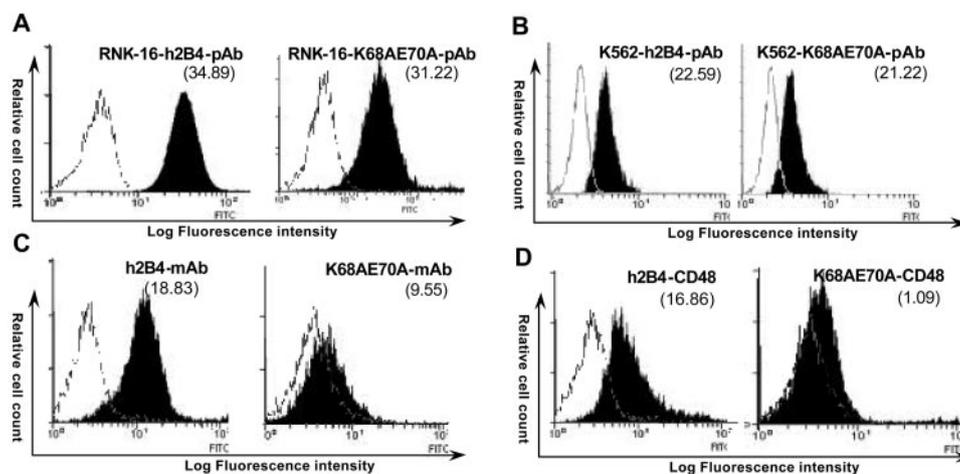
the key amino acids involved in binding with its ligand CD48. The expression pattern of the mutants compared with the wild type is shown in Table II and Figs. 3 and 4. FACS analysis was performed with CD48-Fc fusion protein with wild-type h2B4 and h2B4 mutants. Mean fluorescence intensity of binding of CD48-Fc to h2B4 mutants in comparison with its binding to wild-type h2B4 is also shown in Table II. Statistical analysis using *t* test ( $p < 0.05$ ) showed that K47A, D49A, S50A, K55A, N61A, and T110A did

not significantly alter the CD48 binding, whereas K54A, G114A, and K115A significantly altered the binding. In particular, mutants K68A and E70A were shown to highly significantly alter the binding to CD48. To determine the relative locations of these residues on the V domain and to verify whether these modifications alter the 3-dimensional (3-D) structure of the 2B4, a predicted 3-D structure of 2B4 and its mutants was determined using the 3D-PSSM software ([www.sbg.bio.ic.ac.uk/~3dpssm](http://www.sbg.bio.ic.ac.uk/~3dpssm)) (47). The predicted residues based on computer simulation that affect CD48 binding to h2B4 lie closely together on the surface of the V domain (Fig. 6). Corresponding to CD2/CD58 interaction, E70 lies in the C' strand and K54 lies on the F strand of the predicted  $\beta$  strand, whereas the other amino acids alter the binding map within the predicted loops: two residues in FG loop (G114 and K115) and one residue in C'C' loop (K68). Thus, several charged amino acid residues make relative contributions to 2B4/CD48 interaction.

#### Mutations Lys<sup>68</sup>Ala and Glu<sup>70</sup>Ala abolish 2B4/CD48 interaction

The 2B4 mutants K68A and E70A showed mean fluorescence intensity values of 3.59 and 1.60 with CD48-Fc fusion protein, respectively. This suggests that these amino acids play a significant role in CD48 binding to 2B4. When both the amino acid residues were mutated, it completely abolished the binding of CD48 to 2B4 (Fig. 4 and Table II). The predicted 3-D structure of the double mutant was similar to the wild-type 3-D structure (Fig. 6). More importantly, Glu<sup>70</sup> lies in the  $\beta$  sheet of the Ig domain, implicating that 2B4/CD48 interaction is similar to the face-to-face interaction of CD2 and CD58, which involves  $\beta$  sheets of both counterreceptors. This indicates that amino acid residues Lys<sup>68</sup> and Glu<sup>70</sup> are essential for 2B4/CD48 interaction.

To further confirm the results obtained by transient transfection, we generated stable expression of wild-type and double-mutant K68AE70A h2B4 in RNK-16 and K562 cells. Both wild-type and double-mutant K68AE70A h2B4 showed similar levels of expression on RNK-16 as well as K562 cells (Fig. 5, A and B). The significance of the double mutant was further confirmed when it



**FIGURE 5.** Stable expression of wild-type and double-mutant K68AE70A h2B4 in RNK-16 and K562 cells. Wild-type and double-mutant K68AE70A h2B4 were stably expressed in RNK-16 and K562 cells. *A*, Expression of stably transfected wild-type and double-mutant K68AE70A h2B4 in RNK-16 cells determined by flow cytometry using preimmune sera (open) and anti-h2B4 polyclonal Ab (filled). FITC-conjugated anti-rabbit IgG was used as the secondary Ab. *B*, Expression of stably transfected wild-type and double-mutant K68AE70A h2B4 in K562 cells determined by flow cytometry using preimmune sera (open) and anti-h2B4 polyclonal Ab (filled). FITC-conjugated anti-rabbit IgG was used as the secondary Ab. *C*, Binding of mAb C1.7 to RNK-16 cells stably transfected with wild-type and double-mutant K68AE70A h2B4. Open histogram control Ab and filled histogram mAb C1.7. FITC-conjugated anti-mouse IgG was used to detect C1.7 by flow cytometry. *D*, Stably expressed double-mutant K68AE70A failed to bind CD48. RNK-16 cells stably transfected with wild-type and double-mutant K68AE70A h2B4 were incubated with human sera (open) and soluble CD48-Fc fusion protein (filled) and analyzed by flow cytometry. FITC-conjugated anti-human IgG was used as the secondary Ab. Numbers in parentheses, mean fluorescence intensity.

Table II. Comparison of mean fluorescence intensity (MFI) values for mAb C1.7, polyclonal anti-2B4, and CD48-Fc binding to h2B4 and mutants

	Mutants	MFI for mAb <sup>a</sup>	MFI for Polyclonal pAb	MFI for CD48-Fc
1	h2B4	14.63 (1.68) <sup>a</sup>	23.49 (8.98)	12.75 (0.55)
2	K47A	10.48 (2.96)	17.09 (3.08)	9.96 (1.46)
3	D49A	10.63 (0.66)	11.18 (2.65)	9.94 (1.47)
4	S50A	9.91 (0.27)	12.08 (0.77)	10.61 (0.48)
5	K54A	9.30 (0.73)	11.97 (1.06)	9.33 <sup>b</sup> (0.66)
6	K55A	10.02 (0.25)	13.63 (1.15)	9.87 (1.34)
7	N61A	10.08 (0.06)	11.41 (0.18)	10.10 (1.47)
8	K68A	6.53 (3.12)	20.59 (9.70)	3.59 <sup>b</sup> (1.95)
9	E70A	5.67 (3.84)	14.14 (1.00)	1.60 <sup>b</sup> (0.64)
10	T110A	13.50 (0.48)	13.96 (4.99)	11.36 (1.23)
11	G114A	13.93 (0.15)	15.09 (0.48)	9.45 <sup>b</sup> (0.25)
12	K115A	13.82 (0.16)	15.45 (0.84)	9.35 <sup>b</sup> (0.25)
13	K68AE70A	2.11 (0.54)	24.27 (3.90)	0.30 <sup>b</sup> (0.13)

<sup>a</sup> Numbers in parentheses, SD.

<sup>b</sup> Student's *t* test was used to calculate *p* values; \*, *p* < 0.05.

almost completely abolished the binding of CD48 to 2B4 (Fig. 5D).

#### Mutations Lys<sup>68</sup>Ala and Glu<sup>70</sup>Ala inhibit NK cell function

h2B4 has previously been shown to activate NK cell-mediated cytotoxicity (12, 15, 16, 48, 49). To determine whether interaction through Lys<sup>68</sup> and Glu<sup>70</sup> of h2B4 with CD48 plays any functional role in NK cell activation, we performed cytotoxicity assays as well as calcium flux experiments. For cytolytic function, we used human NK cell line NK92 as effectors against <sup>51</sup>Cr-labeled K562 target cells stably transfected with h2B4 and double-mutant K68AE70A. The expression of wild-type and double-mutant K68AE70A h2B4 in K562 cells was determined by flow cytometry using polyclonal anti-h2B4 (Fig. 5B). Because K562 is a very susceptible target for NK killing, the baseline killing is very high in the absence of any other stimulation, as expected. Expression of wild-type h2B4 in K562 cells increased the cytolytic function of NK92 cells. This may be due to enhanced adhesion of these transfected cells with NK92 as well as activation signals resulting from 2B4/CD48 interactions. The cytotoxic activity of NK92 against K562 transfected with the double-mutant K68AE70A reduced the killing to the basal level (Fig. 7A). Similarly, wild-type and double-mutant K68AE70A h2B4 expressed in the rat NK cell line RNK-16 was able to mediate cellular cytotoxicity of <sup>51</sup>Cr-labeled P815 target cells stably transfected with hCD48. As compared with the wild-type h2B4-RNK-16 transfectants, the double-mutant K68AE70A-transfected RNK-16 cells showed significantly reduced cytolytic activity against P815 cells transfected with hCD48 (Fig. 7B). This suggests that 2B4/CD48 interaction involving the amino acid residues Lys<sup>68</sup> and Glu<sup>70</sup> does play a role in the cytolytic function of NK cells.

We also performed intracellular calcium flux experiments to confirm the functional data obtained in the cytotoxicity assay. Wild-type and the double-mutant K68AE70A h2B4 were expressed in RNK-16 cells, and induction of calcium release was determined upon interaction with soluble CD48-Fc fusion protein. An Ab against NKR-P1, mAb 10/78, was used as a positive control, as it has been shown previously that it stimulates phosphoinositide turnover and induces calcium flux within RNK-16 cells (50–52). Cross-linking of h2B4 using soluble CD48-Fc fusion protein on h2B4 RNK-16 transfectants resulted in a significant calcium flux, as measured by fura 2 over time, whereas cross-

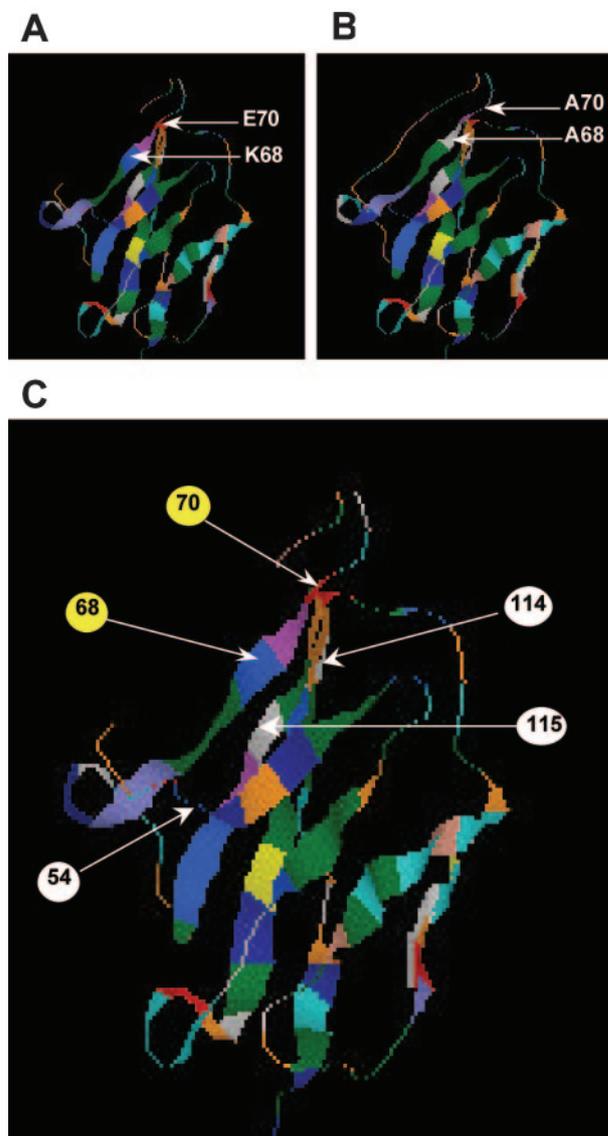
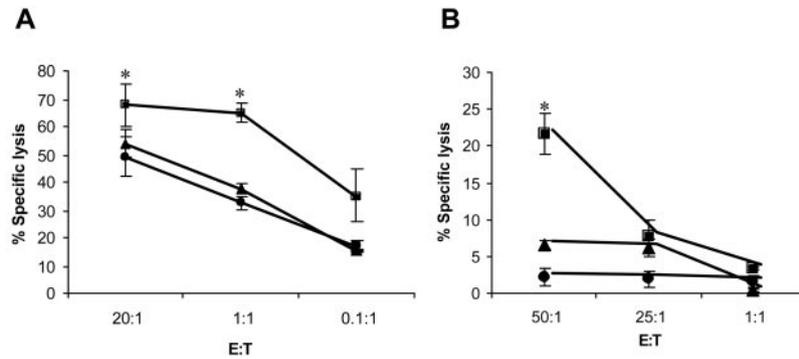


FIGURE 6. Predicted structure of V domains of h2B4 and mutant K68AE70A. A, Predicted ribbon structure of h2B4. B, Predicted ribbon structure of mutant K68AE70A, which has charged residues lysine and glutamic acid, mutated to noncharged residue alanine. The figures were generated using 3D-PSSM program. C, Ribbon structure of V domain of h2B4 showing the amino acid residues, which caused inhibition in binding to soluble CD48-Fc. Residues analyzed by site-directed mutagenesis that caused inhibition in binding to soluble CD48-Fc are shown circled, and their positions on the h2B4 model are shown. Mutants K68A and E70A shown in yellow shaded circles caused maximum inhibition of binding, whereas the other residues partially affected the binding of h2B4 to soluble CD48-Fc.

linking of mutated h2B4 on double-mutant K68AE70A RNK-16 transfectants did not show any discernible calcium mobilization (Fig. 8), which convincingly corroborates with the cytotoxicity assays.

#### Discussion

Direct cell-cell interactions are important for immune cell function to fight against foreign pathogens and tumor cells. Multiple receptor-ligand interactions present on the cell membrane determine the cell functions in both adaptive and innate immunity. In humans, CD58 is the high affinity receptor for CD2 and is the well-studied



**FIGURE 7.** Disruption of 2B4/CD48 interaction due to the double mutation K68AE70A inhibits NK cytotoxicity. *A*, Cytolytic activities of NK92 cells against K562 stably transfected with wild-type h2B4 (squares), double-mutant K68AE70A (triangles), and untransfected K562 (circles) were determined using a standard 4-h  $^{51}\text{Cr}$  release assay. *B*, Cytolytic activities of RNK-16 stably transfected with wild-type h2B4 (squares), double-mutant K68AE70A (triangles), and untransfected RNK-16 (circles) against hCD48-transfected P815 target cells were determined using a standard 4-h  $^{51}\text{Cr}$  release assay. The surface expression of wild-type and double-mutant K68AE70A h2B4 in RNK-16 and K562 cells is shown in Fig. 5, *A* and *B*. Assays were performed in triplicate, and the results are representative of three independent experiments. Each data point is the mean value of the repeated experiments, and the error bars refer to the mean SD generated from the three independent assays. Student's *t* test was used to calculate *p* values; \*, *p* < 0.05.

heterophilic adhesive interaction model. Wang et al. (45) crystallized this receptor-ligand complex and studied the interface of the structure in detail. The CD2/CD58 interactions are predominantly determined by charged amino acids present in the extracellular domain. The nature of the interface is derived from charge complementarity rather than shape complementarity. In support of this view, CD2 has more positively charged residues, whereas its counterreceptor CD58 has more negatively charged residues at the extracellular domain. Like CD2/CD58, 2B4 has seven positively charged and two negatively charged amino acid residues, whereas CD48 has five positively charged and seven negatively charged amino acid residues in the V domain. This strongly suggests that 2B4/CD48 interactions may be charge driven.

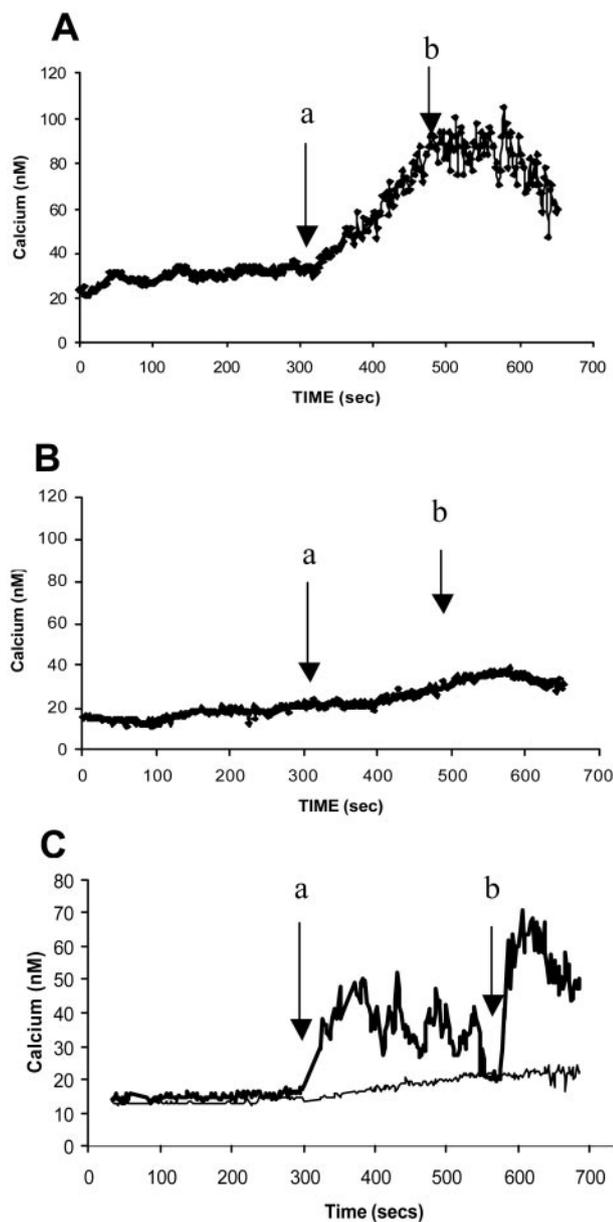
In the CD2/CD58 model, 10 salt bridges and 5 hydrogen bonds form an interdigitated salt bridge network. This array not only ensures high coligand specificity, but also contributes binding energy, because the unfavorable like-charge residues clustering in each of the binding surfaces will be neutralized upon complex formation (44). In our study, mutation at K54, G114, and K115 reduced the CD48 binding, suggesting that these amino acids are involved in initial binding by forming salt bridge as well as hydrophobic bond with CD48. This initial recognition and V domain elasticity help for corecognition of other charged amino acids to form a strong salt bridge between 2B4 and CD48. Changing K68 and E70 to alanine significantly reduces the binding and totally abolishes it in the K68AE70A double-mutated 2B4 protein, suggesting that these two are primarily involved in the CD48 binding (Figs. 4 and 5*D*). Interestingly, our data also identified a critical role for these two amino acid residues in the recognition of h2B4 by mAb C1.7 (Fig. 5*C*). CD48 has a Ser and Lys residue corresponding to positions 68 and 70 in 2B4 (Fig. 2). Therefore, it is tempting to speculate that Glu<sup>70</sup> of 2B4 may form salt bridges (electrostatic interactions) with Lys<sup>70</sup> of CD48. Moreover, E70 is conserved in h2B4 and hCD58, whereas K68 is conserved between h2B4 and hCD2 (Fig. 2). In view of the conservation of these residues in hCD2 and hCD58, it is likely that the corresponding residue in CD48 may be involved in h2B4 interaction. A limited study of h2B4 and hCD48 did not show any polymorphism (14, 16, 34, 48, 49, 53). In mice, both 2B4 and CD48 display polymorphism in their V domains that could provide a basis for 2B4/CD48 interaction in different strains of mice (54).

The poor shape complementarity and the charge-dominated interface in the CD2-CD58 complex create a suitably loose and dy-

namic contact area between T cells and target cells that does not impede subsequent TCR-peptide MHC contact. Charge complementarity between interacting surfaces of CD2 and CD58 facilitates a rapid on-rate, whereas the slight presence of hydrophobic interactions yields a fast off-rate, thereby maintaining an optimal distance between T cell and APC surface membranes for immune recognition (55). It has been shown that rapid receptor-ligand dissociation is responsible for high receptor-ligand mobility at cell-cell contacts (56, 57). This might be the case in 2B4/CD48 interaction between NK cells and tumor cells. In human NK cells, 2B4 is an activating receptor, and upon ligation with CD48, NK cells can lyse a variety of tumor cells (15, 16, 49). It is also shown that not all 2B4-expressing NK clones are activated to the same degree even though the 2B4 expression level is the same in those cell lines (16). This suggests that 2B4 is an activator as well as costimulator by creating a close environment for coengagement of other triggering receptors (58). The 2B4 present on the T cells are not directly involved in the activation of T cells. However, ligation of CD48 with neighboring T cells can augment T cell-mediated cytotoxicity, suggesting that 2B4/CD48 interaction may provide costimulation signals to T cells (35, 37).

The functional significance for the interaction of 2B4 and CD48 was analyzed by cytolytic experiments and calcium flux assays. Several studies have shown that 2B4-CD48 counterreceptors can transduce signals through both receptors (16, 28, 36, 59). We tested the effect of the double mutation K68AE70A on the cytolytic function as well as on induction of intracellular calcium. Our experiments showed decrease in  $\text{Ca}^{2+}$  levels and cytolytic activity with double-mutant K68AE70A compared with wild-type h2B4, indicating that these two charged amino acids are involved in the functional activation of both 2B4 and CD48 receptors. These two charged amino acids may be responsible for forming salt bridges between the two molecules, and may be involved in the functional activation of both 2B4 and CD48 receptors.

In conclusion, we have shown that charged amino acids in the V domain of 2B4 are involved in 2B4/CD48 interaction. We further demonstrated that the amino acid residues Lys<sup>68</sup> and Glu<sup>70</sup> in the V domain of h2B4 play a critical role in physical interaction with CD48, and disruption of this interaction impairs functional activation of NK cells through both receptors. The identification of h2B4-CD48 binding site has implication in designing small molecules that could block/modulate NK and T cell functions resulting from 2B4/CD48 interaction.



**FIGURE 8.** The double mutation, K68AE70A, inhibits calcium mobilization in RNK-16 transfectants. Calcium mobilization determined by the ratio technique fura 2 over time. *A*, RNK-16 cells stably expressing wild-type h2B4; *B*, double-mutant K68AE70A (Fig. 5A) were seeded on coverslips and loaded in 3  $\mu$ M fura 2 dye in a modified Krebs-Ringer buffer solution for 30 min at 37°C. They were treated with 10  $\mu$ g/ml soluble CD48-Fc fusion protein (*a*), followed by 20  $\mu$ g/ml goat anti-human IgG (*b*). *C*, Untransfected RNK-16 were treated either with 5  $\mu$ g/ml anti-NKR-P1A (10/78) mAb (positive control) (thick) or 10  $\mu$ g/ml soluble CD48-Fc fusion protein (thin) (*a*) and subsequently exposed to 20  $\mu$ g/ml goat anti-mouse IgG or goat anti-human IgG (*b*), respectively. The data are representative of three independent experiments.

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### Disclosures

The authors have no financial conflict of interest.

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