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CD94 and a Novel Associated Protein (94AP) Form a NK Cell Receptor Involved in the Recognition of HLA-A, HLA-B, and HLA-C Allotypes

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Summary

Whereas the human killer cell inhibitory receptors (KIRs) for HLA class I are immunoglobulin-like monomeric type I glycoproteins, the murine Ly49 receptors for H-2 are type II homodimers of the C-type lectin superfamily. Here, we demonstrate that human NK cells also express C-type lectin receptors that influence recognition of polymorphic HLA-A, HLA-B, and HLA-C molecules. These receptors are heterodimers composed of CD94 chains covalently associated with novel tyrosine-phosphorylated glycoproteins (94AP). Some NK clones recognize a common HLA-C ligand using both KIRs and CD94–94AP receptors. These findings suggest the existence of human inhibitory MHC class I receptors of the immunoglobulin and C-type lectin superfamilies and indicate overlap in ligand specificity.

Introduction

Natural killer (NK) cells preferentially kill targets lacking major histocompatibility complex (MHC) class I (Ljunggren and Karre, 1985) and express receptors for class I that are responsible for inhibition of cytotoxicity (reviewed by Lanier and Phillips, 1996). While the nature of the interactions triggering NK cell–mediated cytotoxicity are not well defined, receptors for MHC class I have been identified. In the mouse, the *Ly49* genes, members of the C-type lectin superfamily, encode type II membrane glycoproteins that bind H-2 ligands (Brennan et al., 1994; Daniels et al., 1994; Kane, 1994; Karlhofer et al., 1992; Mason et al., 1995; Stoneman et al., 1995). The human killer cell inhibitory receptors (KIRs) responsible for HLA class I recognition are type I membrane glycoproteins with homology to the immunoglobulin superfamily (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995a). Both KIRs and Ly49 receptors are expressed on subsets of NK cells and serve to inhibit cell-mediated cytotoxicity against cells bearing appropriate MHC class I allotypes. Recent studies have implicated the PTP1C tyrosine phosphatase in the inhibitory function of the KIR (Burshtyn et al., 1996; Fry et al., 1996).

The striking structural differences between the KIRs (immunoglobulin superfamily) and Ly49 receptors (C-type lectin superfamily) have presented an intriguing enigma. As yet, human homologs of the *Ly49* genes and murine counterparts of the *KIR* have not been identified, raising the possibility that the inhibitory class I receptors arose independently in the two species as a consequence of MHC divergence (Gumperz and Parham, 1995). The *Ly49* genes are on mouse chromosome 6, linked to the *NKR-P1* genes encoding C-type lectins preferentially expressed on NK cells (Giorda et al., 1992; Ryan et al., 1992; Yokoyama et al., 1990). Members of the C-type lectin superfamily encoding type II proteins expressed on NK cells have been identified on human chromosome 12 (syntenic with mouse chromosome 6), including *CD94* and a human *NKR-P1* gene (Chang et al., 1995; Lanier et al., 1994).

CD94 is expressed on most NK cells and a subset of T lymphocytes (Aramburu et al., 1990, 1991). Studies to examine the function of CD94 have demonstrated that anti-CD94 monoclonal antibodies (MAbs) can either augment, inhibit, or have no effect on NK cell–mediated cytotoxicity and cytokine production (Pérez-Villar et al., 1995). Such differences in behavior are seen comparing NK cell clones established from a single donor (Pérez-Villar et al., 1995). Moretta et al. (1994) have suggested that CD94 is a specific receptor for HLA-B alleles of the Bw6 serotype, based on the observation that certain NK cell clones expressing high levels of cell surface CD94 are able to kill Epstein–Barr virus (EBV)-transformed B lymphoblastoid cell lines expressing HLA-B7 in the presence, but not the absence of anti-CD94 MAb. In contrast, in an extensive analysis we have observed many $CD94^{bright+}$ NK cell clones that fail to recognize HLA-B7 and for which the effect of anti-CD94 MAb on HLA recognition is more complicated. Moreover, the finding that CD94 may be a single gene encoding a glycoprotein essentially lacking a cytoplasmic domain, suggested that CD94 alone may not be capable of signal transduction. This inference led us to propose an alternative model in which CD94 associates with other proteins involved in HLA recognition (Chang et al., 1995). Here, we present evidence in support of this hypothesis.

Results

Effect of Anti-CD94 MAb on NK Cell Recognition of HLA Class I Molecules

NK cell clones kill the class I–deficient 721.221 EBV-transformed B lymphoblastoid cell line, but are heterogeneous in their ability to lyse transfectants expressing different HLA class I molecules (Litwin et al., 1993). While a prior study reported that NK cell clones expressing high levels of CD94 are unable to kill target cells bearing HLA-B7 (Moretta et al., 1994), we were unable to observe a direct correlation between cell surface density of CD94 and HLA-B7 recognition in the analysis of 194 NK cell clones derived from five different blood donors (Chang et al., 1995). However, we have noted that anti-CD94

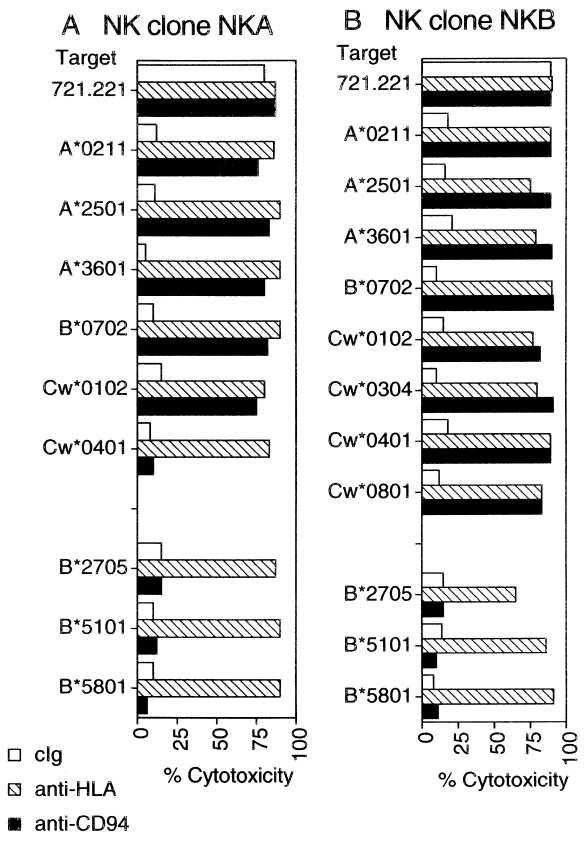

Figure 1. Effect of Anti-CD94 MAb on HLA Class I Recognition

NK cell clones NKA and NKB were assayed for lysis of 721.221 target cells or 721.221 transfectants expressing the indicated HLA-A, HLA-B, or HLA-C alleles in the presence or absence of control IgG1, anti-CD94 ($F(ab')_2$ fragments of HP-3D9), or anti-HLA class I (DX15 and DX17) MAbs. MAbs were used at 2.5 μg/ml. The effector to target cell ratio was 3:1. Clones NKA and NKB were established from an individual with the HLA haplotype A1, A68, B14 (Bw6), B57 (Bw4), Cw6. The KIR phenotype of clone NKA is $NKB1^+$, $GL183^-$, $HP\text{-}3E4^+$, and clone NKB is $NKB1^+$, $GL183^-$, $HP\text{-}3E4^-$. MAb DX9 against NKB1 reversed the protection conferred by the HLA-B alleles of the Bw4 serotype (i.e., HLA-B*2705, HLA-B*5101, and HLA-B*5801) (data not shown). Clone NKA also expresses an $HP\text{-}3E4^+$ KIR recognizing HLA-C*0401, potentially explaining the inability of anti-CD94 MAb to affect lysis of this transfectant (see Figure 2). In addition to the alleles shown in the figure, neither the NKA nor the NKB NK cell clone recognized HLA-B*1501 (Bw6), HLA-B*4601 (Bw6), HLA-B*5401 (Bw6), or HLA-B*5501 (Bw6) (i.e., these transfectants were killed to the same extent as the untransfected 721.221 cell line, data not shown).

MAb affects HLA recognition by several NK cell clones and this prompted us to investigate this activity further. In these experiments, $CD94^+$ NK cell clones were assayed for lysis of the 721.221 cell line or transfectants expressing HLA-A, HLA-B, or HLA-C molecules in the presence or absence of anti-CD94 MAb. With many NK clones, anti-CD94 MAb had no effect on the recognition of any HLA-A, HLA-B, or HLA-C allele analyzed, although all NK clones expressed CD94 on the cell surface. However, certain NK cell clones were able to kill the class I transfectants in the presence, but not in the absence, of anti-CD94 MAb. Representative data from two NK cell clones that were affected by anti-CD94 MAb are shown in Figure 1. NK clones NKA and NKB killed 721.221 target cells, but demonstrated diminished cytotoxicity against transfectants expressing HLA class I molecules. Expression of class I on the transfectants was clearly responsible for protection from NK cell lysis, as addition of anti-HLA class I MAb restored cytotoxicity to levels comparable to killing of the class I–negative 721.221 cells (Figure 1). In the presence of $F(ab')_2$ fragments of anti-CD94 MAb, NK cell lysis of transfectants expressing certain HLA-A, HLA-B, and HLA-C molecules was comparable to killing of the class I–negative 721.221 target. For NK cell clone NKA, the inhibition by HLA-A*0211, HLA-A*2403, HLA-A*3601, HLA-B*0702, HLA-Cw*0102, or HLA-Cw*0401 was reversed by anti-CD94 MAb (Figure 1A). For NK cell clone NKB, anti-CD94 MAb affected recognition of HLA-A*0211, HLA-A*2403, HLA-A*3601, HLA-B*0702, HLA-Cw*0102, HLA-Cw*0304, HLA-Cw*0401, or HLA-Cw*0801. These effects were sensitive to HLA class I polymorphism, because anti-CD94 did not affect target cell protection conferred by HLA-B*2705, HLA-B*5101, or HLA-B*5801 and, in the case of NK clone NKA, did not affect recognition of HLA-Cw*0401 (Figure 1).

Prior studies have shown that NK cell recognition of HLA-Bw4, HLA-A3, and HLA-C are mediated by a family of highly related receptors (Colonna et al., 1993; Dohring et al., 1996; Gumperz et al., 1995; Wagtmann et al., 1995a, 1995b). KIRs recognizing HLA-Bw4 ligands can be detected using the DX9 MAb (Gumperz et al., 1995). HLA-C alleles segregate into mutually exclusive groups according to their recognition by KIRs and expression of either S and N or N and K at amino acids 77 and 80 in the α1 domain of the class I heavy chain (Colonna et al., 1993). KIRs recognizing HLA-C alleles with S77 and N80 are recognized by the GL183 MAb (Moretta et al., 1993), whereas KIRs recognizing HLA-C with N77 and K80 are detected with either the EB6 (Moretta et al., 1993) or HP-3E4 MAb (Lanier et al., 1995). Analysis of the HLA specificity of NK cell clones is complicated by the fact that several KIRs can be expressed simultaneously on the cell surface of an individual NK cell clone (Lanier et al., 1995; Litwin et al., 1993; Moretta et al., 1990). Moreover, KIRs recognizing HLA-B and HLA-C ligands function independently in NK cell clones (Lanier et al., 1995). Our observation that anti-CD94 MAb affected NK cell recognition of several HLA-C alleles raised the possibility that certain NK cell clones might express both a KIR and CD94-associated class I receptor reactive with a common HLA-C allele. Therefore, we selected several KIR $HP\text{-}3E4^+$ NK cell clones and assayed them for lysis of 721.221 cells expressing HLA-Cw*0401 in the presence or absence of either anti-CD94, HP-3E4, or both MAbs. Consistent with prior observations (Lanier et al., 1995), many KIR $HP\text{-}3E4^+$ NK cell clones were unable to kill 721.221 cells expressing HLA-Cw*0401 or HLA-Cw*1503, unless assayed in the presence of HP-3E4 MAb, which permits target cell lysis. Although these NK cell clones express CD94, anti-CD94 MAb had no effect on the lysis of targets cells protected by HLA-Cw*0401 or HLA-Cw*1503. A representative NK cell clone of this phenotype is shown in Figure 2A. However, for certain KIR $HP\text{-}3E4^+$ NK cell clones the HP-3E4 MAb was unable to augment lysis of transfectants expressing HLA-Cw*0401, suggesting that such NK

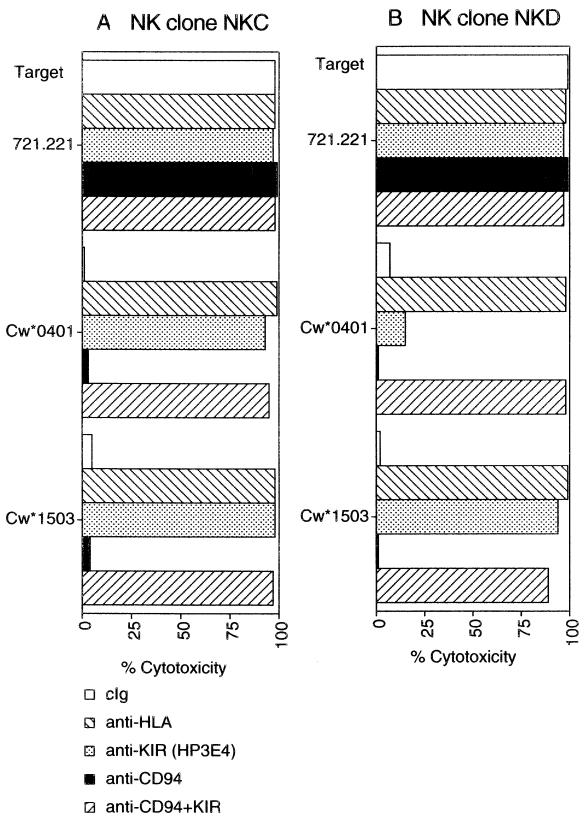

Figure 2. Effect of Anti-CD94 and Anti-KIR on HLA-C Recognition

$CD94^+$, KIR HP-$3E4^+$ NK cell clones NKC and NKD were assayed for lysis of 721.221 target cells or 721.221 transfectants expressing HLA-C*0401 or HLA-C*1503 in the presence or absence of control IgG1, anti-CD94 (DX22), anti-KIR (HP-3E4 MAb), both anti-CD94 plus anti-KIR, or anti-HLA class I (DX15 and DX17) MAbs. MAbs were used at 2.5 μg/ml. The effector to target cell ratio was 3:1. The HLA haplotype of the blood donor of NK clones NKC and NKD was not determined. The KIR phenotypes of NK cell clones NKC and NKD are $NKB1^-$, $GL183^-$, HP-$3E4^+$.

clones possess a second inhibitory receptor for HLA-Cw*0401. These particular NK cell clones were assayed for lysis of 221.Cw*0401 target cells in the presence of anti-CD94, HP-3E4, or both MAbs. The NK clone NKD failed to kill the 221.Cw*0401 transfectant in the presence of either anti-CD94 or HP-3E4 MAb; however, lysis was substantially augmented when both HP-3E4 and anti-CD94 were present (Figure 2B). However, anti-CD94 MAb had no effect on target cell protection conferred by HLA-Cw*1503 (also N77 and K80), whereas HP-3E4 MAb alone permitted lysis of this target (Figure 2B). This differential affect of anti-CD94 MAb on recognition of HLA-Cw*0401 and HLA-Cw*1503 has been observed using several NK cell clones from different blood donors.

To determine whether the effect of anti-CD94 MAb on NK cell recognition of HLA-C molecules requires expression of a KIR, we selected NK cell clones that did not react with MAb HP-3E4, GL183, or EB6. As shown in Figures 3A–3C, NK cell clones NKE, NKF, and NKG (all HP-3E4, EB6, and GL183 negative) were able to kill 721.221 cells expressing HLA-Cw*0102, HLA-Cw*0304, HLA-Cw*0401 and HLA-Cw*0801 in the presence, but not in the absence, of anti-CD94 MAb. These findings demonstrate that NK cell clones lacking the known KIRs reactive with HLA-C can indeed recognize HLA-C molecules and implicate CD94 in the process. In addition, these $EB6^-$, $GL183^-$ NK cell clones were able to recognize certain HLA-A and HLA-B alleles (e.g., HLA-A*0201, HLA-A*0211, HLA-A*2403, HLA-A*3601, HLA-B*0702) and lysis of transfectants expressing those allotypes was augmented in the presence of anti-CD94 MAb or anti-class I MAb (Figure 3).

Evidence for a Tyrosine-Phosphorylated CD94-Associated Glycoprotein

Recently, we cloned a cDNA encoding a type II protein reactive with anti-CD94 MAb (Chang et al., 1995). Given the functional effects of anti-CD94 MAb on NK cells, a notable feature of the CD94 protein was its lack of any significant cytoplasmic domain with which to transduce signals. Because a search for additional CD94 cDNA isoforms encoding a longer cytoplasmic region was unsuccessful, an analysis at the protein level was therefore undertaken. The predicted molecular mass of the polypeptide encoded by the CD94 cDNA is ~20 kDa and included in the amino acid sequence are two potential sites for N-linked glycosylation (Chang et al., 1995). As none of the existing anti-CD94 MAbs are functional in Western blot analysis, we generated a polyclonal rabbit antiserum against the entire extracellular domain of the CD94 protein encoded by the cloned cDNA (Chang et al., 1995). Specific antibodies were purified using a recombinant CD94 fusion protein Sepharose affinity column. The polyclonal anti-CD94 antiserum reacted with murine P815 cells transfected with CD94 cDNA, but not untransfected P815 cells or control transfectants, as determined by immunofluorescence and Western blot analysis (data not shown). Lysates were prepared from NK cell clones and assayed by Western blotting using the anti-CD94 serum. Analysis of 5 NK cell clones and the Jurkat T leukemia cell line (used as a negative control) is shown in Figure 4, findings that represent the results obtained using 25 different NK cell clones established from six individuals. Specific bands of ~25–30 kDa were observed in the NK cell clones, consistent with the size of the CD94 glycoprotein predicted from the cDNA sequence and observed on CD94 transfectants. However, Aramburu et al. (1990) previously demonstrated that ~43 kDa glycoproteins were immunoprecipitated from ^{125}I-labeled NK cells. Because no proteins of ~43 kDa were detected in the Western blots using the polyclonal anti-CD94 serum, this discrepancy prompted us to examine the structural basis for the difference.

NK cell clones were labeled with ^{125}I and lysates were immunoprecipitated with anti-CD94 MAb. Consistent with the prior studies (Aramburu et al., 1990), a predominant ~43 kDa ^{125}I-labeled protein was immunoprecipitated by the anti-CD94 MAb (Figure 5A). After treatment with N-glycosidase F to remove N-linked carbohydrates, this ^{125}I-labeled protein was reduced in size to ~26 kDa (Figure 5A). The relative migration of this protein was unaffected by treatment with O-glycanase, suggesting an absence of O-linked oligosaccharides (data not shown). In contrast, when P815, Jurkat, or COS7 cells

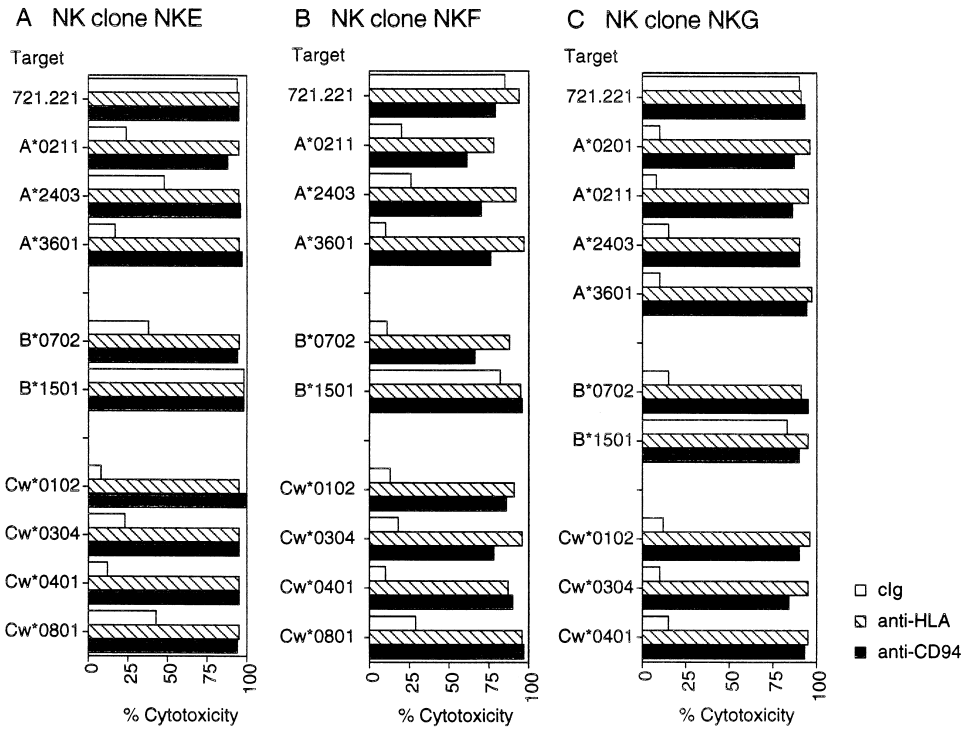

Figure 3. Effect of Anti-CD94 on HLA-C Recognition by KIR GL183- and EB6-Negative NK Cell Clones

NK cell clones NKE, NKF, and NKG (selected on the basis of not reacting with anti-KIR MAb HP-3E4, GL183, or DX9) were assayed for lysis of 721.221 target cells or 721.221 transfectants expressing the indicated HLA-A, HLA-B, or HLA-C alleles in the presence or absence of control IgG1, anti-CD94 (DX22), or anti-HLA class I (DX15 and DX17) MAbs. MAbs were used at 2.5 μg/ml. The effector to target cell ratio was 3:1. Clones NKE, NKF, and NKG were established from an individual with the HLA haplotype A1, A29, B8, B13, Cw6, Cw7. In addition to the alleles shown in the figure, NK cell clones NKE, NKF, and NKG did not recognize HLA-B*4601 (Bw6), HLA-B*5101 (Bw4), HLA-B*5401 (Bw6), HLA-B*5501 (Bw6), or HLA-B*5801 (Bw4) (i.e., these transfectants were killed to the same extent as the untransfected 721.221 cell line, data not shown).

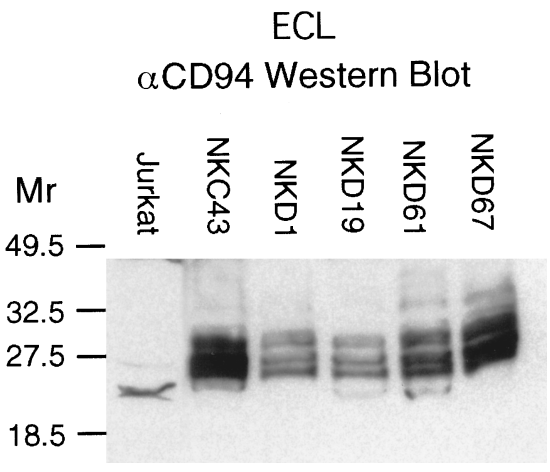

Figure 4. Western Blot Analysis of CD94 on NK Cell Clones

Lysates prepared from five NK cell clones (NKC43, NKD1, NKD19, NKD61, and NKD67) and the Jurkat T leukemia cell line (negative control) were separated by SDS–PAGE using 12% acrylamide gels (5×10^5 cell equivalents of lysate per lane) and transferred to PVDF membranes. CD94 proteins were detected by Western blot analysis using a polyclonal anti-CD94 antiserum and visualized by chemiluminescence. Western blots incubated with normal rabbit serum or HRP-conjugated anti-rabbit immunoglobulin were negative (data not shown).

transfected with CD94 cDNA were ^{125}I labeled and CD94 protein immunoprecipitated with anti-CD94 MAb, ^{125}I-labeled CD94 antigens were not visualized (Figure 5A), unless the autoradiographs were exposed for very prolonged time periods (revealing a faint ~30 kDa band, data not shown). This finding was observed in over 10 independent experiments. Because the CD94$^+$ P815 transfectants expressed CD94 cell surface antigen in amounts equal to or greater than the NK cell clones, this result suggested the protein encoded by the CD94 cDNA inefficiently labels with ^{125}I.

The ^{125}I-labeled protein immunoprecipitated from NK cells by anti-CD94 MAb is a disulfide-bonded dimer, migrating at ~70 kDa under nonreducing conditions and ~43 kDa under reducing conditions (Aramburu et al., 1990). Because the protein encoded by the CD94 cDNA poorly labels with ^{125}I and the apparent molecular mass of the ^{125}I-labeled protein on NK cells is greater than that obtained from CD94 transfectants or predicted from the cDNA sequence, we reasoned that the NK cells might express a heterodimer composed of a CD94 subunit disulfide-bonded to a second glycoprotein of ~43 kDa (~26 kDa after removal of N-glycans) that is readily visualized in ^{125}I-labeled lysates. To test this model, NK cell clones and CD94-transfected P815 cells were labeled with ^{125}I and used for immunoprecipitation with anti-CD94 MAb. After enzymatic removal of N-glycans, proteins were examined by SDS–PAGE and by Western blot

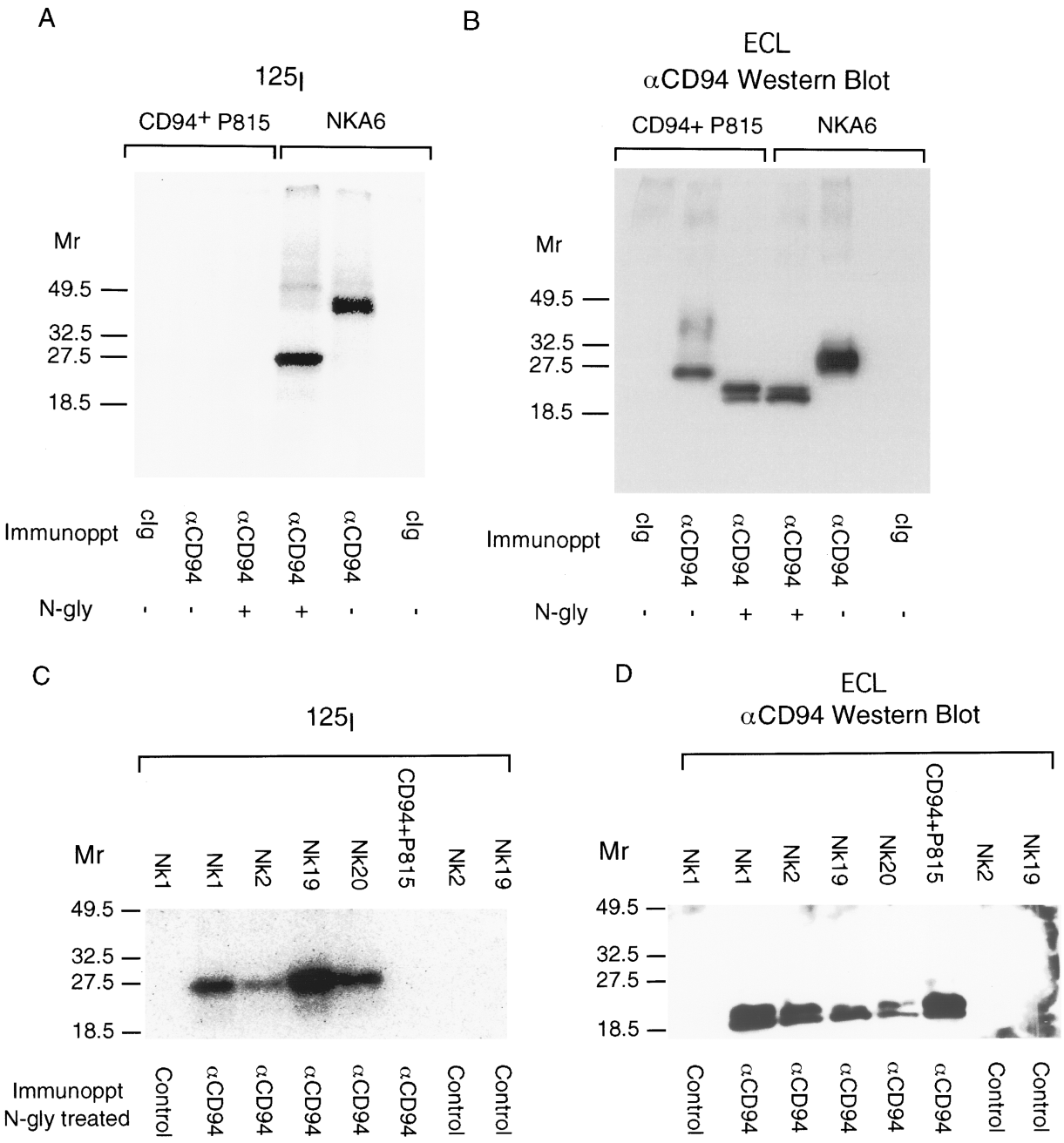

Figure 5. Detection of ^{125}I-Labeled CD94-Associated Proteins

Murine P815 cells stably transfected with human CD94 cDNA (Chang et al., 1995) and NK cell clones were labeled with ^{125}I, lysed, and immunoprecipitation was performed using control immunoglobulin (clg) or anti-CD94 MAb HP-3D9 bound to protein G–Sepharose. Immunoprecipitates were treated with N-glycosidase F (N-gly), as indicated, to remove N-linked oligosaccharides, and then analyzed by SDS–PAGE under reducing conditions using 12% acrylamide gels. Proteins were transferred to PVDF membranes and CD94 proteins were detected by Western blot analysis using an affinity-purified polyclonal anti-CD94 antiserum. ^{125}I-labeled proteins were detected by using a PhosphorImager. Proteins detected by Western blot analysis were visualized by chemiluminescence.

(A) Detection of ^{125}I-labeled proteins immunoprecipitated with anti-CD94 MAb from CD94$^+$ P815 transfectants and NK cell clone NKA6.

(B) Proteins immunoprecipitated with anti-CD94 MAb from CD94$^+$ P815 transfectants and NK cell clone NKA6 were analyzed by Western blot analysis using affinity-purified polyclonal anti-CD94 antiserum.

(C) Detection of ^{125}I-labeled proteins immunoprecipitated with anti-CD94 MAb from CD94$^+$ P815 transfectants and NK cell clones NK1, NK2, NK19, and NK20. All immunoprecipitates were treated with N-glycosidase F before SDS–PAGE.

(D) Proteins immunoprecipitated with anti-CD94 MAb from CD94$^+$ P815 transfectants and NK cell clones NK1, NK2, NK19, and NK20 were treated with N-glycosidase F and then were analyzed by Western blot analysis using affinity-purified polyclonal anti-CD94 antiserum. In (C) and (D), control immunoprecipitates for CD94$^+$ P815 and NK20 are not shown on the figure, but were negative. Note that the CD94 bands of ~20 kDa and ~21 kDa observed in the Western blots were present both in the CD94$^+$ P815 transfectant and the NK cell clones.

analysis using the polyclonal anti-CD94 antiserum. As shown in Figure 5A, no ^{125}I proteins were visualized from CD94 immunoprecipitates prepared from the CD94$^+$ P815 transfectants. However, Western blot analysis using the polyclonal anti-CD94 antiserum (Figure 5B) revealed a predominant ~20–21 kDa band after treatment with N-glycosidase F, demonstrating that the anti-CD94 MAb immunoprecipitated a protein of the expected size. By contrast, anti-CD94 MAb immunoprecipitated an ^{125}I-labeled protein of ~43 kDa (~26 kDa after treatment with N-glycosidase F) from the NK cell clone (Figure 5A). Western blot analysis revealed that this ^{125}I-labeled 26 kDa protein did not react with the polyclonal anti-CD94 antiserum, but that the NK cell clones express a ~20–21 kDa CD94 protein of identical size to that observed in the CD94$^+$ P815 transfectant (Figure 5B). Analysis of four additional NK cell clones gave similar results (Figures 5C, 5D). In all NK clones examined, a ~26 kDa ^{125}I-labeled protein was immunoprecipitated by anti-CD94 MAb; but only a ~20–21 kDa CD94 protein was detected in the immunoprecipitates by Western blot analysis. These results demonstrate that NK cells possess a ~20 kDa CD94 protein corresponding to the predicted size of the CD94 cDNA-translated product and that the ~26 kDa protein visualized by ^{125}I in NK cell clones is not reactive with a polyclonal antiserum generated against the extracellular domain of the cloned CD94 gene product.

Anti-CD94 MAb effects NK cell function despite the lack of a cytoplasmic domain in the CD94 molecule. Therefore, it seems likely that signal transduction may involve the 26 kDa protein associated with CD94 rather than the 20 kDa CD94 subunit. To examine this possibility, a CD94$^+$ NK cell line was stimulated with pervanadate (an inhibitor of protein-tyrosine phosphatases that induces protein-tyrosine phosphorylation; O'Shea et al., 1992), lysed, and immunoprecipitated with an anti-CD94 MAb. Immunoprecipitates were analyzed by Western blot using an anti-phosphotyrosine MAb. As shown in Figure 6, a phosphotyrosine-containing glycoprotein of ~43 kD was detected in the anti-CD94 immunoprecipitate from the NK cell line.

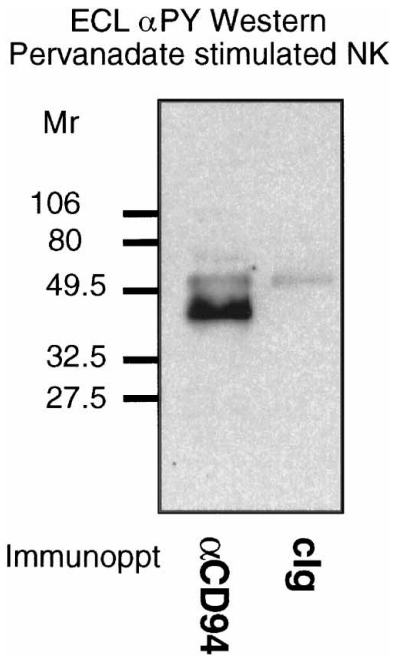

Figure 6. Tyrosine Phosphorylation of CD94-Associated Proteins

A CD94$^+$ NK cell line was stimulated with pervanadate, lysed, and immunoprecipitation was performed using control immunoglobulin (clg) or anti-CD94 MAb (DX22). Immunoprecipitates were resolved by SDS–PAGE under reducing conditions using 4%–20% acrylamide gels. Proteins were transferred to PVDF membranes and tyrosine-phosphorylated proteins were detected by Western blot analysis using HRP-conjugated anti-phosphotyrosine MAb (4G10). Proteins detected by Western blot analysis were visualized by chemiluminescence.

Discussion

The Ly49 receptors are type II membrane glycoproteins encoded by ~8 genes of the C-type lectin superfamily (reviewed by Yokoyama, 1995). Different Ly49 isoforms bind distinct polymorphic H-2 class I molecules (Brennan et al., 1994; Daniels et al., 1994; Kane, 1994), and their diverse cytoplasmic domains suggest that these receptors may transmit positive as well as negative signals (Smith et al., 1994; Wong et al., 1991). Human homologs of the *Ly49* genes have not as yet been found. Although also a member of the C-type lectin superfamily, CD94 has only limited homology with murine Ly49 and unlike Ly49 demonstrates a simple pattern on genomic Southern blot analysis, suggesting that CD94 may be encoded by a single gene (Chang et al., 1995).

While the known structural features of CD94 make it an unlikely candidate for a human Ly49 counterpart, there are intriguing effects of anti-CD94 MAb implicating this receptor in NK cell function. Using NK cell clones established from a single individual, Pérez-Villar et al. (1995) have shown that anti-CD94 MAb can either augment, inhibit, or have no effect on the lysis of Fc receptor–bearing xenogeneic target cells. Additionally, Moretta et al. (1994) have reported that certain NK cell clones kill HLA-B7$^+$ target cells in the presence, but not in the absence, of anti-CD94 MAb. These investigators concluded that CD94 is a specific receptor for HLA-B7 and other class I molecules expressing the Bw6 serotype. However, since essentially all NK cells express CD94 (Lopez-Botet, 1995) and in general only a minor subset of NK cell clones in many individuals recognize HLA-B7 (Litwin et al., 1993), the CD94 molecule expressed on most NK cells is probably not a specific receptor for HLA-B7. Nonetheless, our studies have confirmed that anti-CD94 MAb can affect recognition of HLA-B7 by some NK cell clones, but the apparent involvement of CD94 in HLA recognition is more complex than appreciated, likely due to the limited selection of NK cell clones and target cells previously analyzed.

We present evidence that anti-CD94 MAb can effect NK cell killing of the 721.221 B lymphoblastoid cell line transfected with certain HLA-A, HLA-B, or HLA-C genes. Comparison of the class I alleles recognized by different NK cell clones and affected by anti-CD94 MAb failed to provide a simple explanation for the specificity of this putative class I receptor. In particular, in several NK

cell clones anti-CD94 MAb affected recognition of HLA-B*0702, but not other HLA-B alleles possessing the Bw6 serological epitope (e.g., HLA-B*1501), indicating that CD94 receptors are not specific for Bw6. Neither was there evidence that CD94 receptors recognize the Bw4 motif, because anti-CD94 MAb did not affect recognition of HLA-Bw4 alleles (e.g., HLA-B*5101, HLA-B*5801). Amongst the HLA-C alleles, CD94 receptors failed to discriminate the polymorphism at amino acids 77 and 80 previously defined for the KIR molecules identified by the EB6 and GL183 MAbs (Colonna et al., 1993). However, it should be appreciated that analysis of the HLA specificity of NK cells is complicated by the fact that a single NK clone can simultaneously express two or more KIRs that function independently (Lanier et al., 1995; Moretta et al., 1990; Wagtmann et al., 1995a). Indeed, we have demonstrated that both anti-CD94 MAb and anti-KIR HP-3E4 MAb can affect recognition of HLA-Cw*0401 by an individual NK cell clone, suggesting overlap in ligand specificity of these receptors. The effect of anti-CD94 MAb on recognition of HLA-C was observed even in NK cell clones that do not express KIRs reactive with the EB6, HP-3E4, or GL183 MAbs, indicating that the EB6 and GL183 reactive KIRs are not required for HLA-C recognition. Finally, with many NK cell clones anti-CD94 MAb had no effect on recognition of any class I molecule examined, although if these NK cell clones also expressed KIRs reactive with the same class I molecules, this would complicate the interpretation of these negative results.

The disparate functional effects of anti-CD94 MAb on different NK cell clones might be explained either by structural heterogeneity of the CD94 molecule itself or association with another molecule responsible for HLA binding and signal transduction. We previously demonstrated that the CD94 cDNA lacks a cytoplasmic domain (Chang et al., 1995), and in further experiments we were unable to identify other CD94 isoforms containing a longer cytoplasmic region (unpublished data). Another unexpected finding was an apparent discrepancy in the predicted size of the protein encoded by the CD94 cDNA (Chang et al., 1995) and the protein detected on the surface of ^{125}I-labeled NK cells by immunoprecipitation with anti-CD94 MAb (Aramburu et al., 1990). We now present evidence suggesting that CD94 receptors are heterodimers composed of ~30 kDa CD94 subunits disulfide-bonded to novel CD94-associated proteins (94AP) of ~43 kDa that are tyrosine phosphorylated after NK cell activation (thus implying the existence of a cytoplasmic region and possible involvement in signal transduction). The observation that the ~43 kDa 94AP readily label with ^{125}I, whereas the CD94 proteins are difficult to visualize by ^{125}I labeling suggests the extracellular region of the 94AP also differs from CD94. Furthermore, a polyclonal rabbit antiserum generated against the entire extracellular domain of a recombinant CD94 glycoprotein did not bind to the ~43 kDa 94AP. However, this polyclonal anti-CD94 serum did detect a glycoprotein of the size predicted from the CD94 cDNA in all NK cell lines and clones examined, demonstrating that a CD94 antigen reactive with the polyclonal antiserum is present in these NK cells. It is possible that the ~43 kDa 94AP are isoforms of the CD94 gene generated by alternative splicing; however, if this is the case it is surprising that a polyclonal antiserum against the entire CD94 extracellular domain failed to react with this protein. Alternatively, 94AP may be encoded by a different gene or genes and these proteins may form heterodimers with CD94 subunits. Because it disulfide-bonds to CD94, it seems likely that the 94AP is also a type II glycoprotein, structurally similar to CD94. Several genes of the C-type lectin superfamily encoding type II receptors preferentially expressed on NK and T cells are present in the NK complex on human chromosome 12, making it possible that the gene or genes encoding the 94AP will be found in this region.

^{125}I-labeled CD94-associated glycoproteins have been detected on all NK cell clones examined, irrespective of their class I specificity. While CD94–94AP receptors are clearly involved in recognition of certain class I molecules, it is uncertain whether this complex is directly responsible for HLA recognition or serves as a coreceptor, similar to the role of the CD8$\alpha\beta$ heterodimer in T cell receptor recognition of class I. Comparison of different NK cell clones has revealed subtle biochemical differences in the 94AP molecules, suggesting that the 94AP may be structurally heterogeneous (L. L. L., unpublished data). If the 94AP are heterogeneous and differentially expressed in NK cell clones, this could account for the unexplained functional effects of anti-CD94 MAb on different NK cells and on the recognition of diverse class I molecules. It is tempting to speculate that the 94AP may represent functional homologs of the murine Ly49 receptors; however, this awaits the molecular characterization of the 94AP.

Experimental Procedures

NK Cell Clones

NK cell clones ($CD3^-$, $CD56^+$) were isolated from the blood of healthy adults by single cell cloning using a flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California). Cells were cultured as described previously (Litwin et al., 1993; Yssel et al., 1984).

Antibodies

DX22 (an IgG1 MAb against human CD94) and DX24 (an IgG1 MAb against human KIR also reactive with GL183 MAb) were generated by immunizing BALB/c mice with an NK cell clone and fusing splenocytes with the Sp2/0 myeloma cell line using standard techniques. Anti-KIR MAbs GL183 and EB6 (Moretta et al., 1990) were provided by Drs. L. and A. Moretta. Anti-CD94 MAbs HP-3D9 and HP-3B1 (Aramburu et al., 1990; Lopez-Botet, 1995) and anti-KIR MAb HP-3E4 (Melero et al., 1994) were provided by Dr. M. Lopez-Botet. Anti-HLA class I MAbs DX15 and DX17 (both murine IgG1) were described previously (Gumperz et al., 1995; Phillips et al., 1995). Other MAbs were the gift of Becton Dickinson Immunocytometry Systems. $F(ab')_2$ fragments of murine IgG1 MAb were prepared by digestion with immobilized pepsin (Pierce Chemicals, Rockford, Illinois) (10 mg immunoglobulin in 0.2 M sodium citrate, 0.15 M sodium chloride [pH 3.5] were added to 1 ml immobilized pepsin and incubated at 37°C for 2 hr). Intact antibodies were removed by using protein A affinity chromatography and the purity of the $F(ab')_2$ fragments was determined by SDS–PAGE.

Immunofluorescence and Flow Cytometry

These methods have been described previously (Lanier and Recktenwald, 1991).

Cytotoxicity Assays

Cell-mediated cytotoxicity was measured using a 4 hr ^{51}Cr radioisotope release assay (Lanier et al., 1995). Alternatively, cytotoxicity was monitored using an Alamar blue viability assay. In this assay, effector cells at various concentrations were combined with 4×10^4 target cells in flat-bottomed 96-well microtiter plates (Falcon, Lincoln Park, New Jersey) and incubated at 37° for 18 hr. Alamar blue (Alamar Biosciences, Sacramento, California) was added to each well (25 μl Alamar blue/well) and the plates were incubated at 37°C for an additional 4–6 hr with occasional mixing. After incubation, the absorbance was measured at 570 nm and 600 nm using a 96-well microtiter plat reader (V Max kinetic microplate reader, Molecular Devices, Menlo Park, California). Percent viability was determined according to the following equation: Percent viable target cells =

$$\frac{\text{absorbance (600 nm} - \text{570 nm) of effector + target cells} - \text{absorbance of effector cells only}}{\text{absorbance of target cells alone}} \times 100$$

Controls consisted of target cells incubated in the absence of effector cells and effector cells incubated without target cells.

Polyclonal CD94 Antisera

A chimeric IgG Fc–CD94 fusion protein was produced by inserting a cDNA encoding the extracellular (EC) domain of CD94 (amino acids 44–179) (Chang et al., 1995) into the pMECD8Ig vector (provided by Dr. K. Moore), a derivative of the SRα expression vector (Takebe et al., 1988), containing a CD8 leader segment and a human IgG Fc fragment. Because CD94 is a type II protein, the human IgG Fc–CD94 EC fusion protein was constructed with the IgG Fc fragment at the N terminus and the CD94 EC at the COOH terminus of the fusion protein. This was achieved by using oligonucleotide primers to amplify the EC domain of CD94 that contained an XhoI restriction enzyme site at the 5′ end of the sense primer and a NotI restriction enzyme site after the CD94 termination codon in the antisense primer. The pMECD8IgG vector was digested with XhoI and NotI and the CD94 EC cDNA was inserted by ligation immediately after the IgG Fc fragment. The CD8IgG–CD94 EC cDNA was sequenced to confirm the construction. Recombinant protein was generated by transient expression in COS7 cells and was affinity purified from supernatants using protein A–Sepharose chromatography. Rabbits (Babco, Berkeley, California) were immunized with the IgG Fc–CD94 EC fusion protein and specific antibodies were affinity purified by binding to IgG Fc–CD94 EC fusion protein covalently linked to CNBr-activated Sepharose. Rabbit antibodies were absorbed with human immunoglobulin and serum proteins (50% saturated ammonium sulfate–precipitated proteins from normal human serum) covalently coupled to CNBr–Sepharose. Affinity-purified rabbit anti-CD94 antibodies specifically reacted with human CD94, as determined by immunofluorescence and Western blot analysis using CD94 transfectants.

Transfectants

The MHC class I–deficient EBV-transformed B lymphoblastoid cell line 721.221 (Shimizu and DeMars, 1989) was provided by Drs. R. DeMars. 721.221 cells were stably transfected with HLA-A, HLA-B, and HLA-C cDNA, as described previously (Gumperz et al., 1995; Litwin et al., 1993). Transfectants were routinely monitored for expression of surface HLA class I by flow cytometry. The murine mastocytoma cell line P815 was stably transfected with CD94 cDNA (Chang et al., 1995) in the pBJ expression vector (Lin et al., 1990).

Biochemistry

Cells were labeled with ^{125}I (Amersham, Arlington Heights, Illinois) using glucose oxidase and lactoperoxidase and lysed in 1% NP-40 (Sigma Chemicals, St. Louis, Missouri) lysis buffer (50 mM Tris, 150 mM NaCl [pH 8.0]) containing protease inhibitors (1 mM PMSF and 20 Kallikrein inhibitor U/ml aprotinin) and phosphatase inhibitors (1 mM EGTA, 10 mM sodium fluoride, 1 mM tetrasodium pyrophosphate, 0.1 mM β-glycerophosphate, 1 mM sodium orthovanadate), by the method described previously (Lanier et al., 1988). Cell lysates were incubated on ice for 2 hr with antibody-coated protein G–Sepharose (Sigma) or Pansorbin (Calbiochem, San Diego, California), as described previously (Lanier et al., 1988), and were washed in stringent wash buffer (50 mM Tris, 150 mM NaCl [pH 8.5]) containing 1% Triton X-100 (Sigma), 0.1% SDS (Bio-Rad, Richmond, California), 1% sodium deoxycholate (Sigma), and protease inhibitors. In some experiments, anti-CD94 MAbs were covalently coupled to protein G–Sepharose by using 10 mM dimethyl pimelimidate–2HCl (DMP, Pierce), as described by Schneider et al. (1982). Immunoprecipitates were incubated for 18 hr at 37°C with an optimal concentration of N glycosidase F (Boehringer Mannheim, Indianapolis, Indiana), using the conditions recommended by the supplier to remove N-linked oligosaccharides. Samples were analyzed by SDS–PAGE (Bio-Rad or Novex, San Diego, California) and radioactivity was detected using a PhosphorImager (Molecular Devices, Sunnyvale, California). For Western blot analysis, cell lysates (1×10^8 cells/ml in 1% NP-40 lysis buffer) or immunoprecipitates were solubilized in sample buffer (62.5 mM Tris [pH 6.8], 2.3% SDS, 10% glycerol, with or without 5% 2-mercaptoethanol), separated by SDS–PAGE, and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, Massachusetts) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were incubated overnight at room temperature in blocking buffer (15 mM Tris, 150 mM NaCl [pH 8.0]), containing 0.05% NaN_3, 0.5 g/ml $MgCl_2$, 0.5% Tween 20 (Sigma), and 10% nonfat dry milk (Carnation, Nestle Food Company, Glendale, California) and then were incubated for 2 hr at room temperature with blocking buffer containing primary antibodies (1 μg/ml). Blots were washed three times for 5 min in wash buffer (15 mM Tris, 150 mM NaCl [pH 8.0]), containing 0.5 g/ml $MgCl_2$ and 0.5% Tween 20, and then incubated for 45 min at room temperature in 20 ml wash buffer containing 1/1000 horseradish peroxidase (HRP)-conjugated $F(ab')_2$ donkey anti-rabbit immunoglobulin (Amersham), 3% bovine serum albumin (Sigma), 50 μl pooled human serum (PelFreez, Brown Deer, Wisconsin) and 50 μl fetal bovine serum (Sigma). Blots were washed twice in wash buffer and once in wash buffer containing 0.5 M NaCl and 0.1% SDS and then developed for 1 min at room temperature with SuperSignal CL-HRP substrate (Pierce). Chemiluminescence was detected by autoradiograph (BioMax Film, Kodak).

For detection of phosphorylated proteins, cells were incubated at 37°C for 10 min in phosphate-buffered saline (pH 7.4) containing freshly prepared sodium pervanadate (100 μM sodium orthovanadate and 10 mM H_2O_2, Sigma) (O'Shea et al., 1992) and then lysed in 1% NP-40 Tris-buffered saline (50 mM Tris, 150 mM NaCl [pH 8.0]) with protease inhibitors (1 mM PMSF and 20 Kallikrein inhibitor U/ml aprotinin) and phosphatase inhibitors (1 mM EGTA, 10 mM sodium fluoride, 1 mM tetrasodium pyrophosphate, 0.1 mM β-glycerophosphate, 1 mM sodium orthovanadate) (Lanier et al., 1988). Lysates were precleared and CD94 proteins were immunoprecipitated with immobilized anti-CD94 MAb (Lanier et al., 1988). Immunoprecipitates were electrophoresed on 4%–20% acrylamide gels, blotted to PVDF membranes, and phosphorylated proteins detected by using HRP-conjugated anti-phosphotyrosine MAb PY20 (Zymed, South San Francisco, California) or HRP-conjugated anti-phosphotyrosine MAb 4G10 (UBI, Lake Placid, New York). Blots were incubated at room temperature for 2 hr in 1 μg/ml MAb in 15 mM Tris, 150 mM NaCl, (pH 8.0), containing 0.5 g/ml $MgCl_2$, 0.5% Tween 20, and 5% bovine serum albumin.

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