Tyrosine kinase activity is associated with CD44 in human neutrophils

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Abstract CD44 appears to be involved in signal transduction, however, the mechanism of this function is unclear. Protein kinase activity was detected in neutrophils associated with CD44. Most of the protein kinase activity associated with these antigens was tyrosine kinase activity. Src family kinases lyn and hck were found to account for much of the associated tyrosine kinase activity. The data suggest that associated tyrosine kinase activity may play a role in signal transduction from CD44 in neutrophils to regulate other cell functions.

Key words: Signal transduction; Protein kinase; Neutrophil; CD44; Inflammation; Phagocyte

1. Introduction

CD44, also known as Pgp-1 and Hermes, is a type I transmembrane protein, expressed as a family of isoforms generated by alternative RNA splicing and post-translational modifications, on a variety of cells [1]. CD44 binds hyaluronic acid and osteopontin [2], and appears to play a role in inflammation, leukocyte adhesion and homing, including adhesion to endothelial cells and homophilic leukocyte aggregation, and is also capable of signaling, resulting in T-cell activation [1,3–9]. In addition, CD44 may play a role in metastasis, since CD44 expression is associated with metastatic potential in a variety of malignancies [1,8–14].

The mechanism of signaling via CD44 is unclear [1,15]. Protein phosphorylation is an important mechanism of regulating protein function, and CD44 has potential serine phosphorylation sites in its intracellular domain and is constitutively phosphorylated [1,15]. In addition, recent studies have suggested that CD44 may be associated with the tyrosine kinase lck, and signal via phosphorylation pathways in T-cells [16]. When CD44 was immunoprecipitated from solubilized neutrophils, protein tyrosine kinase activity associated with this antigen was detected in the immunoprecipitates. The src family kinases lyn and hck were found to account for much of this activity. The data suggest that tyrosine kinase activity associated with CD44 may play a role in the transduction of regulatory signals in neutrophils.

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Abbreviations: DFP, diisopropylfluorophosphate; NMS, normal mouse serum; NRS, normal rat serum; NaCl-HEPES, 145 mM NaCl, 20 mM HEPES, pH 7.3; Brij solubilization buffer, 20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mM PMSF, 2 mM $MgCl_2$, 0.02% NaN_3, and 1.0% Brij 58; Brij wash buffer, 20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mg/ml BSA, 0.5% Brij 58, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, 1 mM PMSF, and 0.02% NaN_3; Labeling buffer, NaCl-HEPES, 0.1% Brij 58, 6 mM $MnCl_2$, 40 mM $MgCl_2$, 200 μM Na_3VO_4, 200 μM Na_2MoO_4, and 10 μCi of [γ-^{32}P]ATP; Buffer A, 1 mg/ml BSA, 0.05% NP-40, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.05% NaN_3; Brij-SA buffer, 1 mg/ml BSA, 0.05% Brij 58, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.05% NaN_3; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Cell preparation

Neutrophils were prepared from heparinized (2 units/ml) human venous blood as described [17] and were suspended at the indicated concentrations in 145 mM NaCl, 20 mM HEPES (Gibco, Grand Island, NY, USA), pH 7.3 (NaCl-HEPES). Differential cell counts on Wright-stained cells routinely revealed greater than 95% neutrophils. Viability as assessed by trypan blue dye exclusion was greater than 98%.

2.2. Antibodies and reagents

mAb AHN-12 (IgG1) (CD45) has been previously described [18]. CD44 mAbs were obtained from the Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens including the following CD44 mAbs: CD44-6B6, CD44-9F5, A3D8, HP2/9, BRIC214, and 212.3, as was the CD66de mAb COL-1. The T-cell reactive mAbs 5T-0111 (MEM-24) (IgG1) and 5T-012 (263K 3F4) (IgG2a) were obtained from the T-cell panel of the same Workshop. AHN-17 (IgG1) and AHN-17.1 (IgG1) recognize migration inhibitory related protein -8 and -14, and were described in the same Workshop. Polyclonal antibodies to yes, src, lck, lyn, fyn, hck, and fgr were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Polyclonal antibodies to yes, fyn, src, and fgr were also obtained as a gift from Dr. F. Uckun, University of Minnesota Medical School, Minneapolis, MN, USA. Antiserum to syk was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Normal mouse serum (NMS) and normal rat serum (NRS) were purchased from Sigma (St. Louis, MO, USA).

2.3. Immunoprecipitation and PAGE

Immunoprecipitation was performed as previously described with minor modifications [19,20]. Briefly, 2×10^7 cells were suspended in 1.1 ml of Brij solubilization buffer (20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mM PMSF, 2 mM $MgCl_2$, 0.02% NaN_3, and 1.0% Brij 58 (Pierce)), and incubated on ice for 15 min. The suspensions were then centrifuged at $9800\times g$ for 15 min at 4°C. The resulting supernatants were used for immunoprecipitation or analyzed by SDS-PAGE directly.

For immunoprecipitations, 1 ml of 10% *Staphylococcus aureus* (Pansorbin A, Calbiochem, La Jolla, CA, USA) was mixed with 100 μl of rabbit anti-mouse IgG^{H+L} (Organon Teknika, Westchester, PA, USA), and the mixture was incubated at 4°C for 1 h. One ml of buffer A (1 mg/ml BSA, 0.05% NP-40, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.05% NaN_3) was added and the Pansorbin A-antibody complex was recovered by centrifuging at $2000\times g$ for 10 min at 4°C and resuspended in 390 μl of buffer A and 40 μl of ascites containing the indicated mAb, or normal mouse serum (NMS) was added, and the mixtures were incubated for 2 h at 4°C. The Pansorbin A-antibody complex was recovered by centrifuging at $2000\times g$ for 10 min at 4°C, washed once with 1 ml of buffer A, and resuspended in 1 ml of Brij-SA buffer (1 mg/ml BSA, 0.05% Brij 58, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.05% NaN_3). Cell proteins

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were then immunoprecipitated in reaction mixtures containing the neutrophil extract, Pansorbin A-bound antibody complex, 0.5% Brij 58, 1 mg/ml BSA, 20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, and 1 mM PMSF, in a total volume of approximately 300 μl in 10×75 mm glass tubes. After 1 h at 4°C, the mixture was washed three times by adding 1 ml of Brij-wash buffer (20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mg/ml BSA, 0.5% Brij 58, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, 1 mM PMSF, and 0.02% NaN_3), and centrifuging at 2000×*g* for 12 min at 4°C. The pellet was then suspended in 1 ml of NaCl-HEPES, transferred to a 1.5-ml Eppendorf tube and pelleted at 9800×*g* for 10 min at 4°C.

2.4. Protein kinase assay

Protein kinase activity was detected as previously described [20]. Briefly, immunoprecipitates were suspended in 30 μl of NaCl-HEPES. Thirty μl of labeling buffer (NaCl-HEPES, 0.1% Brij 58, 6 mM $MnCl_2$, 40 mM $MgCl_2$, 200 μM Na_3VO_4, 200 μM Na_2MoO_4, and 10 μCi of [γ-^{32}P]ATP (sp. act. 4500 Ci/mmol, ICN, Irvine, CA, USA) was then added, and the mixtures were incubated for 10 min at 23°C. The reaction was stopped by adding 4× Laemmli sample buffer containing 400 μM ATP, incubated at 100°C for 2 min, and analyzed by SDS-PAGE [21]. Molecular weight standards were purchased from Sigma. Gel slabs were stained, dried, and subjected to autoradiography by using Dupont Cronex film.

2.5. Identification of specific tyrosine kinases

To identify specific tyrosine kinases associated with CD44, neutrophils were solubilized in Brij solubilization buffer, immunoprecipitated with the indicated mAb, and the immunoprecipitates were radiolabeled with [γ-^{32}P]ATP as described above, and ^{32}P-labeled proteins were reimmunoprecipitated as previously described [20]. Briefly, the precipitate was washed once with 600 μl of NaCl-HEPES containing 1 mM Na_3VO_4, 1 mM Na_2MoO_4, and 400 μM ATP, and centrifuged at 9800×*g* for 5 min at 4°C. One ml of NaCl-HEPES containing 1 mM Na_3VO_4, 1 mM Na_2MoO_4, and 400 μM ATP was added and the mixture was transferred to a new Eppendorf tube and centrifuged at 9800×*g* for 5 min at 4°C. Four hundred μl of NaCl-HEPES containing 1% SDS, 200 μM Na_3VO_4, and 200 μM Na_2MoO_4 was added to the precipitate, and the mixture incubated at 100°C for 2 min and then 14 min at 23°C. The mixture was centrifuged at 13 000×*g* for 5 min at 23°C, and the pellet was incubated with 200 μl of NaCl-HEPES containing 1% SDS, 200 μM Na_3VO_4, and 200 μM Na_2MoO_4 for 10 min at 23°C, and recovered by centrifuging at 13 000×*g* for 5 min. The supernatants were pooled and frozen until use. The supernatants were then diluted with NaCl-HEPES containing 1% NP-40, 200 μM Na_3VO_4, and 200 μM Na_2MoO_4 to 0.1% SDS, and immunoprecipitated as described above for 1 h in reaction mixtures containing the indicated antibody bound to 20 μl of protein A-agarose beads (Sigma), and washed four times as described above, except that the beads were centrifuged at 400×*g* for 5 min at 4°C, and the wash buffer consisted of 20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mg/ml BSA, 0.5% NP-40, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, 1 mM PMSF, and 0.02% NaN_3. The resulting immunoprecipitate was analyzed by SDS-PAGE and autoradiography as described above.

2.6. Phosphoamino acid analysis

Phosphoamino acid analyses were performed as previously described [20,22]. Briefly, radiolabeled proteins resolved by SDS-PAGE were transblotted onto Immobilon-P (PVDF) paper (Millipore, Bedford, MA, USA), localized by autoradiography, and excised. The proteins on the PVDF were then hydrolyzed in vacuo in constantly boiling 6 M HCl for 2 h at 110°C. The HCl was removed by evaporation using a SpeedVac (Savant Instruments). The dried, partially hydrolyzed samples were then dissolved in water containing phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) (Sigma) each at 1 mg/ml. The samples were then spotted on aluminum sheets precoated with silica gel (0.2 mm layer thickness, 11 cm height, Merck Laboratories, EM Science, Gibbstown, NJ, USA) and resolved by one-dimensional thin layer chromatography in ethanol/25% NH_4OH (3.5:1.6). The chromatography cycle was repeated three times to achieve optimal separation. Between cycles, plates were air dried followed by chromatography in the same solvent. The radiolabeled phosphoamino acids were detected by autoradiography using X-Omat AR film.

3. Results

3.1. Identification of protein kinase activity associated with CD44

To determine if protein kinase activity was present in the material immunoprecipitated by CD44 mAbs, we analyzed immunoprecipitates from unlabeled neutrophils for their ability to incorporate [γ-^{32}P]ATP into precipitated proteins as described in Section 2. When neutrophils were solubilized as described in Section 2, protein kinase activity was detected in the material immunoprecipitated by CD44 mAbs (Fig. 1). When [γ-^{32}P]ATP was added to the material immunoprecipitated by the CD44 mAb CD44-6B6 (Fig. 1, lane 3) or any of five other CD44 mAbs (CD44-9F5, A3D8, HP2/9, BRIC214, and 212.3, not shown), ^{32}P was incorporated into at least four distinct proteins of ~100–108, 64, 55–60, and 50–52 kDa, that were not present when material was immunoprecipitated by NMS (lane 1), the T-cell reactive mAb 5T-012 (lane 2), or the T-cell reactive mAb 5T-011, the CD45 mAb AHN-12, the CD66de mAb COL-1, or mAbs AHN-17 or AHN-17.1 (not shown).

Each of the four individual ^{32}P-labeled proteins identified in the CD44 immunoprecipitate shown in Fig. 1 were excised from the gel and examined for phosphoamino acid content. Phosphoamino acid analyses of these proteins revealed that

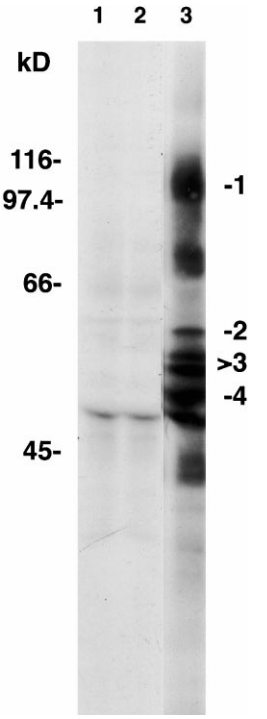

Fig. 1. Co-immunoprecipitation of protein kinases with CD44. Neutrophils were solubilized in Brij solubilization buffer and immunoprecipitated with the indicated antibody, and the immunoprecipitates were then incubated with [γ-^{32}P]ATP as described in the text. The resulting phosphoproteins were resolved by SDS-PAGE and visualized by autoradiography. Immunoprecipitating antibodies: NMS (lane 1), the T-cell reactive mAb 5T-011 (lane 2), and the CD44 mAb CD44-6B6 (lane 3). The numbers at the right indicate the bands subjected to phosphoamino acid analysis. Proteins used as molecular weight standards were: myosin heavy chain, 200 000; *Escherichia coli* β-galactosidase, 116 000; phosphorylase *a*, 97 400; bovine serum albumin, 66 000; and ovalbumin, 45 000. A duplicate experiment gave similar results.

the majority of radiolabel in each of these four proteins was present on tyrosine, demonstrating the presence of tyrosine kinase activity (Fig. 2, lanes 1–4).

3.2. Identification of tyrosine kinases associated with CD44

As expected, several src family kinases, including yes, src, lyn, hck, fgr, and syk, were detected in neutrophils [17]. To identify the tyrosine kinases coprecipitated with CD44, reimmunoprecipitation with antisera to specific tyrosine kinases was employed. The associated kinase assay was performed as in Fig. 1, and the ^{32}P-labeled proteins were dissociated from the immune complexes and reimmunoprecipitated with antisera to specific tyrosine kinases as described in Section 2 (Fig. 3). Reimmunoprecipitation of ^{32}P-labeled proteins generated from immunoprecipitates with the CD44 mAb CD44-6B6 demonstrated the presence of lyn (Fig. 3, lane D) and hck (lane G). No ^{32}P-labeled proteins were detected in the immunoprecipitates with normal rat serum (NRS) (lane A), or antisera to yes (lane B), fyn (lane C), src (lane F), or syk (lane H). ^{32}P-labeled proteins were immunoprecipitated by antisera to fgr (lane E), however, preclearing experiments demonstrated that these ^{32}P-labeled proteins were lyn and hck, reflecting some cross-reactivity of the anti-fgr antisera (not shown).

3.3. Activation of lyn and hck by CD44 antibodies

To determine if CD44 antibody binding to the neutrophil surface could alter the activity of lyn and hck, cells were incubated with the CD44 mAb, CD44-6B6, or NMS for 15 min at 37°C in HBSS, and either solubilized, or incubated another 15 min in the presence of anti-mouse IgG and then solubilized, in cell solubilization buffer. The solubilized cell extracts were then immunoprecipitated with antibodies to lyn and hck as described above, and the kinase activity present in the immunoprecipitate was assayed by radiolabeling with [γ-^{32}P]ATP as described above. No alteration in the kinase activity of lyn or hck following incubation with the mAb was detected (not shown).

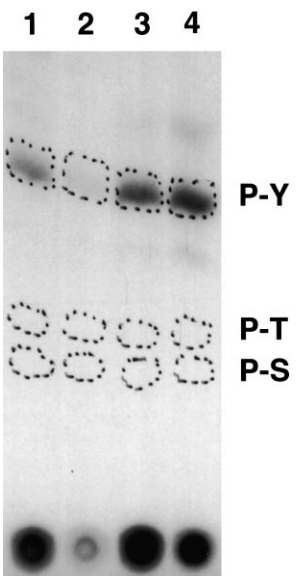

Fig. 2. Phosphoamino acid analysis of proteins co-precipitated by CD44 mAbs and labeled in an in vitro kinase assay. Neutrophils were solubilized in Brij solubilization buffer and immunoprecipitated with the CD44 mAb CD44-6B6 (right) as described in Fig. 1, and the immunoprecipitates were incubated with [γ-^{32}P]ATP. The resulting phosphoproteins were resolved by SDS-PAGE as in Fig. 1, transferred to Immobilon, visualized by autoradiography, excised, subjected to acid hydrolysis, and the resultant phosphoamino acids resolved by thin layer chromatography, and visualized by autoradiography as described in the text. The lane numbers correspond to the phosphoproteins numbered in Fig. 1 (lane 1: ~100–108 kDa; lane 2: ~64 kDa; lane 3: ~55–60 kDa; lane 4: ~50–52 kDa). The positions of migration of authentic phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) are indicated by dotted lines.

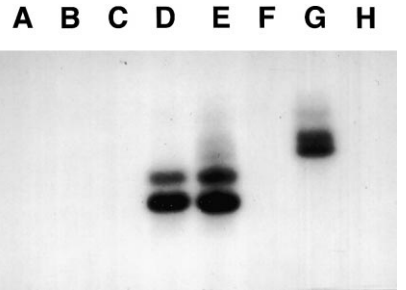

Fig. 3. Immunoprecipitation of tyrosine kinases associated with CD44. Neutrophils were solubilized in Brij solubilization buffer, immunoprecipitated with the CD44 mAb CD44-6B6, and the immunoprecipitates were incubated with [γ-^{32}P]ATP as described in the text and Fig. 1. The immunoprecipitates were then incubated at 100°C for 2 min in a buffer containing 1% SDS, cooled to 23°C, and the supernatant diluted to 0.1% SDS as described in the text. These extracts were then immunoprecipitated with NRS (lane A), yes (lane B), fyn (lane C), lyn (lane D), fgr (lane E), src (lane F), hck (lane G), or syk (lane H), and analyzed by SDS-PAGE and autoradiography as described in the text.

4. Discussion

CD44 appears to function directly as a cell adhesion molecule, but also seems to be capable of transmitting an activation signal that regulates cellular function [1–9,15]. While the details of the 'activation signal' transmitted by CD44 are not known, the findings reported here suggest that protein kinase activity associated with CD44 may play a role in the signal transduction process in neutrophils. Taher et al. recently reported that ligation of CD44 by CD44 mAbs in T-cells transduces signals that result in tyrosine phosphorylation of several intracellular proteins, including ZAP-70 [16]. In addition, cross-linking CD44 induced an increase in kinase activity of lck, and lck was associated with CD44 in T-cells, suggesting that lck is involved in CD44 mediated signaling [16]. In a separate study, both fyn and lck were found to be associated with CD44 in T-cells [23].

CD44 has been identified as a major membrane binding partner for ERM (ezrin/radixin/moesin) proteins, and ERM family members are felt to be involved in the association of actin filaments with the plasma membrane [24–26]. The formation of CD44/ERM complexes, implicated in actin filament reorganization, appears to be regulated in part by the Rho family of small G proteins and phosphatidylinositol turnover [24,25,27].

It is of interest that tyrosine kinase activity, including lyn and hck, were found associated with CD44 in human neutrophils. It was not possible to determine if fgr was also associated due to cross reactivity of the fgr antibodies with lyn and hck. Neutrophils contain several other src family kinases [17]

that were not found associated with CD44. Src kinases have been found to form complexes with several receptors in other cells, including CD50, CD63, and CD66 in neutrophils [17,20,28], and several glycosyl-phosphatidylinositol-anchored proteins in other cells [29]. Protein kinase C was found to be associated with CD44 in murine T-cell lymphoma cells, and may also play a role in cell signaling [15], and some data suggest that phosphorylation of serine in the cytoplasmic domain of CD44 is involved in outside-in signaling [30]. Tyrosine kinase activity associated with CD44 may be involved in signal transduction in neutrophils.

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