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# The Microbial Receptor CEACAM3 Is Linked to the Calprotectin Complex in Granulocytes

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**Engulfment of foreign pathogens is an evolutionary ancient host cell endocytic response. Signaling pathways effecting phagocytosis are divergent and largely depend on the structural features of the cell surface receptor utilized. CEACAM3, a member of the CD66 complex on human neutrophils, has been implicated as a cellular receptor promoting phagocytosis of microorganisms. The cytoplasmic domain of CEACAM3 (CEACAM3$_{cyt}$) contains an immunoreceptor tyrosine-based activation motif. In this study we demonstrate that CEACAM3$_{cyt}$ is phosphorylated by protein kinase C, casein kinase I, and Src-kinase *in vitro*. To identify molecules binding to CEACAM3$_{cyt}$ *in vivo,* we used differentially phosphorylated recombinant expressed CEACAM cytoplasmic domains to isolate CEACAM3$_{cyt}$-associated proteins from granulocyte extracts. Calprotectin, which modulates neutrophil integrin-mediated adhesion and leukocyte trafficking and displays antimicrobial activity, interacts specifically with CEACAM3$_{cyt}$. This interaction is calcium-modulated but independent of phosphorylation of CEACAM3$_{cyt}$. Although tyrosine-phosphorylated CEACAM3$_{cyt}$ binds and stimulates Src-kinases *in vitro,* no CEACAM3$_{cyt}$-associated phosphokinase activity was copurified.** © 2001 Academic Press

***Key Words:*** **CEACAM3; CD66d; CGM1; calprotectin; S100A8; MRP8; S100A9; calgranulin; tyrosine phosphorylation.**

*CEACAM3* belongs to the carcinoembryonic antigen (CEA) gene family and is expressed as a cell-surface immunoglobulin-like glycoprotein. Prior to the revision of the CEA nomenclature (1) CEACAM3 was known as carcinoembryonic gene member 1 (CGM1). It is also designated CD66d since it belongs to the CD66 cluster of highly homologous CEA-related granulocyte differentiation antigens. Although it has been shown that CEACAM3 is involved in activation (2) and integrin $\beta_2$ mediated adhesion (3) of neutrophils its physiologic role remains to be explored. Recent interest in CEACAM3 was generated by reports that members of the CD66 family function as microbial receptors for human pathogens like *Neisseria meningititis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* (4–9). Neisseria interact with CEACAM3 and CEACAM1 (CD66a) on neutrophils, and in HeLa transfectants both antigens promote internalization of bacteria (5, 6, 8).

CEACAM3 and CEACAM1 are structural unique within the CD66 molecules since both contain a transmembrane and a cytoplasmic domain. The cytoplasmic domain of both molecules occurs as a short and large isoform. The long cytoplasmic domain of both, CEACAM1 and CEACAM3, contains two tyrosine residues. But whereas the spacing of the tyrosine residues within CEACAM1 resembles an immunoreceptor tyrosine-based inhibition motif (ITIM) (10), CEACAM3 contains an immunoreceptor tyrosine-based activation motif (ITAM) (11). For CEACAM1 it has been shown that (i) the phosphorylation on one or both of its two tyrosine residues (Tyr-488 and Tyr-515) is triggered by several physiological events (12); (ii) one or both of the tyrosine-phosphorylated residues are involved in the association with protein kinases (PTKs) of the Src-family (13), the protein-tyrosine phosphatases (PTPs) SHP-1 (14) and SHP-2 (15), paxillin (16), and integrin $\beta_3$ (17). Moreover the cytoplasmic domain of CEACAM1 is a target protein of the ubiquitous intracellular $Ca^{2+}$ dependent signaling molecule calmodulin (18).

Several studies implicate that CEACAM3 participates in signal transduction (2, 3, 11) but no CEACAM3 associated proteins have been identified yet. To isolate molecules binding to the cytoplasmic domain of CEACAM3 (CEACAM3$_{cyt}$), we used differentially phosphorylated recombinant expressed CEACAM cytoplasmic domains to purify CEACAM3$_{cyt}$ associated

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proteins from granulocyte extracts. We report here that calprotectin, which displays antimicrobial activity (19) and is known to enhance neutrophil integrin $\beta_2$ expression (20) and extravasation (21, 22) interacts specifically with $CEACAM3_{cyt}$ in a calcium-dependent manner.

## MATERIALS AND METHODS

*Preparation of a mouse monoclonal antibody (mAb) to $CEACAM3_{cyt}$.* A mAb to $CEACAM3_{cyt}$ was generated in collaboration with Eurogentec (Belgium) following standard procedures. The mAb designated TS1 was selected for further use, based on its high immunoreactivity with native and recombinant CEACAM3/$CEACAM3_{cyt}$. Subcloning of clone TS1 generated two mAbs TS1a and TS1b of the $IgG_{1K}$ subtype.

*Northern blots.* For Northern blots a 272-bp PCR fragment corresponding to the large cytoplasmic domain of CEACAM3 was generated. This fragment was also expressed as polyhistidine fusion protein. Premade human multiple tissue Northern (MTN) blots (Clontech, Palo Alto, CA) were hybridized overnight in a hybridization oven (GFL, Braunschweig, Germany) using the standard hybridization oven as suggested by the MTN blot manufacturer. The blots were stripped for rehybridization by boiling in 0.1% SDS for 10 min. Labeling of the probe was done by random priming and approximately $2 \times 10^6$ cpm/ml of the $^{32}$P-radiolabeled probe was used for hybridization at 42°C overnight. After washing the membranes at room temperature twice in 2× SSC/0.05% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) for 5 min each, followed by two stringent washes at 50°C in 2× SSC/0.1% SDS for 20 min, filters were then exposed to x-ray film at −70°C for 24–48 h.

*In vitro tyrosine phosphorylation and immunoaffinity purification of phosphoproteins.* Cloning of the cDNA coding for the wild-type cytoplasmic domain of *CEACAM1* and *CEACAM3* and *in vitro* tyrosine phosphorylation was performed as described (16, 17). Phosphotyrosyl proteins were purified using the PY Immunoaffinity system according to the manufacturers specifications (Oncogene Science). Protein kinase C, casein kinase I, casein kinase II (Biomol, Hamburg) were diluted in kinase dilution buffer according to manufacturer's instructions and added to 500 $\mu$g of purified cytoplasmic CEACAM3 domain in kinase assay buffer. For radioactive labeling 50 $\mu$Ci [$\gamma$-$^{32}$P]ATP (30 Ci/mmol; 1 Ci = 37 GBq) was added.

*Purification of proteins on Ni–NTA immobilized CEACAM3 and CEACAM1 domains.* The purified phosphorylated or unphosphorylated CEACAM3 and CEACAM1 domains were adjusted to 250 $\mu$g and 2 ml of a 50% slurry of Ni–NTA resin was added. After incubation for 2 h at 4°C the suspension was transferred into a column and washed three times in 1 ml wash buffer (0.3 M NaCl, 0.050 M Na-phosphate, pH 7.5). Granulocytes were isolated from buffy coat of normal donors by Ficoll–Paque gradient centrifugation ($d$ = 1.119). Freshly isolated granulocytes were extracted with the same volume of 1% NP-40 diluted in PBS containing proteinase inhibitors. After centrifugation (10,000*g*, 30 min) 5 ml of the NP-40-soluble supernatant (1 mg/ml) was passed over the Ni–NTA immobilized CEACAM1 and CEACAM3 domains. The column was washed with 0.3 M NaCl, 0.05 M Na-phosphate (pH 6.0) and eluted using 0.3 M NaCl, 0.05 M phosphate (pH 3.0). SDS–PAGE, silver staining and Western blots were performed as described (23). N-terminal sequences of eluted proteins were determined by automated Edman degradation (Richter AG, Hamburg).

*Precipitation studies.* For immunoprecipitation extracts from cells containing 500 $\mu$g of protein were incubated with approximately 5 $\mu$g monoclonal antibody for 1 h at 4°C. Subsequently, protein G PLUS/protein A–agarose (50 $\mu$l) was added. After incubation on a rocker platform at 4°C for 24 h, the precipitates were washed four times with antibody (Ab)-wash buffer. Precipitated proteins were boiled in sample buffer, separated by SDS–PAGE electrophoresis and visualized by immunoblotting. Precipitation using the agarose coupled SH2 domains were performed according to the manufacturer's specifications (Oncogene Science). For $CEACAM3_{cyt}$ precipitations 50 $\mu$l of a 50% slurry of Ni–NTA resin was added to 0.1 ml of *in vitro* tyrosine phosphorylated $CEACAM3_{cyt}$ (0.25 mg/ml) in binding buffer (0.3 M NaCl, 0.05 M sodium phosphate, pH 7.8). Precipitates were washed three times in 1 ml wash buffer (0.3 M NaCl, 0.05 M Na-phosphate, pH 6.0) and eluted in 200 $\mu$l elution buffer (0.3 M NaCl, 0.05 M sodium phosphate, pH 3.0).

*Src kinase assay.* The peptide $NH_2$-Arg-Thr-Ala-Ser-Ile-Tyr($PO_3H_2$)-Glu-Glu-Leu-Leu-His-COOH (corresponding to amino acids surrounding Y196 in $CEACAM3_{cyt}$) was synthesized following standard procedures. Src-kinase (200 units/50 ml in 50% ethylene glycol) was diluted 1:20 in 10 $\mu$l kinase dilution buffer and added to 10 $\mu$l kinase assay buffer containing 1 mM phosphopeptide or 5 $\mu$l of recombinant $CEACAM3_{cyt}$. Following a 4-h incubation at 4°C, 10 $\mu$l of ATP label mix (0.15 mM ATP, 30 mM $MgCl_2$) containing 1 $\mu$g of acid denatured enolase and 20 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (30 Ci/mmol; 1 Ci = 37 GBq) was added. After incubation at 30°C for 30 min, reactions were stopped by adding 30 $\mu$l of SDS–PAGE sample buffer and boiling for 5 min.

*In vitro complex formation studies.* Extracts from cells containing 500 $\mu$g of protein precleared by rotating at 4°C with 30 $\mu$l of a 50% slurry of protein G PLUS/protein A–agarose (Dianova, Hamburg, FGR) for 30 min and beads were removed by centrifugation (10,000 rpm). Supernatants were incubated with approximately 5 $\mu$g monoclonal antibody for 1 h at 4°C. Subsequently, protein G PLUS/protein A–agarose (50 $\mu$l) and $CEACAM3_{cyt}$ domains adjusted to 50 $\mu$g were added. After incubation on a rocker platform at 4°C for 2 h, the precipitates were washed four times with antibody (Ab)-wash buffer. Precipitated proteins were boiled in sample buffer, separated by SDS–PAGE electrophoresis, and visualized by autoradiography.

*Antibodies and other reagents.* Monoclonal antiphosphotyrosine antibody PY 20 (ICN Biochemicals, Cleveland, OH) and agarose-conjugated antiphosphotyrosine antibody P-Tyr (Ab-1)-A (Oncogene Science) were used in this study. For immunoprecipitation assays protein G PLUS/protein A–agarose (Dianova, Hamburg, Germany) was used. Monoclonal antibodies to human S100A8 and S100A9 were purchased and used according to the manufacturer's specifications (BMA Biomedicals AG, Augst, CH). All other reagents were from Sigma (Munich, Germany).

## RESULTS AND DISCUSSION

To determine the expression pattern of CEACAM3 a 272-bp PCR fragment corresponding to the large cytoplasmic domain of CEACAM3 was used in Northern blot analysis. A weak specific band of 1.3 kb, corresponding to the full-length CEACAM3 transcript was obtained with RNA of peripheral blood leukocytes. No specific signal was detected in the following tissues: spleen, testis, colon, brain, liver, pancreas, thymus, ovary, placenta, skeletal muscle, prostate, small intestine, heart, lung, and kidney (not shown). These data confirm and extend a previous report (24) that the CEACAM3 gene is specifically expressed in the granulocytic lineage. As shown in Fig. 1 in the membrane preparations from granulocytes, the only antigen identified by a monoclonal antibody generated against the long cytoplasmic CEACAM3 domain was the antigen of $\sim M_r$ 30 kDa. This antigen of $\sim M_r$ 30 kDa is also

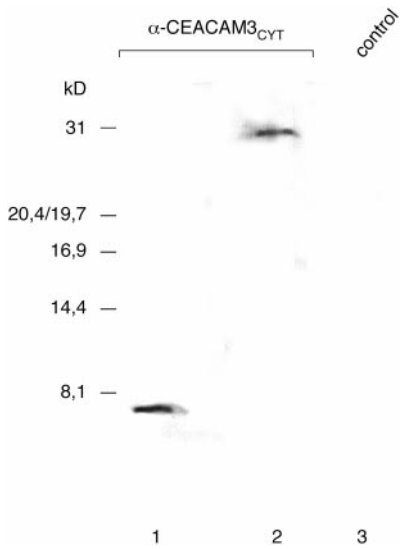


**FIG. 1.** Specificity of TS.1 antibody to CEACAM3$_{cyt}$. Immunoblot using CEACAM3$_{cyt}$ mAb TS.1 with recombinant bacterially expressed CEACAM3$_{cyt}$ (lane 1) and membrane extracts from granulocytes (lane 2). Isotype ($IgG_1$)-matched Ab with granulocyte extracts in lane 3 (control). 17.5% SDS–PAGE, size markers are at the left margin.

recognized by the CEACAM mAb T84.1 which is known to bind CEACAM3 (25). N-terminal sequencing of this band of $\sim M_r$ 30 kDa purified over T84.1 mAb confirmed its identity as CEACAM3 (not shown). This result indicates that the $\sim M_r$ 30,000 granulocyte membrane antigen is the product of the CEACAM3-specific mRNA containing the long 71-amino-acid cytoplasmic domain. Expression of this CEACAM3 isoform in granulocytes is consistent with previous reports (24, 26, 27) on CEACAM3 RNA expression patterns in leukocytes.

In our previous work, we have shown that association of CEACAM**1** with the intracellular proteins depends on tyrosine phosphorylation of the cytoplasmic CEACAM**1** domain (13, 16, 17). As shown in Figs. 2A and 2B both tyrosine residues of CEACAM**3**$_{cyt}$ are tyrosine phosphorylated by Src-kinase *in vitro*. We also confirmed phosphorylation of CEACAM3$_{cyt}$ on serine/threonine residues by protein kinase C and casein kinase I (not shown). These results are in full accordance with sequence-based predictions on tyrosine (28) and serine/threonine phosphorylation (2, 35, 36) of CEACAM3$_{cyt}$.

Following aggregation ITAM bearing receptors are phosphorylated by Src family protein tyrosine kinases. Phosphorylated ITAMs serve as docking sites for cytoplasmic SH2 domain-bearing protein tyrosine kinases and adapter molecules that lead to cell activation (29). Based on putative SH2 binding motifs in CEACAM3$_{cyt}$ (YEEL and YCRM) we hypothesized that CEACAM3 has the potential to assemble, activate and retain Src-kinases via its cytoplasmic domain. As shown in Fig. 3A the *in vitro* tyrosine phosphorylated CEACAM3$_{cyt}$ domain bound to the SH2 domains of Src with no or weak binding to the SH2 domains used as specificity controls. Furthermore, autophosphorylation of Src and phosphorylation of the substrate enolase are enhanced by the addition of CEACAM3$_{cyt}$ (Fig. 3B). This stimulatory effect is dependent on phosphotyrosine 196, since a phosphopeptide of 13 amino acids corresponding to the amino acid sequence containing Y196 also stimulates Src kinase activity (Fig. 3C). These data are in agreement with studies on the regulation of Src-kinase activity (30, 31) and Src-SH2 specificities (32, 33). In conclusion, our results indicate that CEACAM3$_{cyt}$ is a substrate and binding partner for Src-kinases *in vitro*.

Three features hinder *in vivo* isolation of CEACAM3$_{cyt}$ associated proteins: (i) CEACAM3 is expressed on mature granulocytes in very low amounts (24); (ii) CEACAM3$_{cyt}$ is not tyrosine phosphorylated in resting granulocytes (12); (iii) stimuli leading to phosphorylation or dephosphorylation of CEACAM3$_{cyt}$ in granulocytes have not been established. Therefore, in order to identify phosphorylation-dependent and -independent associated proteins of CEACAM3$_{cyt}$ we used tyrosine phosphorylated and unphosphorylated CEACAM3$_{cyt}$ constructs as bait to isolate binding proteins from

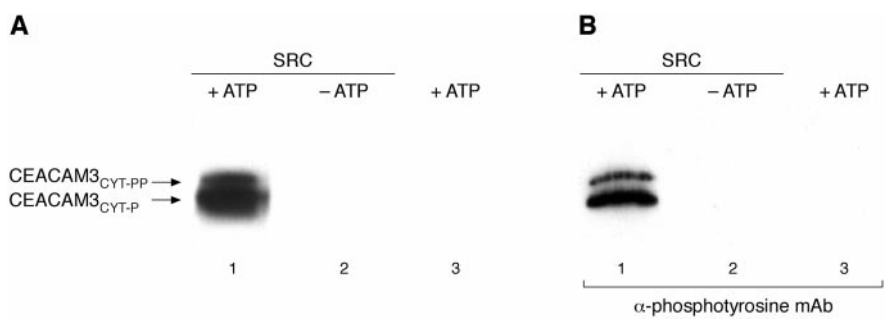


**FIG. 2.** *In vitro* tyrosine phosphorylation of CEACAM3$_{cyt}$. (A) Autoradiogram. 17.5% SDS–PAGE of CEACAM3$_{cyt}$ incubated for 30 min with Src-kinase in the presence of ATP (lane 1), with Src-kinase without ATP (lane 2) and without Src-kinase in the presence of ATP. Size markers are at the left margin. (B) Immunoblot using anti-phosphotyrosine mAb PY 20; lanes as in (A).

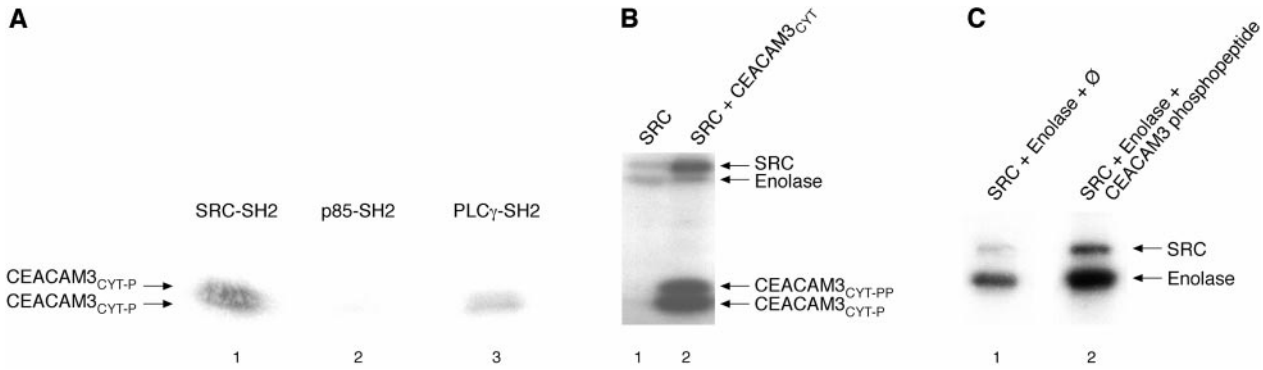


**FIG. 3.** (A) Precipitation of tyrosine phosphorylated $CEACAM3_{cyt}$ by immobilized SH2 domains by the SH2-domain of Src (lane 1), the 85-kDa subunit of phosphatidylinositol-3-kinase (lane 2) and the N-terminal SH2 domain of phospholipase Cγ (lane 3). Positions of the phosphorylated $CEACAM3_{cyt}$ are indicated on the left margin. (B and C) Activation of Src-kinase by $CEACAM3_{cyt}$. Src-kinase was preincubated at 4°C with kinase assay buffer alone (lane 1 in B and C), $CEACAM3_{cyt}$ (lane 2 in B) or 1 mM $CEACAM3_{Y196}$ phosphopeptide (lane 2 in C). *In vitro* kinase reactions were performed with enolase as exogenous substrate. Kinase products were analyzed by SDS–PAGE and autoradiography.

granulocyte extracts. Since the ligand specificity of phosphotyrosyl binding protein modules—such as SH2—depends largely on the local concentration of phosphoproteins we expected unspecific binding of SH2 containing proteins due to the high local concentration of phosphotyrosyl residues. For that reason, we used the cytoplasmic domain of CEACAM1 as a control, since the cytoplasmic domain of CEACAM1 is structurally very similar to $CEACAM3_{cyt}$. Adjusted amounts of—either phosphorylated or unphosphorylated—$CEACAM3_{cyt}$ and $CEACAM1_{cyt}$ were immobilized on Ni–NTA resin. Extracts from granulocytes eluted from immobilized domains were submitted to SDS–PAGE. As shown in Fig. 4A each of the unphosphorylated as well as phosphorylated CEACAM1 constructs (lanes 1 and 2) bound to defined sets of proteins. Two proteins of $\sim M_r$ 14 and ~8 kDa were detectable, which bound to the unphosphorylated as well as phosphorylated cytoplasmic CEACAM**3** domain only (Fig. 4B). These finding suggest that the two proteins of $\sim M_r$ 14 and 8 specifically interact with $CEACAM3_{cyt}$ independent of $CEACAM3_{cyt}$ tyrosine phosphorylation.

Sensitive PTK and PTP activity assays were performed with the eluates of the CEACAM constructs. In contrast to the eluates of $CEACAM1_{cyt\text{-}p}$ no phosphokinase activity was detectable in neither the $CEACAM3_{cyt\text{-}p}$ eluates nor the $CEACAM3_{cyt}$ eluates (not shown). These data are in accordance with a study by Skubitz *et al.* (12) who reported associated tyrosine kinase activity with CEACAM1 but failed to demonstrate associated kinase activity with CEACAM3. To rule out that phosphatases in the granulocyte extract dephosphorylated tyrosine phosphorylated $CEACAM3_{cyt}$ during the purification procedure, we detached the *in vitro* tyrosine-phosphorylated $CEACAM3_{cyt}$ from the Ni–NTA-matrix. Using phosphotyrosine mAb PY 20 in Western blot analysis, we showed that the detached construct was still tyrosine phosphorylated.

Thus, although $CEACAM3_{cyt}$ is a substrate and binding partner for Src kinases *in vitro* we were not able to purify any $CEACAM3_{cyt}$ associated PTK activity from granulocyte extracts. A possible explanation for these differences might be that in the granulocyte extracts binding of the SH2 domain containing PTKs (and/or PTPs) is inhibited by constitutively formed signal complexes binding these signaling molecules and thus preventing them to bind to phosphorylated $CEACAM3_{cyt}$ on the column. However, at least for Src-kinases we have shown binding to $CEACAM1_{cyt}$ using the same experimental setting (16, 17). One likely explanation would be that protein(s) from granulocyte extracts—different from PTKs and PTPS—bound to $CEACAM3_{cyt}$—but not to $CEACAM1_{cyt}$- and inhibited binding of SH2 domain containing PTKs and/or PTPs to phosphorylated $CEACAM3_{cyt}$.

Sequencing of the proteins copurified with $CEACAM3_{cyt}$ revealed for the 8-kDa protein a 16-amino-acid exact match to the amino terminus of the S100 calcium-binding protein A8. The identity of S100A8 was further confirmed in immunoblots by using a commercially available mAb against human S100A8 (Fig. 4C). The ~14-kDa protein could not be sequenced since the $NH_2$-terminus was blocked, but immunoblots revealed its identity as the S100 calcium-binding protein A9 (Fig. 4C). S100A9 is known to contain an N-terminal block (34) and within cells the preferred form is a heterodimer with S100A8 (35, 36). This molecular complex has been designated as calprotectin. Though disulfide-linked complexes can be generated *in vitro* from purified S100A8 and S100A9 proteins, heteromeric complexes formed *in vivo* are known to be noncovalently linked and break down on SDS gels under both reducing and nonreducing conditions (37). The mode of interaction between calprotectin with target proteins differs substantially from the mode of interaction of calmodulin, which binds to the cytoplasmic

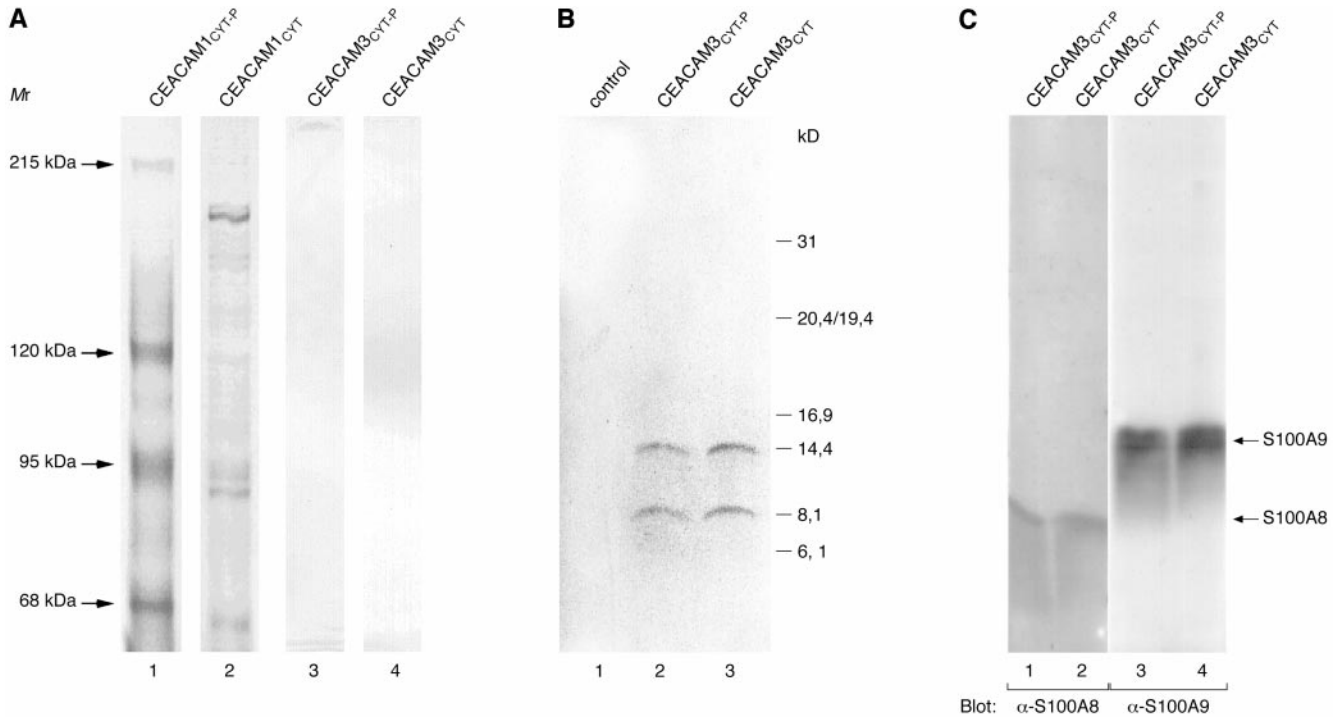


**FIG. 4.** Purification of $CEACAM3_{cyt}$-associated proteins from granulocyte extracts. (A) 7.5% SDS–PAGE and silver staining of eluates from immobilized CEACAM domains. Extracts from granulocytes were subjected to purification on adjusted amounts of immobilized *in vitro* tyrosine phosphorylated $CEACAM1_{cyt}$ (lane 1), unphosphorylated $CEACAM1_{cyt}$ (lane 2), *in vitro* tyrosine phosphorylated $CEACAM3_{cyt}$ (lane 3) and unphosphorylated $CEACAM3_{cyt}$ (lane 4). (B) 17.5% SDS–PAGE and silver staining of eluates from immobilized $CEACAM3_{cyt}$. Extracts from granulocytes were subjected to purification on binding resin only (lane 1) or adjusted amounts of immobilized *in vitro* tyrosine phosphorylated $CEACAM3_{cyt}$ (lane 2) and unphosphorylated $CEACAM3_{cyt}$ (lane 3). (C) Western blot using α-S100A8 mAb (lanes 1 and 2) and α-S100A9 mAb (lanes 3 and 4) of eluates from immobilized $CEACAM3_{cyt}$.

domain of CEACAM1. Whereas the bilobed calmodulin wraps around or clamps target proteins (38, 39) S100 dimers functionally crosslink two homologous or heterologous target proteins (40). Classical S100 proteins contain EF-hand $Ca^{2+}$-binding domains, but $Ca^{2+}$ binding in solution is low under physiologic conditions (40–42). This affinity increases by several orders of magnitude in the presence of S100 target proteins. In many cases the interaction of an S100 protein with a target protein were observed to occur at free $Ca^{2+}$ levels significantly lower than those with $Ca^{2+}$ present in solution (40–44).

Since calprotectin could be purified from granulocyte extracts with the CEACAM3 cytoplasmic domain only, we used recombinant expressed cytoplasmic CEACAM3 constructs for *in vitro* complex formation studies. S100A8 is coimmunoprecipitated with $CEACAM3_{cyt}$ from granulocyte extracts independent of $CEACAM3_{cyt}$ phosphorylation but in a calcium-dependent manner (Fig. 5A). As shown in Fig. 5B, complexes containing calprotectin are not immunoprecipitated with CEACAM1 mAb 12-140-4 but with mAb T84.1, which is known to bind to CEACAM1 and CEACAM3. Supplementing $Ca^{2+}$ to 2 mM prior to precipitation also clearly enhanced coprecipitation of calprotectin with CEACAM3 *in vivo*. These results indicate that *in vivo* CEACAM3 binds calprotectin via its cytoplasmic domain in a $Ca^{2+}$-dependent manner.

Calcium-modulated S100 proteins have been implicated in the regulation of protein phosphorylation, the dynamics of cytoskeleton components, $Ca^{2+}$ homeostasis, and cell proliferation and differentiation (40). Calprotectin is expressed in circulating neutrophils and monocytes (21, 45, 46). Upon neutrophil activation it is translocated to cytoskeleton and to plasma membrane (47). Existing evidence suggests that calprotectin is involved in $Ca^{2+}$ dependent interactions between Type III filaments and membranes during migration of and phagocytosis by activated granulocytes (47). Furthermore, calprotectin has been implicated in leukocyte trafficking (21, 22) and the modulation of integrin $\beta_2$ mediated adhesion of neutrophils (20).

It is worth mentioning, that—likewise to calprotectin—CEACAM3 is also upregulated from intracellular stores to the plasma membrane following stimulation (48–50) and enhances adhesion activity of integrin $\beta_2$ in neutrophils in a calcium dependent matter (2). In the present study we demonstrate that CEACAM3 binds to calprotectin. Since binding of calmodulin to the cytoplasmic domain of CEACAM1 is also depen-

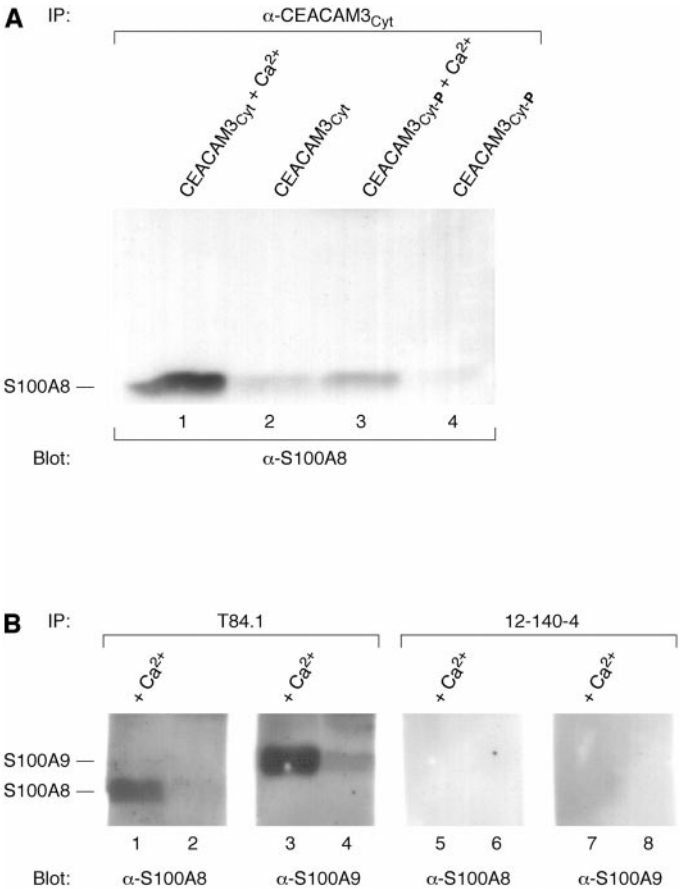


**FIG. 5.** Coimmunoprecipitation of calprotectin with CEACAM3 from granulocyte membrane extracts. (A) Adjusted amounts of unphosphorylated $CEACAM3_{cyt}$ (lanes 1 and 2) or *in vitro* tyrosine phosphorylated $CEACAM3_{cyt\text{-}P}$ (lanes 3 and 4) were incubated with granulocyte extracts for 2 h in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 2 mM $Ca^{2+}$. After immunoprecipitation with $\alpha$-$CEACAM3_{cyt}$ precipitates were resolved with 17.5% SDS–PAGE and immunoblotted with anti-S100A8 mAb. (B) Extracts from granulocytes were subjected to immunoprecipitation with $\alpha$-CEACAM mAb T84.1 (lanes 1–4) or $\alpha$-CEACAM1 mAb 12-140-4 (lanes 5–8) in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, 6, and 8) of 2 mM $Ca^{2+}$. Bound proteins were resolved on 17.5% SDS–PAGE and immunoblotted with anti-S100A8 and anti-S100A9. The positions of anti-S100A8 and -S100A9 are indicated on the left.

dent on $Ca^{2+}$ we envision that signaling via CEACAM1 and CEACAM3 within CD66 complex of granulocytes is greatly influenced by intracellular $Ca^{2+}$ concentrations. Further investigations are now underway to decipher the functional role of these calcium-modulated interactions in biologic events such as phagocytosis, leukocyte trafficking, and integrin regulation.

## REFERENCES


1. Beauchemin, N., Dveksler, G., Gold, P., Gray-Owen, S., Grunert, F., Hammarstrom, S., Holmes, K. V., Karlsson, A., Kuroki, M., Lin, S-H., Lucka, L., Najjar, S. M., Neumaier, M., Obrink, B., Shively, J. E., Skubitz, K. M., Stanners, C. P., Thomas, P., Virji, M., von Kleist, S., Wagener, C., Watt, S., and Zimmermann, W. (1999) Redefined nomenclature for members of the carcinoembryonic antigen family. *Exp. Cell Res.* **25,** 243–249.
2. Skubitz, K. M., Campbell, K. D., and Skubitz, A. P. (1996) CD66a, CD66b, CD66c, and CD66d each independently stimulate neutrophils. *J. Leukocyte Biol.* **60,** 106–117.
3. Stocks, S. C., Ruchaud-Sparagano, M. H., Kerr, M. A., Grunert, F., Haslett, C., and Dransfield, I. (1996) CD66: Role in the regulation of neutrophil effector function. *Eur. J. Immunol.* **26,** 2924–2932.
4. Virji, M., Watt, S. M., Barker, S., Makepeace, K., and Doyonnas, R. (1996) The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae. Mol. Microbiol.* **22,** 929–939.
5. Virji, M., Makepeace, K., Ferguson, D. J., and Watt, S. M. (1996) Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol. Microbiol.* **22,** 941–950.
6. Chen, T., and Gotschlich, E. C. (1996) CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins. *Proc. Natl. Acad. Sci. USA* **93,** 14851–14856.
7. Chen, T., Grunert, F., Medina-Marino, A., and Gotschlich, E. C. (1997) Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. *J. Exp. Med.* **185,** 1557–1564.
8. Gray-Owen, S. D., Dehio, C., Haude, A., Grunert, F., and Meyer, T. F. (1997) CD66 carcinoembryonic antigens mediate interactions between Opa-expressing *Neisseria gonorrhoeae* and human polymorphonuclear phagocytes. *EMBO J.* **16,** 3435–3445.
9. Virji, M., Evans, D., Griffith, J., Hill, D., Serino, L., Hadfield, A., and Watt, S. M. (2000) Carcinoembryonic antigens are targeted by diverse strains of typable and non-typable *Haemophilus influenzae. Mol. Microbiol.* **36,** 784–795.
10. Obrink, B. (1997) CEA adhesion molecules: Multifunctional proteins with signal-regulatory properties. *Curr. Opin. Cell Biol.* **9,** 616–626.
11. Chen, T., Bolland, S., Chen, I., Parker, J., Pantelic, M., Grunert, F., and Zimmermann, W. (2001) The CGM1a (CEACAM3/CD66d)-mediated phagocytic pathway of *Neisseria gonorrhoeae* expressing opacity proteins is also the pathway to cell death. *J. Biol. Chem.* **276,** 17413–17419.
12. Skubitz, K. M., Campbell, K. D., Ahmed, K., and Skubitz, A. P. (1995) CD66 family members are associated with tyrosine kinase activity in human neutrophils. *J. Immunol.* **155,** 5382–5390.
13. Brummer, J., Neumaier, M., Gopfert, C., and Wagener, C. (1995) Association of pp60c-src with biliary glycoprotein (CD66a), an adhesion molecule of the carcinoembryonic antigen family downregulated in colorectal carcinomas. *Oncogene* **11,** 1649–1655.
14. Beauchemin, N., Kunath, T., Robitaille, J., Chow, B., Turbide, C., Daniels, E., and Veillette, A. (1997) Association of biliary glycoprotein with protein tyrosine phosphatase SHP-1 in malignant colon epithelial cells. *Oncogene* **14,** 783–790.
15. Huber, M., Izzi, L., Grondin, P., Houde, C., Kunath, T., Veillette, A., and Beauchemin, N. (1999) The carboxyl-terminal region of biliary glycoprotein controls its tyrosine phosphorylation and association with protein-tyrosine phosphatases SHP-1 and SHP-2 in epithelial cells. *J. Biol. Chem.* **274,** 335–344.
16. Ebrahimnejad, A., Flayeh, R., Unteregger, G., Wagener, C., and Brummer, J. (2000) Cell adhesion molecule CEACAM1 associates with paxillin in granulocytes and epithelial and endothelial cells. *Exp. Cell Res.* **260,** 365–373.
17. Brummer, J., Ebrahimnejad, A., Flayeh, R., Schumacher, U., Loning, T., Bamberger, A. M., and Wagener, C. (2001) cis interaction of the cell adhesion molecule CEACAM1 with integrin beta(3). *Am. J. Pathol.* **159,** 537–546.

18. Edlund, M., Blikstad, I., and Obrink, B. (1996) Calmodulin binds to specific sequences in the cytoplasmic domain of C-CAM and down-regulates C-CAM self-association. *J. Biol. Chem.* **271,** 1393–1399.

19. Steinbakk, M., Naess-Andresen, C. F., Lingaas, E., Dale, I., Brandtzaeg, P., and Fagerhol, M. K. (1990) Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. *Lancet* **336,** 763–765.

20. Newton, R. A., and Hogg, N. (1998) The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. *J. Immunol.* **160,** 1427–1435.

21. Hogg, N., Allen, C., and Edgeworth, J. (1989) Monoclonal antibody 5.5 reacts with p8,14, a myeloid molecule associated with some vascular endothelium. *Eur. J. Immunol.* **19,** 1053–1061.

22. Kerkhoff, C., Eue, I., and Sorg, C. (1999) The regulatory role of MRP8 (S100A8) and MRP14 (S100A9) in the transendothelial migration of human leukocytes. *Pathobiology* **67,** 230–232.

23. Stoffel, A., Neumaier, M., Gaida, F. J., Fenger, U., Drzeniek, Z., Haubeck, H. D., and Wagener, C. (1993) Monoclonal, anti-domain and anti-peptide antibodies assign the molecular weight 160,000 granulocyte membrane antigen of the CD66 cluster to a mRNA species encoded by the biliary glycoprotein gene, a member of the carcinoembryonic antigen gene family. *J. Immunol.* **150,** 4978–4984.

24. Nagel, G., Grunert, F., Kuijpers, T. W., Watt, S. M., Thompson, J., and Zimmermann, W. (1993) Genomic organization, splice variants and expression of CGM1, a CD66-related member of the carcinoembryonic antigen gene family. *Eur. J. Biochem.* **214,** 27–35.

25. Kuroki, M., Arakawa, F., Matsuo, Y., Oikawa, S., Misumi, Y., Nakazato, H., and Matsuoka, Y. (1991) Molecular cloning of nonspecific cross-reacting antigens in human granulocytes. *J. Biol. Chem.* **266,** 11810–11817.

26. Thompson, J. A., Mauch, E. M., Chen, F. S., Hinoda, Y., Schrewe, H., Berling, B., Barnert, S., von Kleist, S., Shively, J. E., and Zimmermann, W. (1989) Analysis of the size of the carcinoembryonic antigen (CEA) gene family: Isolation and sequencing of N-terminal domain exons. *Biochem. Biophys. Res. Commun.* **158,** 996–1004.

27. Thompson, J. A., Grunert, F., and Zimmermann, W. (1991) Carcinoembryonic antigen gene family: Molecular biology and clinical perspectives. *J. Clin. Lab. Anal.* **5,** 344–366.

28. Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **294,** 1351–1362.

29. Cambier, J. C. (1995) Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J. Immunol.* **155,** 3281–3285.

30. Bjorge, J. D., Jakymiw, A., and Fujita, D. J. (2000) Selected glimpses into the activation and function of Src kinase. *Oncogene* **19,** 5620–5635.

31. Brown, M. T., and Cooper, J. A. (1996) Regulation, substrates and functions of src. *Biochim. Biophys. Acta* **1287,** 121–149.

32. Arold, S. T., Ulmer, T. S., Mulhern, T. D., Werner, J. M., Ladbury, J. E., Campbell, I. D., and Noble, M. E. (2001) The role of the Src homology 3–Src homology 2 interface in the regulation of Src kinases. *J. Biol. Chem.* **276,** 17199–17205.

33. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., *et al.* (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell* **72,** 767–778.

34. Kerkhoff, C., Klempt, M., and Sorg, C. (1998) Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). *Biochim. Biophys. Acta* **1448,** 200–211.

35. Hunter, M. J., and Chazin, W. J. (1998) High level expression and dimer characterization of the S100 EF-hand proteins, migration inhibitory factor-related proteins 8 and 14. *J. Biol. Chem.* **273,** 12427–12435.

36. Propper, C., Huang, X., Roth, J., Sorg, C., and Nacken, W. (1999) Analysis of the MRP8–MRP14 protein–protein interaction by the two-hybrid system suggests a prominent role of the C-terminal domain of S100 proteins in dimer formation. *J. Biol. Chem.* **274,** 183–188.

37. Teigelkamp, S., Bhardwaj, R. S., Roth, J., Meinardus-Hager, G., Karas, M., and Sorg, C. (1991) Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. *J. Biol. Chem.* **266,** 13462–13467.

38. Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) Target enzyme recognition by calmodulin: 2.4 A structure of a calmodulin–peptide complex. *Science* **257,** 1251–1255.

39. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Solution structure of a calmodulin–target peptide complex by multidimensional NMR. *Science* **256,** 632–638.

40. Donato, R. (2001) S100: A multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int. J. Biochem. Cell Biol.* **33,** 637–668.

41. Zimmer, D. B., Cornwall, E. H., Landar, A., and Song, W. (1995) The S100 protein family: History, function, and expression. *Brain Res. Bull.* **37,** 417–429.

42. Schafer, B. W., and Heizmann, C. W. (1996) The S100 family of EF-hand calcium-binding proteins: Functions and pathology. *Trends Biochem. Sci.* **21,** 134–140.

43. Wang, C. K., Mani, R. S., Kay, C. M., and Cheung, H. C. (1992) Conformation and dynamics of bovine brain S-100a protein determined by fluorescence spectroscopy. *Biochemistry* **31,** 4289–4295.

44. Zolese, G., Giambanco, I., Curatola, G., De Stasio, G., and Donato, R. (1993) Time-resolved fluorescence of S-100a protein in the absence and presence of calcium and phospholipids. *Biochim. Biophys. Acta* **1162,** 47–53.

45. Lagasse, E., and Clerc, R. G. (1988) Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol. Cell. Biol.* **8,** 2402–2410.

46. Odink, K., Cerletti, N., Bruggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R., and Sorg, C. (1987) Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* **330,** 80–82.

47. Roth, J., Burwinkel, F., van den Bos, C., Goebeler, M., Vollmer, E., and Sorg, C. (1993) MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. *Blood* **82,** 1875–1883.

48. Tetteroo, P. A., Bos, M. J., Visser, F. J., and von dem Borne, A. E. (1986) Neutrophil activation detected by monoclonal antibodies. *J. Immunol.* **136,** 3427–3432.

49. Ducker, T. P., and Skubitz, K. M. (1992) Subcellular localization of CD66, CD67, and NCA in human neutrophils. *J. Leukocyte Biol.* **52,** 11–16.

50. Kuroki, M., Yamanaka, T., Matsuo, Y., Oikawa, S., Nakazato, H., and Matsuoka, Y. (1995) Immunochemical analysis of carcinoembryonic antigen (CEA)-related antigens differentially localized in intracellular granules of human neutrophils. *Immunol. Invest.* **24,** 829–843.