Internalization and recycling of ALCAM/CD166 detected by a fully human single-chain recombinant antibody

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Summary

Activated leukocyte cell adhesion molecule (ALCAM/CD166), a member of the immunoglobulin superfamily with five extracellular immunoglobulin-like domains, promotes heterophilic (ALCAM-CD6) and homophilic (ALCAM-ALCAM) cell-cell interactions. Here we describe a fully human single-chain antibody fragment (scFv) directed to ALCAM/CD166. We selected the I/F8 scFv from a phage display library of human V-gene segments by cell panning and phage internalization into IGROV-I human ovary carcinoma cells. The I/F8 specificity was identified as ALCAM/CD166 by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) peptide mass fingerprinting of the I/F8-immunoprecipitated protein. The I/F8 scFv reacts with the human, monkey and murine ALCAM/CD166 molecule, indicating that the recognized epitope is highly conserved. The I/F8 scFv completely abolished binding of both ALCAM/Fc and CD6/Fc soluble ligands, whereas it did not compete with the anti-ALCAM/CD166 murine monoclonal antibodies J4-81 and 3A6 and therefore recognizes a different epitope. Engagement through I/F8 scFv, 3A6 monoclonal antibody or CD6/Fc ligand induced ALCAM/CD166 internalization, with a kinetics slower than that of transferrin in the same cells. Newly internalized I/F8-ALCAM complexes colocalized with clathrin but not with caveolin and we demonstrated, using surface biotinylation and recycling assays, that endocytosed ALCAM/CD166 recycles back to the cell surface. Such an endocytic pathway allows the efficient delivery of an I/F8 scFv-saporin immunotoxin into tumor cells, as the conjugates are able to selectively kill cell lines expressing ALCAM/CD166. Altogether these data provide evidence of the suitability of the I/F8 scFv for further functional analysis of ALCAM/CD166 and intracellular delivery of effector moieties.

Key words: Recombinant antibodies, ALCAM/CD166, Endocytosis, Recycling

Introduction

The feasibility of antibody-based immunotherapy in humans by means of targeted delivery of therapeutics may require specific human or humanized internalizing antibodies. Large non-immune phage antibody libraries have been developed to serve as a single source for the rapid generation of fully human antibodies (Marks et al., 1991; Nissim et al., 1994; Vaughan et al., 1996; Pini et al., 1998). In the search for internalizing antibodies, Poul and co-workers devised a selection strategy that took advantage of both normal cell surface receptor biology and phage antibody mimicry capacity. They demonstrated that specific phages endocytosed upon binding could be successfully recovered from the cell cytosol after stringent removal of non-specific phage from the cell surface (Poul et al., 2000). In this study we report the successful application of their strategy to select a single-chain antibody fragment (scFv) internalizing into human tumor cells from a fully human scFv library displayed monovalently on phage (Viti et al., 2000). This scFv is specific for the activated leukocyte cell adhesion molecule (ALCAM/CD166).

ALCAM/CD166 is a member of the immunoglobulin gene superfamily belonging to a subgroup with five extracellular immunoglobulin-like domains ($VVC_2C_2C_2$), which includes MCAM/CD146/MUC18 and B-CAM/Lutheran. These molecules are involved in the development and maintenance of tissue architecture, neurogenesis, hematopoiesis, immune responses and tumor progression. ALCAM/CD166 mediates cell-cell clustering through homophilic (ALCAM-ALCAM) and heterophilic (ALCAM-CD6) interactions. Its pattern of expression in human tissues and cells is broad and includes epithelia, neurons, lymphoid and myeloid cells, hematopoietic and mesenchymal stem cells (reviewed by Swart, 2002). The relevance of the engagement of the heterophilic pair CD166-CD6 for optimal activation of T cells has been recently demonstrated (Hassan et al., 2004). Moreover, developmental biology studies showed that ALCAM/CD166 expression

generally occurs in cells undergoing proliferation, and that it acts both in the formation of neuromuscular contacts during development and in guiding non-radial cell migration during brain development (Fournier-Thibault et al., 1999; Heffron and Golden, 2000) (reviewed by Swart, 2002). ALCAM/CD166 is also present in a number of carcinoma cells and cell lines, and in the invasive cells of melanocytic skin lesions where its expression correlates with tumor progression (Degen et al., 1998; van Kempen et al., 2000; Tomita et al., 2000; Swart, 2002; Kristiansen et al., 2003). On the other hand, overexpression of a truncated ALCAM form in melanoma cells, which is unable to support homotypic cell clustering, relieves the constraints for tumor cell migration and apparently promotes tissue invasion (van Kempen et al., 2001). Collectively these reports support the hypothesis that expression of ALCAM/CD166 introduces a more general switch in cell programs, controlling the transition between cell clustering and cell movement.

Several lines of evidence indicate that some members of the cell adhesion molecule (CAM) family are not simply stable residents on the cell surface. Instead, CAMs can be dynamically regulated by various mechanisms, such as lateral oligomerization, endocytosis and recycling to the cell surface, proteolytic ectodomain shedding and transcriptional regulation of expression (Kamiguchi and Lemmon, 2000a). ALCAM/CD166 lateral oligomerization and ectodomain shedding have been recently described (van Kempen et al., 2001; van Kempen et al., 2004), although little is known about ALCAM/CD166 endocytosis besides a previous description of its 'modulation' (Pesando et al., 1986).

Our present finding that phages displaying anti-ALCAM/CD166 specificity could be selected from an internalized phage pool, suggests that ALCAM/CD166 is also capable of ligand-induced internalization. We demonstrate that ALCAM/CD166 functional dynamics include ligand-induced endocytosis and recycling to the cell surface. The endocytic pathway is clathrin-dependent, but the internalization kinetics are slower than those of transferrin. Finally, we show that such an endocytic pathway allows the efficient delivery of an anti-ALCAM/CD166 scFv-saporin immunotoxin into tumor cells, suggesting that this scFv could be of use for the targeted delivery of effector moieties into ALCAM/CD166-positive cells.

Materials and Methods

Cells, antibodies and reagents

The human cell lines IGROV-I and A2774 (ovarian carcinoma, from J. Bénard, Institute Gustave Roussy, Villejuif, France), N592 and GLC-4 [small cell lung cancer (SCLC), from J. Minna (National Cancer Institute, Bethesda, MD), and from E. De Vries and L. De Leij (Utrecht, The Netherlands) respectively], SK-N-BE and GI-ME-N (neuroblastoma, from S. Carrel, Lausanne, Switzerland, and from the Laboratory of Oncology, Istituto G. Gaslini, Genova, Italy, respectively) were grown in RPMI 1640 (Sigma, St Louis, MO), supplemented with L-glutamine, 10% heat inactivated FCS (BioChrome KG, Berlin, Germany), and antibiotics at 37°C in a 5% CO_2 incubator. Culture medium for the murine cell lines L929 (connective tissue; ECACC, Salisbury, UK), F1F (fibrosarcoma; from M. P. Colombo, Istituto Nazionale Tumori, Milano), Neuro2a (neuroblastoma; ATCC, Rockville, MD), and for the COS-7 monkey kidney cell line (ATCC) was DMEM (Sigma) with supplements and culture conditions as above. T lymphoblasts were derived as described (Renard et al., 2002). Monoclonal anti-ALCAM/CD166 antibodies J4-81 and 3A6 were purchased from Antigenix America (New York, NY) and BD Pharmingen (Becton Dickinson, San Diego, CA), respectively. FITC-labeled anti-clathrin and anti-caveolin (IgM, clone C060) antibodies were from BD Pharmingen. Anti-FLAG (clone M2) antibody was from Sigma. Recombinant soluble human ALCAM/Fc, murine ALCAM/Fc and human CD6/Fc chimeras were purchased from R&D Systems (Minneapolis, MN). The anti-NIP scFv (Nissim et al., 1994), a kind gift from G. Winter (MRC, Cambridge UK), was modified by adding the FLAG-tag sequence and used as a control. The anti-saporin monoclonal antibody (mAb) CY12.14 has been previously described (Tazzari et al., 1993).

Selection of internalizing scFv by phage display

The non-immune scFv phage antibody library ETH-2 (Pini et al., 1998), kindly provided by D. Neri, ETH Zürich and Philogen/Philotec, Siena, Italy, and L. Zardi, Istituto G. Gaslini, Genova, Italy, was selected by panning on IGROV-I human ovary carcinoma cells following a modification of a protocol described (Poul et al., 2000). Briefly, 5×10^6 subconfluent adherent IGROV-I cells were incubated for 1.5 hours rocking at 4°C, with 1×10^{11} colony forming units of ETH-2 library, in the presence of 25×10^6 human red blood cells, to reduce non-specific binding. The cells were then extensively washed with PBS and incubated with pre-warmed medium at 37°C for 15 minutes to allow endocytosis of surface-bound phage. To remove phage bound to the extracellular matrix or to the culture plate, adherent cells were trypsinized for 10 minutes at 37°C and washed with culture medium at 4°C. Subsequently, to remove phage bound to the cell surface, the cells were stripped three times with low pH glycine buffer (150 mM NaCl, 100 mM glycine pH 2.5). Internalized phages were recovered from within the cells by lysing with 100 mM triethylamine for 4 minutes at 4°C, and neutralizing with 0.5 M Tris-HCl pH 7.4. The cell lysate was used to infect TG1 *Escherichia coli* to prepare phage for the next round of selection. A total of five rounds of selection were performed. To obtain soluble scFv, phages recovered from the fifth panning were used to infect HB2151 *E. coli*. Antibodies binding IGROV-I cells were identified by cell ELISA (Poul et al., 2000) using native soluble scFv expressed from randomly picked single colonies. Cell-bound scFv was detected via its FLAG-tag using the anti-FLAG M2 mAb (Sigma) followed by a HRP-conjugated goat anti-mouse antibody (Caltag Laboratories, Burlingame, CA). The diversity of positive clones was analyzed by PCR amplification and DNA fingerprinting the scFv gene with *Bst*N1 as described (Marks et al., 1991).

Immunoprecipitation and purification of antigen using I/F8 scFv

Single-chain Fv I/F8 was purified from the culture broth of HB2151 *E. coli* cells using a Sepharose Protein A column (Amersham Biosciences, Uppsala, Sweden), eluted with low pH, 100 mM glycine and dialyzed against PBS for cell culture applications, as described (Viti et al., 2000).

The I/F8 antigen was characterized by immunoprecipitation using scFv antibodies bound to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences). If required, cell surface proteins were labeled with Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) as described for cells growing in suspension (De Rossi et al., 1993) and for adherent cells (Le Bivic et al., 1989). Cells were lysed with lysis buffer: 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Brij97 (Sigma), protease inhibitors (Complete C Mini, Roche Diagnostics, Mannheim, Germany), and nuclei were discarded after centrifugation at 400 ***g***. After one pre-clearing cycle with anti-NIP apten scFv coupled to Sepharose beads, specific absorption was performed by incubating cell extracts with I/F8-Sepharose beads, rotating for 3 hours at 4°C. Sepharose was then thoroughly washed with lysis buffer, and bound material was eluted in SDS-PAGE sample buffer. Immunoprecipitated proteins were fractionated by SDS-

electrophoresis on 10% polyacrylamide gels under reducing and non-reducing conditions. Biotin-labeled proteins were analyzed by western blotting with HRP-conjugated streptavidin (DakoCytomation, Carpinteria, CA), according to standard procedures.

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) peptide mass fingerprinting was performed on the silver-stained protein excised from a preparative SDS-PAGE, as described (Shevchenko et al., 1996). Analysis of tryptic fragments was performed on a Voyager-DE STR time-of-flight instrument (Applied Biosystems, Framingham, MA), equipped with a nitrogen laser operating at 337 nm. Mass spectra of tryptic peptides were acquired operating in the positive ion, delayed extraction and reflectron modes using α-cyano-4-hydroxycinnamic acid (saturated solution in 50% acetonitrile with 0.1% trifluoroacetic acid) as the UV-absorbing matrix. Experimental conditions were as follows: accelerating voltage, 20,000 V; grid voltage, 94%; guide wire voltage, 0.050%; delay, 100 ns; laser power, 2487. Spectra were obtained by combining 400 scans, applying mass calibration, baseline correction, noise filtering and de-isotoping procedures using the spectrometer software (Data Explorer version 4.0, Applied Biosystems). Monoisotopic peptide masses were submitted to ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) and Mascot (http://www.matrixscience.com/) for comparison with entries in the National Center for Biotechnology Information (NCBI) sequence database.

Binding and internalization of ALCAM/CD166 antibodies and ligands

Immunofluorescence was performed incubating 10^5 viable cells with 2 μg/ml scFv I/F8 plus 2 μg/ml anti-FLAG mAb, or with 2 μg/ml anti-ALCAM/CD166 mAbs for 40 minutes on ice. Recombinant soluble ALCAM/CD166 ligands CD6/Fc and ALCAM/Fc chimeras were used at 1 μg/ml and 10 μg/ml, respectively. As secondary reagents, FITC-conjugated goat anti-mouse Ig and anti-human Ig (Caltag Laboratories) were used. The competition between ALCAM/CD166 antibodies and ligands CD6/Fc and ALCAM/Fc was assessed by flow cytometry on SCLC N592 cells. Subsaturating amounts of binder molecule were challenged with varying amounts of I/F8 scFv in a 30-minute incubation on ice with 10^5 viable N592 cells. The cells were then washed twice with PBS 2% FCS and incubated with the appropriate FITC-conjugated secondary antibody. After washes, cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). The amount of binder molecules bound to the cells was measured as mean fluorescence intensity (MFI).

To induce internalization, 2 μg/ml I/F8 scFv were allowed to bind to tumor cells monolayers in the presence or in the absence of an equal amount of anti-FLAG mAb, for 40 minutes on ice. When required, 25 μg/ml RITC- or FITC-labeled human transferrin (Molecular Probes, Eugene, OR), or 5 μg/ml FITC-labeled cholera toxin B (Sigma) were added. As a negative control, an anti-NIP apten scFv was used. After scFv binding, cells were extensively washed with cold PBS and then incubated at 37°C for the indicated times, to allow internalization. Surface-bound scFv were stripped by three washes with cold low pH glycine buffer, and cells were then fixed for 5 minutes on ice with 4% paraformaldehyde (PFA) in PBS pH 7.4 and permeabilized with 0.5% Triton X-100 in PBS. Endocytosed scFv was then detected by staining with anti-FLAG mAb, when required, followed by fluorochrome-conjugated goat anti-mouse Ig. When required, cell nuclei were counterstained with a 15-minute incubation at 37°C with a 1 μg/ml propidium iodide (Sigma), 10 μg/ml RNase A (Sigma) solution. The cells were mounted onto slides with Mowiol and analyzed by confocal fluorescence microscopy. Alternatively, the incubation with the primary antibody was followed by a 40-minute incubation on ice with FITC- or RITC-labeled secondary antibody, and the samples were then incubated at 37°C to allow internalization of the immunocomplexes. Surface-bound scFv were stripped as described above and the cells were fixed and analyzed. When the colocalization with other molecules was investigated, after fixation the samples were extensively washed with PBS and the secondary antibody free valences were saturated by a 30-minute incubation with mouse IgG. A second round of PFA fixation followed, and finally the cells were incubated with FITC-labeled anti-clathrin, or with anti-caveolin antibody. Anti-caveolin was detected with a RITC-labeled goat anti-mouse IgM antibody. Isotype-matched negative controls were included. Results were analyzed using an Olympus (Olympus Optical, Tokyo, Japan) laser-scanning microscope FV500 equipped with an Olympus IX81 inverted microscope and Argon ion 488 nm, He-Ne 543 nm, and He-Ne 633 nm lasers. Digital images were acquired through PLAPO 40× and 60× objectives, with the Fluoview 4.3b software program. Images were acquired sequentially as single transcellular optical sections, archived in TIFF and mounted using Photoshop.

Internalization and recycling

ALCAM/CD166 endocytosis and recycling was evaluated by its protection from cleavage by a membrane impermeable reagent based on published pulse-chase protocols (Fabbri et al., 1999; Garza and Birnbaum, 2000), with minor modifications. Subconfluent cells plated on 60 mm diameter dishes were washed twice in PBS and incubated for 30 minutes on ice in the presence of 0.5 mg/ml NHS-SS-biotin (Pierce) in PBS. Labeling reaction was then stopped by two PBS washes, followed by a 10-minute incubation on ice in 150 mM NaCl containing 10 mM glycine. Labelled cells were then allowed to react with 5 μg/ml anti-ALCAM/CD166 3A6 mAb for 40 minutes on ice, and subsequently were incubated at 37°C to allow internalization for the indicated times. The samples were then returned to ice and treated with three successive reductions of 15 minutes on ice with a reducing solution containing 50 mM GSH, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2% BSA, 75 mM NaOH and 75 mM NaCl, pH 8.6. Cells were washed carefully in PBS and either lysed or chased by re-incubation at 37°C for various times in duplicate samples. After the incubation, only one of the two samples was again reduced to quantify the amount of protein that recycled back to the plasma membrane. The samples were then collected, lysed and the protein content of lysates was measured by Micro BCA protein assay kit (Pierce). Equal amounts of proteins were then processed for immunoprecipitation with I/F8-Sepharose as described above.

Immunotoxin and toxicity test

Type 1 single-chain RIP saporin S6 and the I/F8 scFv were conjugated via a disulfide bond between chemically inserted sulfhydryl groups. The resulting I/F8/RIP conjugate was separated from the unreacted reagents and from RIP homopolymers by affinity chromatography with rProtein A-Sepharose (Amersham Biosciences). The immunotoxin was then separated from the unconjugated scFv by gel filtration on a Sephacryl S200 column as described (Tazzari et al., 2001). After conjugation, the toxin:scFv molar ratio was 1.4 and the immunotoxin inhibitory activity of protein synthesis, assayed in a rabbit reticulocyte lysate system, showed an IC_{50} of 4.87 ng/ml. The effects of the immunotoxin on cell lines were evaluated as inhibition of protein synthesis and measured as [^{3}H]leucine incorporation. Cells (10^4/well) were seeded in 96-well microtiter plates and immunotoxin was added to final concentrations ranging from 10^{-11} to 10^{-7} M, as RIP. Parallel samples were run with RIP alone, I/F8 scFv alone, and a mixture of unconjugated scFv and RIP. After incubation for 48 hours, 74 kBq L-[4,5-^{3}H]leucine (Amersham Biosciences) per well was added. Eighteen hours later, the cells were harvested with an automatic cell harvester onto glass fiber paper, and the radioactivity incorporated was measured with a liquid scintillation analyzer Tri-Carb 2100TR (PerkinElmer, Boston, MA). Each experiment was conducted in triplicate. IC_{50} was calculated by linear regression analysis.

Immunotoxin entering into the cells was visualized by indirect immunofluorescence. Briefly, 0.2 μg/ml I/F8/RIP or free RIP were allowed to bind to tumor cells monolayers in the presence of the anti-RIP mAb CY12.14 at 0.5 μg/ml, for 40 minutes on ice. The samples were then incubated at 37°C to allow internalization of the immunocomplexes, surface-bound toxins were stripped, and the cells were fixed, permeabilized, stained with FITC-labeled secondary antibody and analyzed as described above.

Results

Isolation and characterization of the I/F8 scFv anti-ALCAM/CD166

The non-immune scFv phage antibody library ETH-2, was selected for internalizing phage antibodies by panning on the IGROV-I human ovary carcinoma cell line. Five rounds of selection were performed. Selections were monitored, starting from round 3, by titrating the number of phage bound to the cell surface and recovered in the first glycine wash (wash 1, required to remove phage bound to the cell surface) and the number of endocytosed phage recovered from within the cell (cell lysate). Data shown in Table 1 suggest that phage were selected on the basis of endocytosis into IGROV-I cells. Thus, the wash 1:input phage ratio increased by a factor of 10, whereas the cell lysate:input phage ratio showed a 500-fold increase. Screening by ELISA on cells, using native soluble scFv expressed from randomly picked single colonies, showed that 45 out of 96 clones (46.9%) bound IGROV-I cells, and gave no signal above background on human long term cultured T lymphoblasts. The cell-bound scFv were detected via their FLAG-tag using an anti-FLAG monoclonal antibody. The diversity of positive clones was analyzed by PCR amplification and DNA fingerprinting of the scFv gene with *Bst*N1 (Marks et al., 1991). scFv were then grouped on the basis of their digestion pattern. Further investigation of scFv specificity is here limited to one representative clone: the I/F8 scFv. The CDR3 nucleotide sequence insert of the I/F8 scFv DP47 heavy chain is GGGTATGTGGCT and the one of the I/F8 scFv DPL16 light chain is CCCCCTTTTAGTGCGGAG.

Immunofluorescence analysis on a panel of tumor lines, showed that the I/F8 antigen is broadly distributed in human tumors derived from various tissues, although with great differences in the level of surface expression (not shown). Immunoprecipitation from biotin surface-labeled IGROV-I tumor cells, showed that the I/F8 antigen is a 116 kDa monomer (Fig. 1A). Similar protein bands were detected in I/F8-immunoprecipitated molecules from various human tumor cells (Fig. 1B). The 116 kDa protein bands present in I/F8 immunoprecipitates from two IGROV-I cellular extract preparations were subsequently subjected to in-gel digestion with trypsin and protein identification with MALDI-TOF mass spectrometry and peptide mass fingerprinting. A database search revealed identity with ALCAM/CD166 or MEMD protein with high *Z*-scores (2.31 and 2.41, respectively) and a good sequence coverage (ProFound). The MEMD protein is identical to ALCAM/CD166 (Degen et al., 1998). The immunoprecipitates from the other cell lines, although displaying slightly different molecular weights, were again identified as ALCAM/CD166. The differences in molecular weight are probably due to tissue specific variation in N-glycosylation pattern (Pourquié et al., 1992; Denziger et al., 1999).

Comparative analysis of the I/F8 scFv and of the murine monoclonal antibody J4-81 (specific for human ALCAM/CD166) by immunofluorescence indicated that the two reagents display an identical pattern of reactivity on a panel of human

Table 1. Phage antibody library selection on IGROV-I cells indicates the enrichment of internalizing phages

		Phage output		Output/ input ratios ($\times10^{-3}$)	
Round	Phage input	Wash 1	Cell lysate	Wash 1	Cell lysate
3	2.4×10^{9}	2.0×10^{7}	7.5×10^{5}	8.3	0.3
4	3.3×10^{9}	1.2×10^{8}	0.9×10^{8}	36.3	27.2
5	8.0×10^{8}	7.0×10^{7}	1.5×10^{8}	87.5	187.5

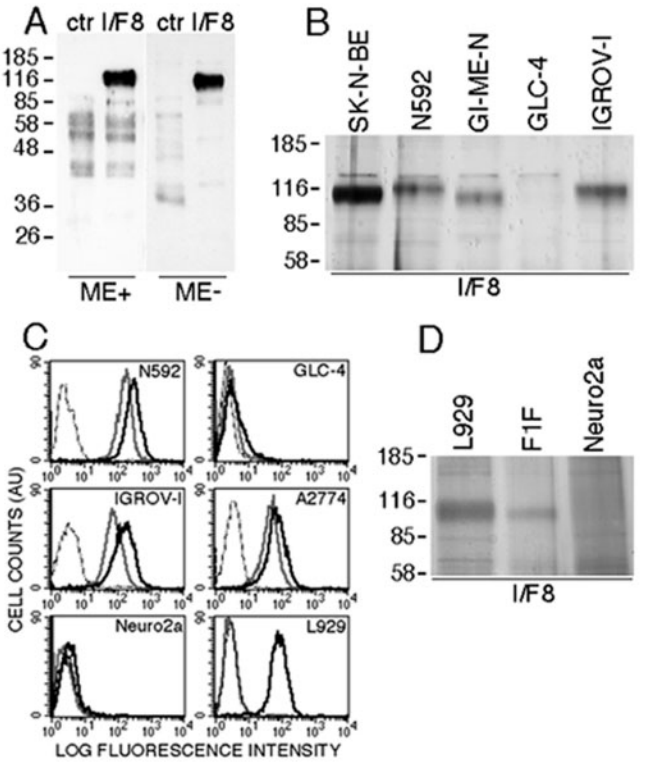

Fig. 1. Characterization of the I/F8 antigen. (A) Immunoprecipitation from Sulfo-NHS-LC-biotin surface-labeled IGROV-I tumor cells using the I/F8 scFv coupled to CNBr-activated Sepharose beads. Immunoprecipitates were resolved by SDS-PAGE on a 10% polyacrylamide gel, and revealed by western blot analysis using HRP-conjugated Streptavidin and chemiluminescence. The I/F8 antigen is a 116 kDa monomer under both reducing (ME+) and non-reducing (ME–) conditions. Immunoprecipitates using an irrelevant scFv coupled to Sepharose beads were run as negative controls (ctr). (B) Silver-stained preparative immunoprecipitation and SDS-PAGE analysis of the I/F8 antigen. I/F8 immunoprecipitates from several tumor cell lines were resolved by SDS-PAGE on a 10% polyacrylamide gel under reducing conditions, and revealed by staining with silver nitrate. Bands were then excised from the gel and processed for MALDI-TOF analysis. (C) Immunofluorescence analysis of I/F8 (black line) and J4-81 (gray line) reactivity on various human and murine tumor cell lines. Broken line represents negative control with an irrelevant scFv. (D) Silver staining of I/F8 immunoprecipitated molecules from three murine tumor cell lines, resolved by SDS-PAGE on a 10% polyacrylamide gel. Numbers on left-hand side of blots indicate molecular weight markers in kDa.

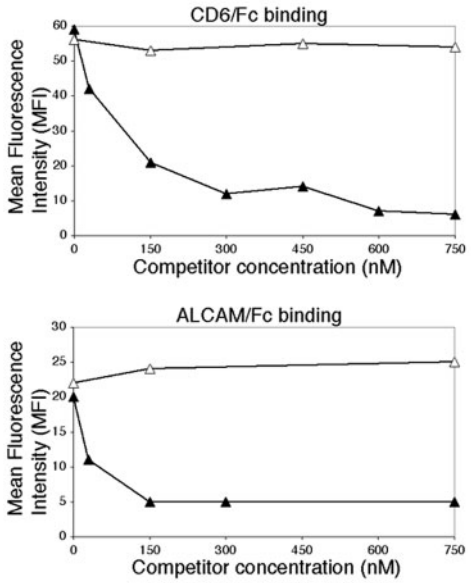

Fig. 2. Cell surface binding interference experiments between ALCAM/CD166 ligands and the I/F8 scFv, measured by flow cytometry on N592 SCLC cells. Ligand binding in the presence of I/F8 is indicated by black triangles and binding in the presence of the irrelevant anti-NIP scFv is indicated by empty triangles. CD6/Fc and ALCAM/Fc chimeras were used at 10 and 100 nM respectively.

tumor cell lines (Fig. 1C). In addition, mouse F1F fibrosarcoma cells and L929 connective tissue cells (Fig. 1C) and monkey COS-7 kidney cells also showed reactivity with I/F8, indicating that the I/F8 scFv recognizes a phylogenetically conserved epitope, whereas the J4-81 antibody does not react with murine cells (Fig. 1C). Consistent with the immunofluorescence data, a 110 kDa protein was immunoprecipitated by I/F8 also from murine cells (Fig. 1D). The anti-ALCAM/CD166 specificity of the I/F8 scFv was further confirmed by ELISA on both recombinant soluble human ALCAM/Fc and murine ALCAM/Fc chimeras (not shown). ELISA on the recombinant soluble human ALCAM/Fc chimera also showed that 42 of the 45 scFv selected on the base of their reactivity with IGROV-I cells were ALCAM/CD166-specific. The three clones whose scFv did not recognize ALCAM/Fc showed indeed BstN1 digestion patterns different from the DNA fingerprint of the I/F8 scFv gene and from each other. Their antigen specificity is currently under investigation.

The ALCAM/CD166 epitope recognized by I/F8 was partly characterized by cell surface binding interference experiments with both ALCAM/CD166 ligands, measured by flow cytometry on the SCLC N592 cell line, that grows in suspension and is more suitable for this type of analysis. I/F8 scFv competed with both CD6/Fc and ALCAM/Fc binding to the cell surface (Fig. 2). The J4-81 antibody inhibited only the CD6/Fc binding (not shown), as expected (van Kempen et al., 2001). These data suggest that the I/F8 epitope is located in the V_1 domain, which is involved in ligand binding (van Kempen et al., 2001). The difference between the ALCAM/Fc and the CD6/Fc concentration in the binding/competition assay (tenfold difference) reflects the difference in affinity between the homophilic and the heterophilic interactions recently reported (Hassan et al., 2004).

Table 2. I/F8 scFv does not compete with either J4-81 or 3A6 binding to ALCAM/CD166 expressed on the surface of SCLC N592 cells

		Mean fluorescence intensity	
Competitor	nM	J4-81 (3 nM)	3A6 (3 nM)
PBS	-	40	38
I/F8 scFv	3	37	41
I/F8 scFv	30	45	47
I/F8 scFv	300	44	59
I/F8 scFv	600	54	63
NIP scFv	600	38	38

In addition, I/F8 failed to compete with both J4-81 and 3A6 antibodies for the binding to ALCAM/CD166 expressed on the surface of N592 cells (Table 2), indicating that the I/F8 scFv recognizes a different epitope.

ALCAM/CD166 is endocytosed slowly via a clathrin-dependent pathway

As the I/F8 phage antibody was selected on the basis of endocytosis into IGROV-I cells, we first investigated by confocal immunofluorescence microscopy whether the ALCAM/CD166 molecule could be internalized upon ligand binding. Live IGROV-I cell monolayers were allowed to bind I/F8 scFv at 4°C in the presence or absence of the anti-FLAG mAb as dimerizing agent. Cells were then moved to 37°C for the indicated times to induce internalization and were subsequently acid-washed to strip the surface-bound molecules. As control of the efficiency of surface stripping by acid washing, one sample was acid-treated directly after incubation at 4°C. Samples were then fixed, stained with labeled secondary antibodies and visualized by confocal microscopy. The residual staining detected in the stripped samples therefore corresponded to the endocytosed molecules. At 4°C the cells bind the antibody (Fig. 3A) but do not internalize, as surface-stripping completely removes the signal (Fig. 3B). On the other hand, upon I/F8 scFv binding ALCAM/CD166 was endocytosed after a 30-minute incubation at 37°C (Fig. 3C), and dimerization by anti-FLAG mAb appeared to substantially increase the internalization process (Fig. 3D). ALCAM/CD166 internalization was also induced by the ALCAM/CD166 soluble ligand CD6/Fc chimera, and by the murine anti-ALCAM/CD166 mAbs 3A6 and J4-81 (not shown).

The kinetics of ligand-induced ALCAM/CD166 endocytosis were then investigated and compared with that of transferrin, a well-established marker of the endosomal pathway, by triggering I/F8-induced internalization in the presence of transferrin-FITC. ALCAM/CD166 is internalized in a time-dependent manner at long time intervals (Fig. 4). After incubation at 4°C, I/F8 staining was associated with the cell surface and was completely removed by stringent acid washes (Fig. 4A, a). After 10 minutes at 37°C, I/F8 staining was still associated primarily with the cell surface, as it was efficiently stripped, but it assumed a ‘punctate’ pattern (Fig. 4A, c). After 30 minutes a more vesicular pattern was observed close to the cell surface and acid wash-resistant staining appeared, indicating presence of internalized molecules (Fig. 4A, e). At 60 minutes, the intracellular vesicular staining was more

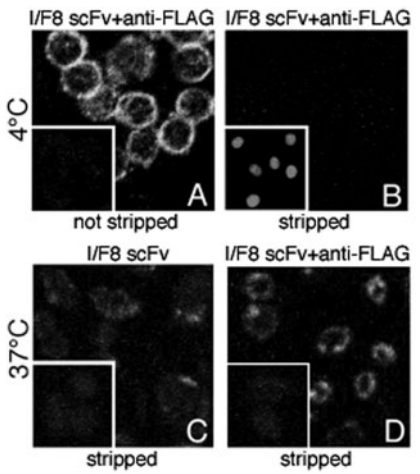

Fig. 3. I/F8-induced internalization of the ALCAM/CD166 molecule. IGROV-I cells were incubated with I/F8 scFv at 4°C in the presence (A,B,D) or in the absence (C) of the anti-FLAG-tag mAb as dimerizing agent. Cells were then placed on ice (A,B) or moved to 37°C for 30 minutes to induce internalization (C,D), and were subsequently untreated (A) or acid-washed to strip the surface-bound molecules (B-D). Samples were then fixed, permeabilized, stained with FITC-labeled secondary antibodies and visualized by using an Olympus confocal laser microscope system. Insets show staining with the irrelevant anti-NIP scFv as a negative control, except B where the Propidium Iodide-counterstained nuclei present in the same field are visualized. The residual staining detected in the stripped samples therefore corresponds to the endocytosed molecules. Magnification, 400×.

pronounced (Fig. 4A, g), but the label formed small 'spots' that, unlike the transferrin signal, distributed in the submembrane region and did not cluster toward the perinuclear area (Fig. 4B). As the kinetics of ALCAM/CD166 endocytosis seems much slower than that of transferrin (Fig. 4A, b,d,f,h), the little colocalization between the two signals could be attributed to asynchrony of the internalization process. Alternatively, the two molecules could follow different endocytic pathways. To address this issue, the ALCAM/CD166 internalization route was studied by sequential immunofluorescence using both the I/F8 scFv and the J4-81 mAb, and anti-clathrin or anti-caveolin-1 antibodies on A2774 cells, which display high caveolin-1 expression. Internalized ALCAM/CD166 colocalized primarily with clathrin (Fig. 5A,B) and not with caveolin-1 (Fig. 5D,E), showing that it is indeed routed to early endosomes via clathrin-coated pits. As positive controls, molecules colocalizing with clathrin and caveolin-1, such as, respectively, transferrin and cholera toxin B, were investigated (Fig. 5C,F).

Endocytosis may dynamically control the amount of cell surface expressed molecules under certain physiological conditions (Minana et al., 2001; Kamiguchi and Lemmon, 2000b). We then examined the internalization of ALCAM/CD166 in cells undergoing division. In A2774 human ovary carcinoma cells the ligand-triggered internalization of the ALCAM/CD166 molecule is maximal at the cleavage furrow during cytokinesis (Fig. 6B,C), suggesting that ALCAM/CD166 internalization may play a role in the rearrangement of cell-cell contacts. This phenomenon was frequently observed in low-density cell cultures, where 55±5% of cell doublets appeared as in Fig. 6 in two different experiments.

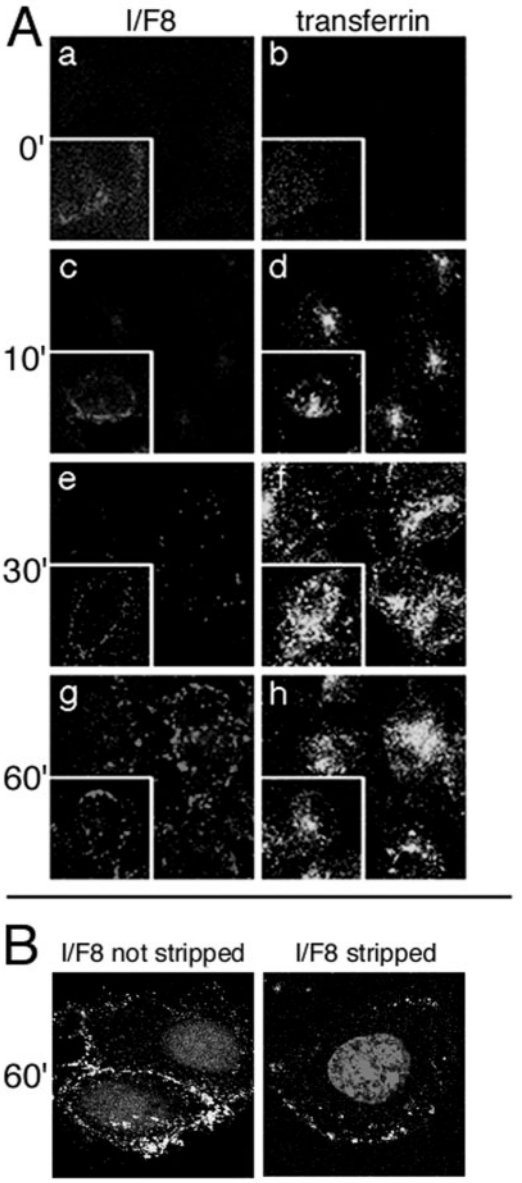

Fig. 4. Internalization kinetics of ALCAM/CD166 compared to transferrin. (A) ALCAM/CD166 internalization is time dependent and slower than transferrin. Confocal microscopic imaging of IGROV-I cells incubated with I/F8 scFv and anti-FLAG mAb in the presence of transferrin-FITC. The samples were allowed to internalize for the indicated times (in minutes) before acid-stripping of the surface-bound molecules, fixing, permeabilizing and staining with RITC-labeled secondary antibody. Images were acquired sequentially as single transcellular optical sections, and the same section is shown stained with I/F8 (left panels) and with transferrin (right panels). Insets show the non-stripped samples. (B) Internalized ALCAM/CD166 distributes in the sub-membrane region and does not cluster toward the perinuclear area. Confocal microscopic imaging of IGROV-I cells stained with I/F8 plus anti-FLAG mAb, allowed to internalize for 60 minutes, acid-washed to strip the surface if required (right panel), fixed, permeabilized and stained with FITC-labeled secondary antibody. Nuclei were counterstained with Propidium Iodide. Magnification, 600×.

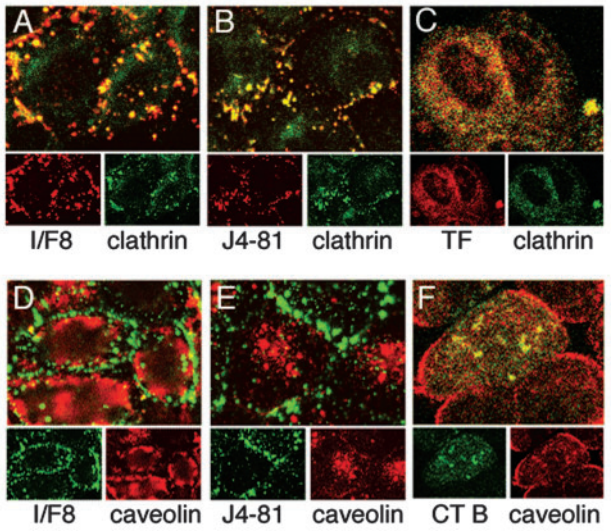

Fig. 5. Internalized ALCAM/CD166 colocalizes primarily with clathrin. A2774 cells were stained either with I/F8 scFv plus anti-FLAG mAb (A,D) or with J4-81 mAb (B,E), allowed to internalize for 40 minutes at 37°C, acid-washed to remove the surface-bound antibody, fixed, permeabilized and stained with fluorochrome-labeled secondary antibody. As the colocalization with other molecules was investigated, the secondary antibody free valences were saturated by a 30-minute incubation with mouse IgG, followed by a second round of PFA fixation, and finally the cells were incubated with FITC-labeled anti-clathrin or with anti-caveolin antibodies. Anti-caveolin was then detected with RITC-labeled goat anti-mouse IgM antibody. As colocalizing positive controls, transferrin-RITC (TF, panel C) and cholera toxin B-FITC (CT B, panel F) were allowed to internalize for 10 and 40 minutes, respectively, at 37°C. Cells were then acid-washed, fixed, permeabilized and processed for staining with anti-clathrin and anti-caveolin antibodies. Double immunofluorescence was visualized by confocal microscopy. Single-color immunofluorescence images are shown in the small panels and the corresponding merged images are shown in the large panels. Original magnification, 600×.

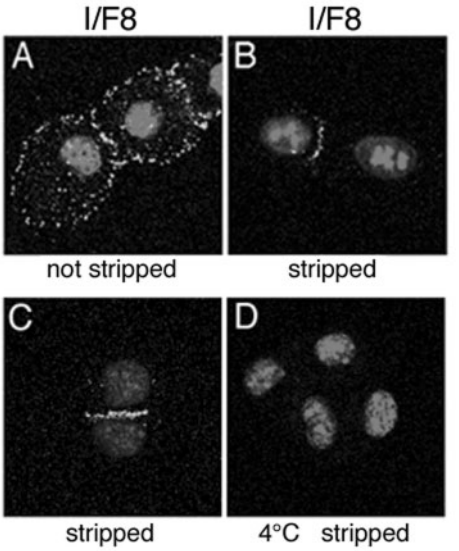

Fig. 6. Ligand-triggered internalization of the ALCAM/CD166 molecule is maximal at the dividing cell contact region. Confocal microscopic imaging of A2774 cells stained with I/F8 plus anti-FLAG mAb, kept on ice (A,D) or allowed to internalize for 40 minutes (B,C), acid-washed to strip the surface if required (B,C,D), fixed, permeabilized and stained with FITC-labeled secondary antibody. Nuclei were counterstained with Propidium Iodide (original magnification 600×).

Internalized ALCAM/CD166 recycles to the plasma membrane

The ability of ALCAM/CD166 to internalize and recycle from the endocytotic compartment back to the cell surface was then biochemically analyzed. Cells were labeled with NHS-SS-biotin on ice, incubated with the anti-ALCAM/CD166 murine mAb 3A6 for various times at 4°C as controls or at 37°C to allow internalization, and then exposed to the membrane impermeable reducing agent glutathione (GSH). The endocytosed pool of proteins was thus protected from reduction and remained biotinylated. The cell samples were then either lysed and immunoprecipitated, or chased at 37°C for various time periods. At each time point, cells were re-exposed to GSH to remove biotin from proteins that had recycled back to the cell surface, lysed and immunoprecipitated. Equal amounts of proteins from cell lysates were subjected to immunoprecipitation using the I/F8 scFv coupled to sepharose, whose binding to ALCAM/CD166 molecules is not blocked by the 3A6 mAb. The antibody-induced internalization of ALCAM protein was detected (Fig. 7A) and showed a time-dependent increase, with a maximum at 40 minutes of incubation (Fig. 7B). Such slow kinetics are consistent with the fluorescence microscopy data (Fig. 4). Chase experiments showed that internalized ALCAM/CD166 molecules could recycle back to the cell surface. The pool of biotinylated ALCAM/CD166 was indeed decreased after a 20-minute chase (Fig. 7C) and was almost undetectable after a 30-minute chase (Fig. 7D). These data indicate that internalized ALCAM/CD166 molecules are not all directed to the degradative pathway but can be recycled to the cell surface, where they become susceptible to the reducing effect of GSH. We can therefore conclude that surface oligomerization of ALCAM/CD166 molecules triggers an endocytosis and recycling process that proceeds at a slow rate.

The ALCAM/CD166 internalization pathway is suitable for intracellular drug delivery

Ribosome-inactivating proteins (RIPs) are *N*-glycosylases from plants that cleave one or more adenine molecules from ribosomal RNA, thus damaging ribosomes in an irreversible manner. Thus the cytotoxicity of RIPs strictly depends on their entrance into the cell. We then evaluated the potential of the scFv I/F8 as an RIP carrier by producing an immunotoxin formed by I/F8 linked to the type 1 RIP saporin via an artificial disulfide bond. The activity of the I/F8/RIP immunotoxin was assayed on both human and murine ALCAM/CD166-positive and -negative cell lines by a protein synthesis inhibition assay. As shown in Table 3, different ALCAM/CD166-positive cells showed different sensitivity to free saporin or to the I/F8/RIP conjugate, but the toxicity of the immunotoxin was always at least a 100-fold higher than that of the free RIP. For example, the I/F8/RIP had an IC_{50} of 3.19×10^{-9} M with saporin on the

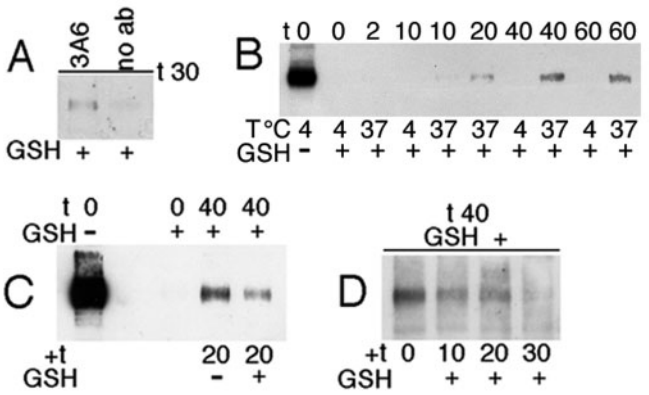

Fig. 7. Ligand-induced internalization and recycling of ALCAM/CD166 molecules. (A) A2774 cells were surface-labeled with NHS-SS-biotin, incubated for 30 minutes at 37°C with or without the anti-ALCAM/CD166 mAb 3A6, treated with the reducing agent GSH, lysed and immunoprecipitated with I/F8 scFv coupled to Sepharose beads. Residual biotin-labeled molecules identify internalized proteins. (B) The kinetics of 3A6 mAb-triggered ALCAM/CD166 internalization was evaluated at different time points prior to GSH treatment and lysis. Optimal receptor internalization is at 40 minutes. The efficiency of surface NHS-SS-biotin stripping by GSH was evaluated at different time points on 3A6-treated cells maintained at 4°C. (C) To analyze receptor recycling, biotin-labeled and 3A6-treated A2774 cells were allowed to internalize ALCAM/CD166 for 40 minutes and were treated with GSH. Cells were then re-incubated at 37°C for 20 minutes to chase internalized receptors, and treated with (+) or without (–) the reducing agent to remove the label from molecules re-expressed to the cell surface. Cell lysis and I/F8-sepharose bead immunoprecipitation followed. Internalized ALCAM/CD166 recycles to the cell surface. (D) Kinetics of recycling. NHS-SS-biotin-labeled internalized receptors were chased by re-incubation at 37°C for different time points (t) before the second GSH treatment and immunoprecipitation. Immunoprecipitates were resolved by SDS-PAGE and revealed by western blot analysis with HRP-streptavidin and chemiluminescence.

L929 murine cell line, whereas free saporin mixed with scFv was toxic only at 3.22×10^{-7} M concentration. Surface ALCAM/CD166 negative cells, as the murine Neuro2a neuroblastoma line, were not affected.

The kinetics of I/F8/RIP entering into the cell could also be visualized (Fig. 8). Live A2774 cell monolayers were allowed to bind either the I/F8/RIP immunotoxin or RIP alone at 4°C in the presence of the anti-RIP mAb CY12.14. Cells were then kept on ice as a control or at 37°C for the indicated times to induce internalization and were subsequently acid-washed to strip the surface-bound molecules. Samples were then fixed, permeabilized, stained with FITC-labeled secondary antibody, counterstained with Propidium Iodide and visualized by confocal microscopy. The staining detected in the stripped samples therefore corresponded to the endocytosed molecules. At 4°C the cells bind but do not internalize the immunotoxin, as surface stripping completely removes the signal (Fig. 8A,B), whereas at 37°C they internalize it in a time-dependent manner at longer time intervals (Fig. 8C-E). Altogether, these data demonstrate that the I/F8 scFv can deliver moieties inside the cell through ALCAM/CD166 internalization.

Table 3. Inhibitory effect of immunotoxin and a mixture of free saporin and I/F8 on protein synthesis by target cell lines

Cell line	IC_{50} (nM)*	
	I/F8/RIP	I/F8+RIP
IGROV-I	5.06	491.0
N592	2.41	252.0
A2774	4.00	458.0
L929	3.19	322.0
Neuro2a	56.30	61.5

*Results are representative of two different experiments per cell line, each performed in triplicate.

Discussion

In this study we describe the selection and characterization of a human anti-ALCAM/CD166 scFv, its use in the study of the internalization and recycling of cell surface ALCAM/CD166, and its potential use for the immunotargeting of therapeutics into ALCAM/CD166-positive cells.

The I/F8 scFv was isolated from a large non-immune phage antibody library. Most of the antibodies isolated from such combinatorial libraries expressed on phage have been selected using immobilized purified antigens or peptides, facing the risk that the selected antibodies do not recognize the native protein in its physiological context. On the other hand, attempts to select non-immune libraries on native antigens expressed on living cells have been successful in only a few cases (Marks et al., 1993; de Kruif et al., 1995; Vaughan et al., 1996; Wong et al., 2001). The limiting factor in the selection by cell panning is related to the high background binding of non specific phages compared to the relatively low binding of phages specific for a given antigen (Pereira et al., 1997; Watters et al., 1997; Becerril et al., 1999). Here we show the advantage of selecting for antibodies that trigger a biological function, such as receptor-mediated endocytosis, to reduce the high background binding when selecting phage antibodies by panning on living cells. In addition, this approach may allow the selection of antibodies capable of mimicking natural ligand functions, a property that may have practical applications. In the case of I/F8, the capacity of being endocytosed as a phage-displayed scFv was retained by the monomeric soluble scFv, albeit with a limited efficiency when compared with its dimerized form. However, the internalizing properties of the scFv monomer were sufficient for the intracellular delivery of a drug that requires entry into the cell for its activity (Stirpe, 2004).

Endocytosis is involved in several cellular processes including regulation of cell-surface expression of signaling molecules, and proceeds through a variety of clathrin-dependent and clathrin-independent endocytic pathways. Our present data suggest that ALCAM/CD166 can be internalized following soluble ligand engagement primarily through a clathrin-dependent pathway. Similarly clathrin-dependent internalization has been reported for other members of the CAMs family, such as N-CAM (Minana et al., 2001) and L1 molecules (Kamiguchi et al., 1998). However, kinetic studies revealed that ALCAM/CD166 internalization proceeds at a slower rate than that of other well known examples of clathrin-mediated endocytosis, such as the transferrin. Slow

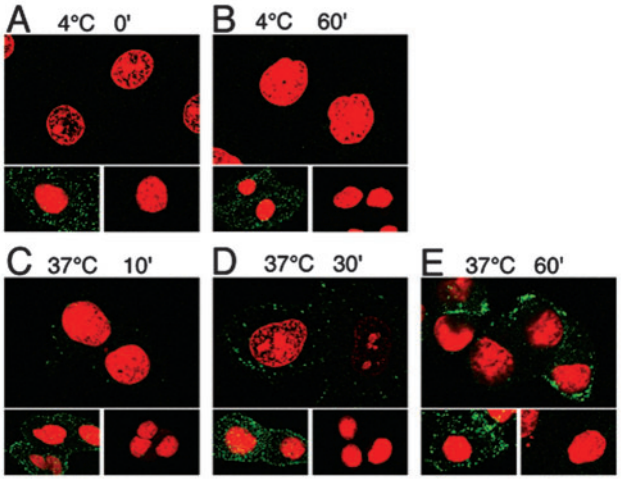

Fig. 8. Internalization kinetics of the I/F8/RIP immunotoxin. A2774 cells were incubated with either I/F8/RIP (large and small left-hand panels) or RIP alone (small right-hand panels) at 4°C in the presence of the anti-RIP mAb CY12.14 as dimerizing agent. Cells were then left on ice (A,B) or transferred to 37°C for different times to induce internalization (C,D,E), and were subsequently untreated (small panels) or acid-washed to strip the surface-bound molecules (large panels). Samples were then fixed, permeabilized, stained by FITC-labeled secondary antibody and nuclei were counterstained with Propidium Iodide. Samples were visualized by using an Olympus confocal laser microscope system. Crosslinking the immunotoxin with anti-RIP mAb allowed the visualization of the internalized fraction at concentrations as low as 1×10^{-8} M I/F8/RIP (about 0.2 μg/ml). This amount of immunotoxin was used, as it is the highest concentration that still shows a 100-fold difference in toxicity when compared to free RIP. Original magnification, 600×.

internalization kinetics have also been described also for other receptors internalized through clathrin-dependent pathways (Innamorati et al., 2001; Bronfman et al., 2003). In addition, unlike transferrin, internalized ALCAM/CD166 molecules appeared to localize predominantly in a sub-membrane compartment even at later time points.

In the well-known clathrin-dependent endocytic pathway, internalized molecules are delivered to the early endosome and then trafficked either to the recycling endosome or to the late endosome-lysosome pathway (reviewed by Mellman, 1996; Sorkin and von Zastrow, 2002). As the internalized ALCAM/CD166 molecule seemed to reside in the sub-membrane region, we investigated whether it could recycle back to the cell surface. A biochemical assay based on NHS-SS-biotin labeling of surface molecules and treatment with a non-cell-permeable reducing agent (GSH) able to cleave the SS-biotin link, allowed us to demonstrate that ALCAM/CD166 is indeed endocytosed and recycled back to the cell membrane within 30 minutes of chasing with GSH.

Several lines of evidence indicate that CAMs do not simply act as an inert glue that mediates static cell-cell interactions but their surface expression and adhesivity is dynamically controlled to respond to different environmental requirements. For example, cell adhesion can be spatially regulated by the polarized internalization and recycling of CAMs (reviewed by Murase and Schuman, 1999; Kamiguchi and Lemmon, 2000a; Kamiguchi, 2003). As cytoskeletal constraints are known to regulate ALCAM/CD166 lateral mobility (Nelissen et al., 2000), and during cytokinesis actin filaments are dynamically reconstructed and concentrated to the cleavage furrow (Straight and Field, 2000), we investigated ALCAM/CD166 internalization in epithelial cells undergoing cytokinesis. Our present data indicate that ligand-induced ALCAM/CD166 internalization does indeed appear concentrated at the cleavage furrow, where cell-cell contacts must be modified. This observation suggests that ALCAM/CD166 internalization, which requires ligand binding, is also dependent on cytoskeleton dynamics.

In addition to the ability to trigger ALCAM/CD166 internalization, which is shared with other soluble ligands, two remarkable features characterize the I/F8 scFv: the reactivity across different species (human, mouse, monkey) and the efficient competition with soluble ligand binding, both CD6/Fc and ALCAM/Fc chimeras. It is well known that phage display libraries potentially lead to a different spectrum of epitopes from natural immune systems, also generating reagents against the conserved and non-immunodominant regions of a given antigen (Nissim et al., 1994), as is for the case for the I/F8 scFv. A previous targeted mutagenesis study mapped the protein surface structure involved in heterophilic interaction in the N-terminal Ig (V_1) domain of the ALCAM/CD166 molecule (Skonier et al., 1996). The homophilic interaction site was also located in the same V_1 domain by protein truncation experiments (van Kempen et al., 2001). The finding that the I/F8 scFv can efficiently compete with binding of both soluble ALCAM/Fc and CD6/Fc chimeras, strongly suggests that the I/F8 epitope also maps to the V_1 domain of the ALCAM/CD166 molecule.

The CD6 binding site in ALCAM/CD166 is highly conserved in different homologs and mediates cross-species heterophilic binding, whereas the β-sheet face, opposite the CD6 binding site, is less conserved. This observation provided a molecular rationale for the apparent inability to obtain murine monoclonal antibodies against the CD6 binding site (Skonier et al., 1996; Bowen et al., 1997). Only the J4-81 murine antibody blocked heterophilic ALCAM/CD166-CD6 interactions (Bowen et al., 1996), but its reactivity was limited to human ALCAM/CD166. As the I/F8 scFv is reactive across different species, and blocks soluble ALCAM/CD166 ligands binding, we hypothesize that its epitope is mapped within the conserved CD6-binding protein surface. Several studies involved the ALCAM/CD166 molecule in the process of cell spreading, and in particular in the metastatic behavior of different tumors, such as human melanoma (van Kempen et al., 2000) or murine fibrosarcoma (Choi et al., 2000). Thus the availability of a reagent such as I/F8, which is capable of blocking ALCAM/CD166 functions, may provide a useful tool to interfere in ALCAM functions and analyze its functional role in the metastatic process.

The fully human origin of the I/F8 scFv, together with the demonstration that it can be used for the delivery of drugs into a cell, suggests potential applications in tumor targeting. However, the expression of the ALCAM/CD166 molecule on several normal tissues (Uchida et al., 1997; Cortés et al., 1999; Swart, 2002) may hamper the systemic delivery of I/F8 conjugated to highly toxic molecules. Nonetheless, the possible use of I/F8 conjugates in local tumor therapy, or for targeting of less toxic compounds to ALCAM/CD166-positive

cells, may represent an attractive possibility. The recycling experiments show that not all the ALCAM/CD166 molecules are involved in the internalization process, but that a relevant amount of ALCAM/CD166 is still available at the cell surface. These molecules may indeed represent a suitable target for the delivery of other biological modifiers such as immune-stimulating agents.

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