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# Fractionation of polyclonal antibodies to fragments of a neuroreceptor using three increasingly chaotropic solvents

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**Abstract**

We have developed specific antibodies against fragments of anaplastic lymphoma kinase (ALK) in order to develop tools for characterizing the expression and biological function of this orphan receptor. The first fragment consisted of residues 280 to 480 of the murine extracellular domain, was expressed in *Escherichia coli* (*E. coli*), purified in the presence of urea from the pellet of mechanically lysed cells and injected into rabbits as an unfolded protein in urea. The second fragment consisted of residues 1519 to 1619 of the murine sequence, corresponding to the C-terminal side of the kinase domain. It was expressed in *E. coli* as a soluble glutathione-S-transferase fusion protein, purified from the supernatant of broken cells and injected into rabbits as a folded protein. Both antisera were purified using antigen affinity chromatography, with the polyclonal antibodies eluted stepwise using three different buffers, 0.1 *M* glycine, pH 2.9, followed by 7 *M* urea, pH 4, followed by 6 *M* guanidine–HCl (GdnHCl), pH 4. Antisera prepared against either antigen contained antibodies that eluted in each of the three pools, indicating that solvents more chaotropic than acid were required to elute antibody populations that were tightly bound to the antigen column. All three antibody pools were reactive towards their respective antigens upon Western blot analysis. Purified polyclonal antibodies (pAbs) to both fragments also recognized the full-length protein expressed in Chinese hamster ovary cells. In every case, the pAbs eluting in GdnHCl were the most sensitive for detecting full-length ALK. 

*Keywords:* Polyclonal antibodies; Anaplastic lymphoma kinase

## 1. Introduction

Anaplastic lymphoma kinase (ALK) is an orphan tyrosine kinase receptor that was initially isolated as a dimeric nucleophosmine-fusion protein [1]. ALK is a member of the insulin receptor family and has significant sequence homology to leukocyte tyrosine kinase and a molecular mass of over 200 000 [1]. The expression of ALK is localized to neuronal cells in specific parts of the brain, such as the thalamus, the midbrain, etc. [2]. The size of the full-length protein has made it very difficult to obtain folded, purified ALK, and yet much about its functional role in vivo remains unknown. We have therefore gener-

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ated polyclonal antibodies (pAbs) against *E. coli*-derived ALK fragments to use as tools in the characterization of the functional role and biological activity of ALK. Two fragments of ALK were used to immunize rabbits, a peptide consisting of residues 280 to 480, corresponding to part of the extracellular region, and a fragment consisting of residues 1519 to 1619, corresponding to the C-terminal side of the kinase domain [2]. The latter was expressed as a soluble glutathione-S-transferase (GST)-fusion protein.

Although immunological experiments can be done using impure antisera, the many contaminating proteins may interfere with the specific interactions between antigen and Ab. Superior results are often obtained using purified antigen-specific Abs. Purification is usually performed using antigen affinity chromatography on a commercially available activated gel matrix to which the antigen has been covalently bound. Specific antibodies are eluted with conventional elution buffers, such as acid or salt [3], but often with a low recovery [4,5]. In fact, when an initial attempt was made to purify antibodies specific to these ALK fragments using antigen affinity chromatography and acid elution alone, the recovery of pAbs was very low, with much of the antibody remaining bound on the column. We have developed a three-step chaotropic elution procedure, which, when coupled with a procedure for refolding the eluted pAbs [6,7], results in increased recovery of total Ab and also in fractionation of the specific antibodies into subpopulations of differing reactivities. We have applied this technique to ALK, resulting in increased recovery of specific pAbs, which will subsequently be used to characterize the functional role of ALK.

## 2. Experimental

### *2.1. Cloning and expression of ALK fragments in E. coli*

The DNA fragment coding for murine ALK 1519–1619 (referred to as GST–muALK) was cloned into Pharmacia's GST-fusion expression vector pGEX-2T. The expression of the GST–ALK fusion protein is under the control of the *tac* promoter. Upon induction with isopropyl β-D-thiogalactoside (IPTG) at 30°C, the expression of a soluble protein with a molecular mass of about 43 000, corresponding to an intact GST–muALK fusion protein, was achieved.

The DNA fragment coding for murine ALK 280–480 (referred to as ALK280) was polymerase chain reaction (PCR) amplified from a cDNA clone and incorporated into Amgen's autoinducible proprietary expression vector pAMG21. Upon induction with the autoinducer at 37°C, the expression of a protein with a molecular mass of about 23 000, which was sequestered as aggregates in inclusion bodies, was achieved.

### *2.2. Purification of ALK fragments*

The cells expressing ALK280 were broken in double distilled water with a microfluidizer (Microfluidics, Newton, MA, USA) and centrifuged at 14 000 *g*, to pellet insoluble cellular debris. The inclusion bodies of ALK280 were solubilized in 8 *M* urea and the protein was purified by ion-exchange chromatography in the presence of the urea.

*E. coli* cells expressing GST–muALK were broken mechanically in the presence of 1 m*M* dithiothreitol (DTT), and Triton X-100 was added to the lysate to give a final concentration of 1%. The supernatant after centrifugation was incubated with glutathione Sepharose (Pharmacia Biotech, Uppsala, Sweden) for several hours at 4°C. The resin was washed with binding buffer (BB) containing 20 m*M* Tris–HCl, 1 m*M* DTT, 1% Triton X-100, pH 7.5. The GST–muALK protein was subsequently eluted with BB containing 50 m*M* glutathione (GSH) at neutral pH. The purified protein was dialyzed against BB to remove the reduced glutathione. GST was also expressed in *E. coli* as a soluble protein and purified with glutathione Sepharose chromatography, as described for GST–muALK. SDS–PAGE analysis confirmed the size and purity of the proteins, while N-terminal sequencing confirmed that the purified protein was indeed the desired ALK fragment.

### *2.3. Generation of rabbit polyclonal antisera to E. coli-derived ALK fragments*

Rabbits were immunized with the purified GST–ALK fusion protein (1 mg/rabbit) suspended in Freund's complete adjuvant, followed by boost injections with the protein (1 mg/rabbit/injection) in

incomplete adjuvant at 14-day intervals. For ALK280, the rabbits were injected with a solution of the unfolded protein in 8 *M* urea (0.5 mg/rabbit), followed by boost injections of the same material at 14-day intervals.

### *2.4. Immunoaffinity purification of ALK280 and GST–muALK polyclonal antibodies*

ALK antigen columns were prepared using CNBr-activated Sepharose 4B (Pharmacia Biotech), according to the manufacturer's instructions, with one exception: 8 *M* urea was included in the coupling buffer (0.1 *M* sodium bicarbonate, 0.5 *M* NaCl, pH 8.3) for the immobilization of ALK280, as it was insoluble in the coupling buffer alone. After coupling, both antigen columns were washed extensively with phosphate-buffered saline (PBS). ALK280 was coupled at a ligand density of 1 mg/ml resin and GST–muALK was coupled at a density of 2 mg/ml resin. GST was also coupled at a ligand density of 2 mg/ml of resin using CNBr-activated Sepharose. Antisera from rabbits immunized with ALK280 protein were incubated with the ALK280–Sepharose for at least 1 h at 4°C. The flow-through from the immunoaffinity resin was collected by vacuum filtration and the resin was packed into a glass column. After washing the column with PBS until a baseline of UV absorbance was achieved, antibodies were eluted with the sequential application of three increasingly chaotropic solvents: 0.1 *M* glycine, pH 2.9 (elution buffer 1, E1), followed by 50 m*M* sodium acetate, 7 *M* urea, pH 4.0 (E2) and, finally, with 50 m*M* sodium acetate, 6 *M* GdnHCl, pH 4.0 (E3). Following elution, the antibody pools were refolded by dialysis using a previously published procedure [7]. GST–muALK antibodies were purified with the same protocol after the antisera had first been passed over a GST–Sepharose column to remove antibodies made against the GST portion of the fusion protein. The flow-through from the GST–Sepharose column was then incubated with GST–muALK–Sepharose and eluted from the column using the immunoaffinity procedure described for the ALK280 antibodies.

### *2.5. Protein determination*

Protein concentrations were determined by measuring the absorbance at 280 nm using a Beckman DU650 spectrophotometer and assuming $\epsilon_{280}^{0.1\%}$ of 1.1 for ALK280, 1.3 for GST–muALK, 1.6 for GST and 1.5 for the purified antibody pools.

### *2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)*

SDS–PAGE was performed using 4–20% precast Tris–glycine gels (Novex, San Diego, CA, USA) on a Mighty Small II apparatus (Pharmacia Biotech) at a constant voltage of 150 V. Samples were prepared for SDS–PAGE by mixing them with sample buffer containing β-mercaptoethanol. Different loading volumes were used depending on the objective. Molecular mass ($M_W$) markers were obtained from BioRad and Novex.

### *2.7. Western blotting analysis*

Three different analyses were performed. In all cases, antigen was mixed with SDS–PAGE reducing sample buffer, boiled for 5 min and loaded onto a 4–20% Tris–glycine gel. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane overnight at 10V in transfer buffer (25 m*M* Tris, 192 m*M* glycine, 20% methanol) using a mini trans-blot cell (BioRad, Hercules, CA, USA). All subsequent steps were carried out in PBS containing 0.05% Tween-20 (PBS-T) at room temperature with gentle rocking. After blocking for at least 30 min with PBS-T+5% nonfat milk, the nitrocellulose was incubated for 1 h with antibody at various concentrations (discussed below). The nitrocellulose was washed three times with PBS-T and then incubated with an anti-rabbit Ig–horseradish peroxidase (HRP) conjugate (Amersham, Arlington Heights, IL, USA) for 30 min. Following three washes with PBS-T, protein bands were visualized using the ECL system (Amersham).

To examine the distribution of specific antibodies in the three eluent pools, a nitrocellulose membrane onto which 100 ng of the *E. coli*-derived ALK fragment had been transferred was probed using either the E1, E2 or E3 pools, normalized to represent a 1:500 (v/v) dilution of the original antisera.

To assess the sensitivity of the various antibodies to their respective ALK antigens, 50 and 100 ng of antigen were used. The various Ab pools were used

as primary antibody, at an Ab concentration of 50 ng/ml.

In the third type of Western blot analysis, the ability of the different antibody pools to recognize full-length ALK was investigated. Chinese hamster ovary (CHO) cells transfected with either the ALK receptor gene or empty vector (vector control) were lysed with RIPA buffer (25 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 2 m*M* Pefabloc) at a cell density of $1\times10^7$ cells/ml. A 20-μl volume of the lysate was mixed with reducing sample buffer and electrophoresed. The various antibody pools at a concentration of 5 μg/ml were used as the primary antibody.

## 3. Results and discussion

### *3.1. Antibody purification*

Because we did not have any full-length ALK that expressed in *E. coli*, we made several fragments with the hope that Abs generated against one of these could be used as a reagent to study the full-length protein in vivo. This strategy did not require that these fragments be either folded or active. Several fragments from the ALK were generated. The protein fragment from residues 280 to 480, which was used to generate the anti-ALK280 antibodies, was arbitrarily selected, and does not correspond to any predicted domain structure. It was expressed as inclusion bodies in *E. coli*, and purified in the unfolded form in urea. All attempts to remove the urea at neutral pH resulted in precipitation of the ALK280; therefore, a solution of the ALK280 in urea was used to generate antibodies. Three different rabbits were inoculated with this solution of denatured ALK280 antigen in urea, and the different antisera obtained were fractionated (individually) using the procedure described in Section 2. Nearly all of the specific antibodies that were present in the antisera bound to the ALK280 antigen columns, as demonstrated by Western blot analysis of the column flow-throughs (data not shown). Although ALK280 was injected in the denatured state in 8 *M* urea, the majority of the pAbs bound to the ALK280 antigen column in PBS (this was made by conjugating the ALK280 in the presence of 8 *M* urea followed by extensive washing with PBS). This suggests that either the conformation of ALK280 upon injection into rabbits is similar to that of the protein on the Sepharose column, or, alternatively, that the antibodies recognize linear epitopes, which are accessible on ALK280 under both denaturing and physiological conditions. Antibodies eluted in each of the three pools, E1, E2 and E3. The distribution of the antibodies in the different pools is summarized in Table 1. There are differences between the antibody distribution and the total amount of ALK280 antibody recovered from the individual rabbits, but in every case, a substantial percentage of the total

Table 1
Recovery of antibodies from immunoaffinity columns

| | 0.1 *M* glycine, pH 2.9 | | 50 mM NaAc, 7M urea, pH 4.0 | | 50 m*M* NaAc, 6 *M* GdnHCl, pH 4.0 | | Total protein (mg) |
|---|---|---|---|---|---|---|---|
| | Protein recovered (mg) | Percentage of total protein recovered | Protein recovered (mg) | Percentage of total protein recovered | Protein recovered (mg) | Percentage of total protein recovered | |
| αALK280 | | | | | | | |
| Rabbit 1 | 0.74 | 53 | 0.4 | 28 | 0.27 | 19 | 1.41 |
| Rabbit 2 | 0.63 | 37 | 0.8 | 47 | 0.27 | 16 | 1.7 |
| Rabbit 3 | 1.5 | 31 | 2.7 | 56 | 0.63 | 13 | 4.83 |
| αGST–ALK | | | | | | | |
| Rabbit 1 | 2.7 | 43 | 1.6 | 25 | 2.0 | 32 | 6.3 |
| Rabbit 2 | 1.3 | 37 | 0.9 | 26 | 1.3 | 37 | 3.5 |
| Rabbit 3 | 2.0 | 29 | 1.8 | 26 | 3.2 | 45 | 7.0 |

antibodies eluted in both the urea (E2) and the GdnHCl (E3) fractions. SDS–PAGE analysis of the purified antibody pools indicated that the purity is greater than 80%, with albumin as the major contaminant (Fig. 1).

In contrast to the ALK280 protein, the GST–muALK construct was chosen with the hope that it represented an intact structural domain located C-terminal to the kinase domain. Its expression as a soluble GST-fusion protein, rather than as an insoluble protein sequestered in inclusion bodies, may reflect the fact that this ALK domain can fold individually. Folding could also be facilitated by the presence of a stable dimeric GST structure. However, the exact conformational state of this protein was not determined, as it was not the focus of these experiments. The antisera from the three rabbits immunized with GST–muALK were fractionated, with results similar to those obtained with ALK280 (shown in Fig. 1 and Table 1). Again, antibodies eluted in all three pools, with the distribution varying between rabbits. It appears that the anti-GST–muALK eluents contain less contamination. This could be due to the fact that many of the proteins that bind non-specifically to the antigen column may have been removed by the preceding GST affinity chromatography step.

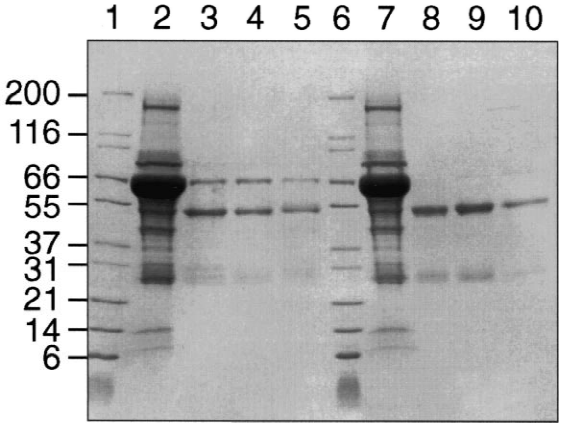


Fig. 1. SDS–PAGE analysis of ALK antibodies eluted from antigen affinity chromatography. A volume equivalent to 2 μg of purified antibody fraction or antisera was run on reducing SDS–PAGE as described in Section 2. Lane 1: Mark 12 markers; lane 2, anti-ALK280 antisera; lane 3, anti-ALK280 E1; lane 4, anti-ALK280 E2; lane 5, anti-ALK-280 E3; lane 6, Mark 12 markers (Novex); lane 7, anti-GST–muALK; lane 8, anti-GST–muALK E1; lane 9, anti-GST–muALK E2; lane 10, anti-GST–muALK E3. Molecular mass markers (in kg/mol) are indicated at the left margin.

The antisera made against both antigens contained polyclonal antibody populations that required strongly chaotropic solvents for elution from the antigen column. These antibodies would have been lost if only the 0.1 *M* glycine, pH 2.9, had been employed as eluent. A third ALK fragment, ALK25, consisting of residues 25 to 250 from the extracellular region of murine ALK, was also purified from *E. coli* as a denatured protein and injected into rabbits. When the resulting antisera was purified as described in Section 2, all of the bound antibodies eluted in the 0.1 *M* glycine, pH 2.9, step. This antisera could also be used for Western blot analysis with its antigen (data not shown). For this antigen, there was no antibody population that required the more chaotropic solvents to elute it. There appears to be no a priori rule to correlate which solvents the antibodies will elute in with the state or fragment of antigen used. No high affinity antibodies appear to have been generated against the ALK25, while both ALK280 and GST–muALK result in the generation of pAbs requiring more strongly denaturing solvents to disrupt the antigen–antibody binding. While the exact distribution of antibodies between the three different eluent pools varies from rabbit to rabbit, the presence or absence of protein in a given pool is a constant and is dependent on the antigen used.

### *3.2. Antibody characterization*

Western blot analysis using ALK280 ($M_r$=23 000) as an antigen was carried out for the three eluents obtained from the ALK280 antisera (E1, E2 and E3). In order to compare the amount of reactive Ab in each of the three eluents, the E1, E2 and E3 pools were used as primary antibody, with the volume of antibody normalized to represent an equivalent amount of the original antisera. As shown in Fig. 2A, E1, E2 and E3 all showed strong reactivity to the ALK280 band, indicating that they all bind the denatured antigen quite avidly. Since this analysis necessitated using different volumes of the three pools, the intensity of staining present reflects both the total amount of reactive Ab present as well as the

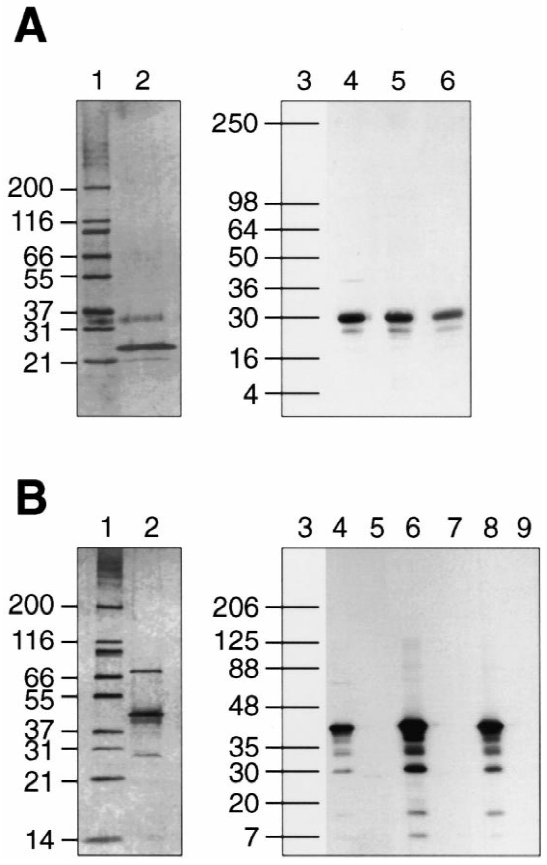


Fig. 2. Western blot characterization of eluted antibodies. (A) Anti-ALK280 antibody. A 100-ng amount of ALK280 antigen was run on SDS–PAGE gels under reducing conditions and then transferred onto nitrocellulose (lanes 4–6). The membrane was probed with the E1, E2 or E3 antibody pools, normalized to correspond to the same volume of antisera. An additional reducing SDS–PAGE was run with 1 μg of ALK280 and this gel was stained with silver to show all protein bands present in the purified antigen preparation. Lane 1, Mark 12 standards (in kg/mol); lane 2, silver stained ALK280 preparation; lane 3, See Blue standards (Novex) (in kg/mol); lane 4, ALK280 probed with E1; lane 5, ALK280 probed with E2; lane 6, ALK280 probed with E3. (B) Anti-GST–muALK antibody. GST–muALK or GST were loaded on a reducing SDS–PAGE gel at 100 ng/lane, electrophoresed and transferred onto nitrocellulose (lanes 4–9). The E1, E2 or E3 antibody pools normalized to correspond to the same volume of antisera were used as a primary antibody. A second SDS–PAGE gel was run with 1 μg of GST–muALK antigen. Protein bands were detected with silver staining. Lane 1, Mark 12 markers; lane 2, silver stained GST–muALK preparation; lane 3, prestained broad $M_w$ markers (BioRad); lane 4, GST–muALK probed with E1; lane 5, GST probed with E1; lane 6, GST–muALK probed with E2; lane 7, GST probed with E2; lane 8, GST–muALK probed with E3; lane 9, GST probed with E3.

affinity of the antibody for the antigen. Thus, the strong reactivity of the antibody pools to ALK280 could either be the result of more total protein (antibody) present in the pool to react with the antigen and/or the presence of that pool of antibodies with a high affinity to the antigen. It is interesting to note that the pAbs in E1, but not in E2 and E3, react weakly to a protein with an apparent molecular mass of 40 000, which is not visible in the silver-stained gel of the ALK280 antigen preparation. All three antibody pools also react with a protein with an apparent molecular mass of 21 000, which is probably a degraded form of ALK280. The molecular masses were determined from the silver stained gel, as the See Blue markers run anomalously when compared to the Mark 12 standard proteins used for the SDS–polyacrylamide gels. However, they can be used for comparing bands between blots.

The anti-GST–muALK pools E1, E2 and E3, when adjusted to equal volume, also bound avidly to the antigen (the GST–muALK) they were originally raised against, detecting the GST–muALK band ($M_r$=43 000) and lower-molecular-mass bands upon Western blot analysis, as shown in Fig. 2B. As the GST-specific Abs were removed from the antisera by affinity chromatography on GST–Sepharose, the lower bands observed most likely reflect degraded products of GST-fusion proteins containing at least a part of the ALK1519–1619 sequence that was fused to GST. In this case, E2 appeared to have the highest reactivity to the antigen followed by E3, then E1. None of the antibody pools reacted with the 70 000 molecular mass band seen on the silver-stained gel. This protein is most likely DnaK, which often co-purifies with GST fusion proteins when a glutathione column is used during the purification [8].

The comparison described above, using volumes equivalent to the same amount of original antisera, depends on both the amount of Ab in the pool and the sensitivity of Ab in each pool. In order to assess antibody sensitivity, Western blot analysis was performed using a fixed concentration of each of the antibody pools, E1, E2 or E3 as the primary antibody (Fig. 3). For anti-ALK280, the E2 pool had the highest sensitivity to ALK280, followed by E1, strongly detecting both 50 and 100 ng amounts of ALK280. The reactivity of E3 towards the ALK280 is very low, suggesting that antibodies eluted with

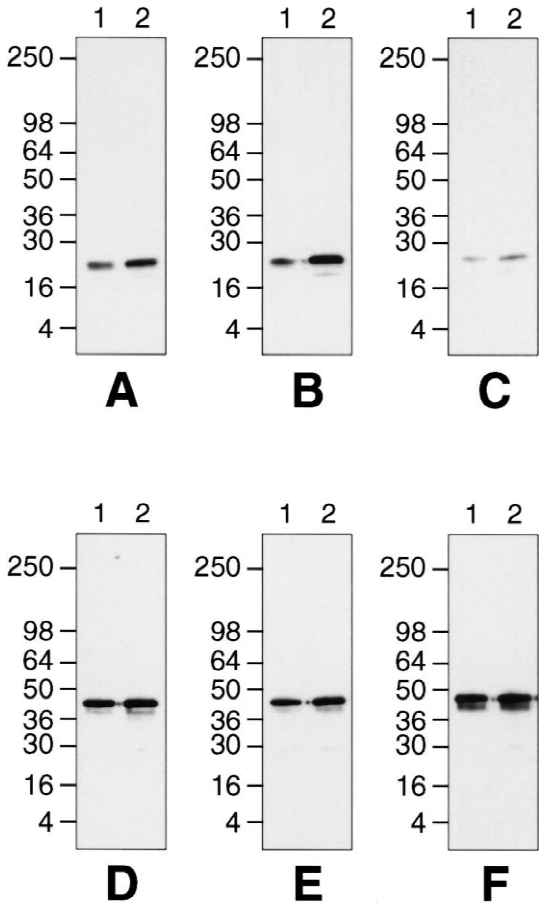


Fig. 3. Western blot analysis of the sensitivity of the antibody fractions towards their antigens. Two different amounts (50 and 100 ng) of the ALK280 or GST–muALK antigens were subjected to reducing SDS–PAGE, and then transferred onto nitrocellulose. The E1, E2 or E3 antibody pools were used as the primary antibody at a concentration of 50 ng protein/ml. Panels A–C. Reactivity of ALK280 antibody pools E1, E2 and E3, respectively, to ALK280. Lane 1, ALK280 (50 ng); lane 2, ALK280 (100 ng). Panels D–F. Reactivity of GST–muALK antibody pools E1, E2 and E3, respectively, to GST–muALK. Lane 1, GST–muALK (50 ng); lane 2, GST–muALK (100 ng). Molecular mass markers (kg/mol) are indicated at the left margin.

the more denaturing conditions do not recognize the denatured ALK fragment as well as antibodies eluted with glycine or urea. It should also be pointed out that the GdnHCl-eluted Ab is the most difficult to refold and least stable and, hence, the observed weak staining for the E3 could be due to conversion to an inactive form (such as an aggregated species) during storage at 4°C.

In the case of the GST–muALK Ab pools, E3 has the greatest sensitivity to GST–muALK, followed by E2 and E1. Again, the results are similar with both 50 or 100 ng of antigen. In this case as well, the concentration of active Ab in E3 may be lower than estimated. It is interesting to note that, in antisera generated against a denatured ALK fragment (ALK280), Abs showing the strongest sensitivity to the antigen are eluted under milder conditions than those Abs generated against a folded soluble protein (GST–muALK). This suggests that the ALK280 Abs bind with less avidity than the GST–muALK Abs to their respective antigen columns. This may be due to differences in conformation of the ALK280 antigen following folding of the protein during injection into rabbits versus folding following immobilization of the protein onto the resin. As the urea-solubilized ALK280 is injected into the animal, protein folding is accomplished by the rapid dilution of both the urea and protein concentrations. This could result in a conformation that is different from that generated when ALK280 refolds after it has been conjugated with the resin during the preparation of the affinity column. Thus, antibodies made against the conformation that ALK280 assumes during injection might show a weaker affinity and, therefore, elute earlier, when exposed to a conformation of ALK280 as it exists as an immobilized protein. It is impossible to determine the actual conformation of ALK280 as it exists once it is in circulation within the rabbits.

The ability of the different antibody pools to recognize unfolded full-length ALK was tested using Western blot analysis, with lysate from ALK-transfected CHO cells as the antigen source (Fig. 4). For this experiment, the identical concentration of antibody in each pool was used to compare their sensitivities. Lysate from CHO cells that had not been transfected with ALK was included as a control. Fig. 4A shows the results with anti-ALK280. The three eluents reacted with a protein band with an apparent molecular mass of 200 000. There are several lower-molecular-mass bands that were also stained with the E1, E2 and E3 pools. With the exception of the 60 000 $M_r$ band present in both the ALK-transfected CHO lysate and the control lysate,

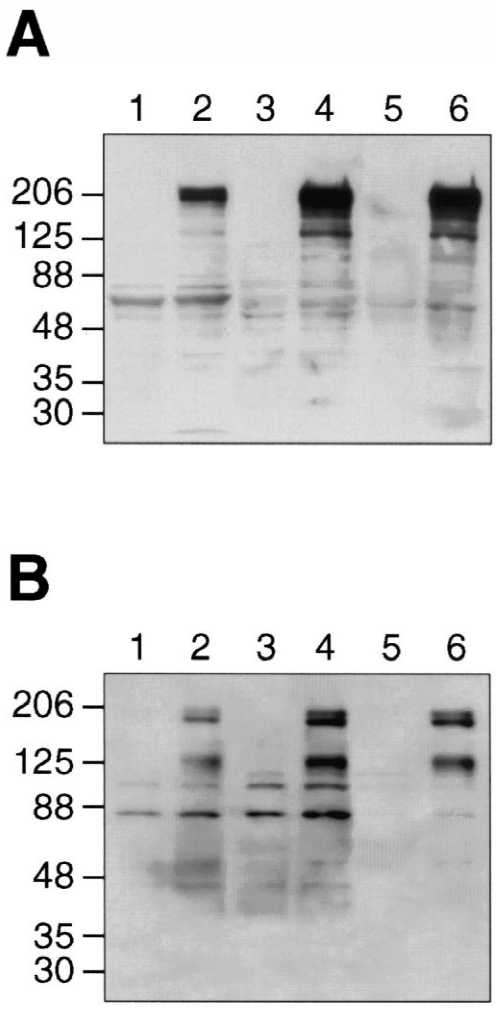


Fig. 4. Reactivity of E1, E2 and E3 antibody pools to full-length ALK receptor. Lysate from CHO cells transfected with full-length ALK (ALK/CHO) was used as a source of antigen, subjected to SDS–PAGE, and transferred onto nitrocellulose. Western blotting was performed with either ALK280 antibody pools (A) or with GST–muALK antibody pools (B). Antibodies were used at a concentration of 5 μg/ml. A lane containing CHO cells transfected with the vector only (V/CHO) was included to assess antibody specificity. Lane 1, V/CHO probed with E1; lane 2, ALK/CHO probed with E1; lane 3, V-CHO probed with E2; lane 4, ALK/CHO probed with E2; lane 5, V/CHO probed with E3; lane 6, ALK-CHO probed with E3. Molecular mass markers (in kg/mol) are indicated at the left margin.

these low-molecular-mass bands indicate post-translational modification or processing occurring at the N-terminus and/or the C-terminus of ALK. The E2 and E3 appear to react more strongly with the full-length ALK than does the E1; this is different than their order of sensitivity to the ALK280 antigen (Fig. 3). Thus, there is a difference in the sensitivity of some of the antibody pools to the full-length protein versus the antigen fragment. This could indicate that there is a difference in the degree of exposure of epitopes for the E1, E2 and E3 pools on the ALK280 fragment compared to the CHO-derived full-length ALK. The epitopes recognized by the E1 antibodies appear to be well exposed on ALK280 following SDS–PAGE and transfer, but are less accessible in the CHO-derived ALK. The opposite is true for the E3, since it showed the weakest sensitivity to ALK280, while exhibiting the strongest sensitivity to CHO-derived ALK.

Fig. 4B shows the reactivity of the GST–muALK pAbs with the full-length ALK. All three eluents reacted with both the ALK band at 200 000 and also with a band with an apparent molecular mass of 130 000. This smaller protein is also recognized by the anti-ALK280, although to a much lesser extent. This suggests that the 130 000 dalton protein results from a truncation in the extracellular domain of the full-length ALK, the region of the protein that is recognized by anti-ALK280. As is the case for the anti-ALK280, the E2 and E3 pools from the anti-GST–muALK showed stronger reactivity to CHO ALK than the E1 pool.

Antisera from other rabbits immunized with either ALK280 or GST–muALK were fractionated and analyzed for reactivity against the full-length CHO ALK and, in every case, the E3 pool had the greatest reactivity towards the full-length molecule. It thus appears that the pool of pAbs that binds the most avidly to the antigen affinity column contains the population of pAbs that have the highest affinity for epitopes present and accessible on the full-length CHO ALK. Omitting the GdnHCl elution would thus have resulted in the loss of the pAbs, which could prove to be the most useful reagent for characterizing the activity and localization of the native ALK.

The reactivity of these antibody pools to the antigen fragment and to the full-length molecule varies, indicating that the solvents have fractionated the antisera into subpopulations of antibodies with different binding properties. Thus, ALK280 E3 is a poor reagent for Western blot analysis of the ALK280 fragment, but a very good reagent for the

full-length CHO ALK, while GST–muALK E2 and E3 are both excellent reagents for Western blot analysis of both GST–muALK and the CHO ALK. Using the impure antisera as a reagent would avoid the problem of low recovery, but would add the complication of reactions with the many contaminating proteins present in sera. The differential reactivity of the three Ab eluent pools also suggests that reagents for a specific application can be generated with this chromatographic procedure.

From the results reported here, it thus appears that by utilizing protein fragments as antigens, followed by antigen affinity chromatography with the three eluents as described, it is possible to obtain antibody reagents that can be used to detect the full-length protein. This procedure not only results in the increased recovery of antibodies, but also results in the fractionation of pAbs into subpopulations, which may be useful for different applications.

## 4. Abbreviations

| | |
|---|---|
| Ab | Antibody |
| pAbs | Polyclonal antibodies |
| ALK | Anaplastic lymphoma kinase |
| *E. coli* | *Escherichia coli* |
| SDS–PAGE | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis |
| GST | Glutathione-S-transferase |
| GSH | Glutathione |
| CNBr | Cyanogen bromide |
| PBS | Phosphate-buffered saline |
| GdnHCl | Guanidine–hydrochloride |
| HRP | Horseradish peroxidase |
| NC | Nitrocellulose |
| Gly | Glycine |
| DTT | Dithiothreitol |
| IPTG | Isopropyl β-D-thiogalactoside |
| Ig | Immunoglobulin |

## References

[1] S.W. Morris, M.N. Kirsten, M.B. Valentine, K.G. Dittmer, D.N. Shapiro, D.L. Saltman, A.T. Loo, Science 263 (1994) 1281.
[2] T. Iwahara, J. Fujimoto, D. Wen, R. Cupples, N. Bucay, T. Arakawa, S. Mori, B. Ratzkin, T. Yamamoto, Oncogene 4 (1996) 439.
[3] P. Cuatrecasas, Biochem. Biophys. Res. Commun. 35 (1969) 531.
[4] P. Cuatrecasas, C.B. Anfinsen, in: W.B. Jakoby (Ed.), Methods in Enzymology, Vol. XII, Academic Press, New York, 1971, p. 345.
[5] J.L. Casey, P.A. Keep, K.A. Chester, L. Robson, R.E. Hawkins, R.H.J. Begent, J. Immunol. Methods 179 (1995) 105.
[6] L.O. Narhi, D.J. Caughey, T. Horan, Y. Kita, D. Chang, T. Arakawa, Anal. Biochem. 253 (1997) 236.
[7] L.O. Narhi, D.J. Caughey, T. Horan, Y. Kita, D. Chang, T. Arakawa, Anal. Biochem. 253 (1997) 246.
[8] M. Yu-Sherman, A.L. Goldberg, EMBO J. 11 (1992) 71.