Biochimica et Biophysica Acta, 799 (1984) 68–79
Elsevier

BBA 21738

HAPTEN-MEDIATED IMMUNOPURIFICATION OF MEMBRANE PROTEINS LABELED WITH FLUORESCEIN DERIVATIVES

GREGG G. GUNDERSEN * and BENNETT M. SHAPIRO **

Department of Biochemistry, University of Washington, Seattle, WA 98195 (U.S.A.)

(Received September 15th, 1983)
(Revised manuscript received February 1st, 1984)

Key words: Immunoadsorbent; Fluorescein derivative; Membrane protein; Hapten

The behavior of cell surface components labeled with fluorochromes can be studied by fluorescence microscopy and spectroscopy; further structural analyses would be facilitated by purification of the labeled components. We have developed a protocol for identifying the targets for labeling with fluorescein derivatives, by using ^{125}I-diiodofluorescein isothiocyanate (^{125}IFC) and for isolating the labeled components with anti-IFC immunoadsorbents. Anti-IFC antibodies obtained from rabbits immunized with IFC-hemocyanin were purified by affinity chromatography and coupled to CNBr-activated Sepharose 4B. The anti-IFC immunoadsorbents could then be used to isolate the entire set of ^{125}IFC-proteins from crude detergent extracts of labeled sea urchin sperm, with a 70% yield and a purification of more than 250 fold. Nonspecific binding of unlabeled proteins to the immunoadsorbent was insignificant. When the immunoadsorbent IFC-protein complex was used directly as an immunogen, antibodies were obtained that reacted with the underivatized proteins that were targets for IFC labeling, as indicated by immunoblotting after gel electrophoresis. The antibodies also reacted with the surface of unlabeled sperm as shown by immunofluorescence. Thus, by treating the IFC-sperm proteins as a class, we obtained antibodies that recognized the unlabeled proteins in situ or in cell extracts. This approach should be generally useful in obtaining reagents directed against specific cell surface components.

Introduction

The labeling of cellular components with fluorescein derivatives has served as a versatile and powerful tool for analyzing molecular behavior by fluorescence spectroscopy and microscopy. For example, fluorescein conjugates of proteins are being used in studies of protein-protein interaction [1–4], of the fate of specific components microinjected into cells [7–11], as specific inhibitors of cellular ATPases [5,6], and, more generally, fluorescent antibodies or lectins are indicators of the behavior of their cellular ligands (e.g. Refs. 12–16). A particularly interesting application of fluorochrome labeling has been the direct reaction with cell surfaces, to provide a system for the exploration of lateral and rotational mobility of membrane components [17–22] or of their fates after membrane fusion [23,24]. Since fluorescein derivatives are photosensitizers, the labeling of specific classes of membrane proteins may be used as a tool to cause specific, temporally controlled photodamage to cells [23,25], a phenomenon that may inadvertantly lead to crosslinking in photo bleaching

* Present address: Department of Biology, UCLA, Los Angeles, CA 90024, U.S.A.
** To whom correspondence should be addressed.
Abbreviations: IFC, diiodofluorescein isothiocyanate; FITC, fluorescein isothiocyanate.

experiments (see Refs. 26–28 for a consideration of this issue).

The physiological behavior of fluorescein-labeled cell surface molecules could be extended to a biochemical level with appropriate structure-function correlations, if the labeled compounds could be isolated and purified. In order to identify the molecular species labeled with fluorescein derivatives, we have used the radioactive congener of fluorescein isothiocyanate (FITC), ^{125}I-diiodo-fluorescein isothiocyanate (^{125}IFC), which can be prepared to high specific activity [29]. FITC- and ^{125}IFC-labeled sperm have allowed an analysis of the fate of sperm membrane components following fertilization [24,30–32]. Whenever altered cellular components are studied, as in the case of fluorescein-labelling of cell surfaces, one must determine whether the labeled components behave similarly to their underivatized counterparts. Toward this end we have developed a technique to purify ^{125}IFC-labeled sperm proteins by virtue of the hapten they bear, expanding upon an approach for the isolation of labeled proteins from complex mixtures [33]. In this paper we report on the preparation of anti-IFC immunoadsorbents and characterize their effectiveness in recovering ^{125}IFC-labeled proteins from crude detergent extracts of labeled sperm. Antisera obtained from rabbits injected with the purified, IFC-proteins reacted with the proteins that carried IFC, whether or not they were labeled. The antisera reacted with whole sperm, lending support to our previous data [29,30] indicating that proteins that are targets for FITC-labeling are on the sperm surface. This approach to the isolation of membrane polypeptides should be applicable to other cell surface labeling systems in order to allow identification and analysis of the labeled components and to provide a means for studying them in their native state.

Methods

Materials. IFC and its radioactive derivative, ^{125}IFC, were synthesized as previously described [29]. Affigel Blue was obtained from Bio-Rad Laboratories (Richmond, CA). *Limulus polyphemus* hemocyanin and polylysine were purchased from Sigma Chemical Co. (St. Louis, MO). Nonidet P-40 and sodium dodecyl sulfate (SDS) were obtained from BDH Chemicals Ltd. (Poole, U.K.). Goat anti-rabbit IgG conjugates (FITC, alkaline phosphatase, and horseradish peroxidase) were from Miles Laboratories, Inc. (Elkhart, IN). Freund's complete adjuvant and normal goat serum were purchased from Gibco Laboratories (Grand Island, NY). Na^{125}I, ^{125}I-Protein A (2–10 μCi/μg), and [^{3}H]succinimidyl propionate (91 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Nitrocellulose, type BA 85, 0.45 μm, was from Schleicher and Schuell, Inc. (Keene, NH).

Sperm from the sea urchin, *Strongylocentrotus purpuratus,* were obtained as previously described [34].

IFC-labeling of proteins. Labeling of lysine, bovine serum albumin, and hemocyanin with IFC were as previously described for porcine γ-globulin [30]. Free IFC was removed by gel filtration over Sephadex G-25. Acetone precipitation of the purified, labeled proteins showed less than 1% free IFC.

IFC- and ^{125}IFC-labeling of sperm and preparation of sperm extracts. Labeling of sea urchin sperm with IFC or ^{125}IFC and extraction with Nonidet P-40 were as previously described [30]. The Nonidet P-40 extraction yielded approximately 90% of the sperm radioactivity and resulted in a 4-fold increase in specific activity (cpm ^{125}I/mg protein). In order to concentrate the ^{125}IFC-labeled proteins in the Nonidet P-40 extract and remove low molecular weight, non-protein radioactive species, a 30% ethanol precipitation, at $-15\,^{\circ}$C, was performed. The ethanol precipitate was either dissolved directly in SDS sample buffer for analysis by SDS-polyacrylamide gel electrophoresis or redissolved in 0.1% Nonidet P-40, 20 mM Tris, pH 8.0, 1 mM dithiothreitol, 10 μg/ml aprotinin, and 0.2 mM phenylmethylsulphonyl fluoride (buffer 1) and dialyzed vs. the same buffer at 4°C. This preparation contained approx. 90% of the protein [35] in the Nonidet P-40 extract and 25% of the radioactivity. Analysis by SDS-polyacrylamide gel electrophoresis showed that all the ^{125}IFC-labeled polypeptides in the Nonidet P-40 extract were recovered in the ethanol precipitate. This redissolved ethanol precipitate was used in the characterization of the antibody resins described below and is referred to as the concentrated, detergent-

extracted ^{125}IFC-sperm proteins.

Labeling of the concentrated detergent extract (prepared identically to that described above but omitting the labeling of sperm with ^{125}IFC) with 8 mM [^{3}H]succinimidyl propionate was at room temperature for 90 min in buffer 1 with Hepes, pH 7.5, substituted for Tris at a final protein concentration of 2.4 mg/ml. Following exhaustive dialysis to remove unreacted [^{3}H]succinimidyl propionate, greater than 95% of the cpm were trichloroacetic acid-precipitable.

Preparation and purification of rabbit anti-IFC antiserum. Preparation of antiserum to IFC was essentially as previously described except that *Limulus polyphemus* hemocyanin was used as carrier for IFC rather than porcine γ-globulin [30]. 10 mg of IFC-labeled hemocyanin was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites on the hind limbs of the rabbit. Intravenous booster injections of 1 mg of IFC-hemocyanin were given at 3 and 6 weeks to obtain high titer antiserum against IFC. A γ-globulin fraction of both preimmune and immune serum was prepared by 40% $(NH_4)_2SO_4$ fractionation. The antiserum specificity was analyzed with double immunodiffusion on 1% agarose [36].

Anti-IFC antibodies were purified by first preparing IgG from the $(NH_4)_2SO_4$ fraction by passing it over DEAE-Sephacel equilibrated with 20 mM $NaPO_4$, pH 8.0. Under these conditions IgG passed through the column while other serum proteins remained bound. The eluted IgG fraction was then placed directly on an Affigel Blue column equilibrated in the same buffer. Affigel Blue was used instead of a resin containing the hapten IFC, since IFC-specific antibodies eluted from Affigel Blue under much milder conditions (see Results and Fig. 1).

Preparation of anti-IFC immunoadsorbents. The $(NH_4)_2SO_4$ and affinity purified fractions of rabbit anti-IFC were coupled to CNBr-activated Sepharose 4B [37] by adding 2–2.5 mg protein per ml resin. Coupling was 90% complete and yielded resins with approx. 2 mg protein/ml settled resin.

The anti-IFC resins were then washed extensively with: 0.5 M HOAc, 0.5 NaCl; 2 M urea, 0.5 M NaCl; 1 M NaCl, pH 10.3; buffer 1; and finally 10 mM Tris-buffered saline, pH 7.4. The immunoadsorbent retained binding capacity for over a year if stored at 4°C in Tris-buffered saline containing 0.1% NaN_3.

The specificity and capacity of the immunoadsorbents was assessed in a microbinding assay. Generally, a small amount of resin (25–50 μl settled volume) was incubated with the concentrated detergent extract of ^{125}IFC-sperm protein (10^5 cpm; 5–20 nm IFC) in 150–200 μl of buffer 1 for 2 h at room temperature. The resin was then washed with 1 ml each of buffer 1 (twice); 1 M NaCl, pH 10.3 (3 times); 8 M urea (3 times); and finally buffer 1. The radioactivity in the pellet (resin) was taken as bound radioactivity and the sum of the radioactivity in the washes as the unbound material. Total recovery of radioactivity with this assay was greater than 90%. Elution of bound ^{125}IFC-sperm proteins with dissociation agents was performed on immunoadsorbents containing approx. 5×10^4 cpm bound ^{125}IFC after washing 3 times with buffer 1; 1 ml of each dissociation agent was added for 15 min at room temperature followed by two 15 min washes with the dissociation agent. The radioactivity recovered in the sum of the three washes was taken as the amount removed by the reagent.

Production of rabbit anti-sperm protein antibodies by injection of IFC-sperm protein: immunoadsorbent complexes. Antibodies were prepared against the IFC-sperm proteins by directly injecting rabbits with a complex of IFC-sperm proteins bound to the anti-IFC immunoadsorbents. The preparation of this complex followed the protocol used in the microbinding assays, except that larger amounts of anti-IFC immunoadsorbents were used and saturating amounts of cold, detergent solubilized IFC-sperm proteins were incubated with the resins. The volume of resin used for each injection was 2 ml for the immunoadsorbent prepared from the affinity purified anti-IFC antibody (injected into rabbit B) and 4.5 ml for the immunoadsorbent prepared from the $(NH_4)_2SO_4$ fraction of anti-IFC antiserum (injected into rabbit D). Immediately prior to injection the immunoadsorbent complex was washed twice with sterile Tris-buffered saline and emulsified to whipped cream consistency by adding 1 volume of Freund's complete adjuvant and 1 volume of sterile Tris-buffered saline and then sonicating and pipetting up and

down with an 18 gauge needle. The emulsion was injected subcutaneously with a 20 gauge needle into multiple sites in the rabbit's shoulder and upper back. Booster injections with the same amount of resin complex were given 2, 6, and 12 weeks after the initial injection. The rabbits were bled at intervals, and the serum obtained was $(NH_4)_2SO_4$ fractionated and assayed for reactivity against sperm proteins.

Immunological assays. An enzyme linked immunosorbent assay (ELISA) [38] was used to quantify the reactivity of the antisera towards the sperm proteins, essentially as described [39]. Each well of the microtiter plate was coated with 200 μl of a concentrated detergent extract of unlabeled sperm proteins (prepared identically to that prepared with the labeled proteins) diluted to 40 μg/ml in Tris-buffered saline containing 0.1% Nonidet P-40 and 0.02% NaN_3 (higher concentrations of the concentrated detergent extract gave identical results, while with lower concentrations the backgrounds were higher).

To determine the specificity of the antisera a western blot [40] analysis was performed as described previously [41]. After SDS-polyacrylamide gel electrophoresis [42] proteins from gradient gels (7.5–15% acrylamide) were transferred to nitrocellulose at 10°C in a Transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA) with 8 V/cm (about 0.5 A) for 2 h (see below). For quantitation of the extent of transfer, the radioactivity in 5 mm slices of the gel and in the paper was determined. Immunological detection was with either ^{125}I-protein A or goat anti-rabbit IgG conjugated to horseradish peroxidase, both according to Towbin et al. [41], except that 4-chloro-1-naphthol at 1 mg/ml was used as a substrate for the horseradish peroxidase.

An initial characterization of the efficiency of electrophoretic transfer of detergent-extracted ^{125}IFC-sperm proteins to nitrocellulose paper showed that most labeled components transfered equivalently, although species below 20 and above 200 kDa did not transfer as well as species between these molecular weights. The low molecular weight components did not bind well to nitrocellulose, whereas proteins above 200 kDa were not eluted from the polyacrylamide gel efficiently. The total transfer time had little effect on the transfer of species between 20 and 200 kDa; increasing the transfer from 1 to 8 h increased the amount bound to nitrocellulose from 50 to 65%. With long transfer times there was a decreased recovery of total radioactivity in the gel and the nitrocellulose, in part due to migration of some components away from the nitrocellulose paper toward the cathode (as shown by placing a piece of nitrocellulose on the cathodic slide of the gel). Because quantitative transfer of the species to the nitrocellulose paper was unobtainable even for long transfer times, we used a convenient 2 h transfer protocol which gave 50–60% transfer of components between 20 and 200 kDa.

Indirect immunofluorescence of fixed sperm was used to localize the antigens detected by the antisera. Sperm were washed twice (10 min; $1000 \times g$) in Millipore filtered seawater and then fixed in 3% paraformaldehyde in Millipore filtered seawater MFW for 1 h at 10°C. After fixation the sperm were washed 3 times with Millipore filtered seawater and then twice with tris-buffered saline containing 0.02% NaN_3 and stored at 4°C. The fixed sperm could be used for a week with little loss in the quality of immunofluorescent staining. Acetone permeabilization of the fixed sperm was the same as reported for hydroid sperm [43], except that the cells were not air dried. The fixed sperm were placed on coverslips coated with 1% polylysine for the antibody incubations. A 30 min preincubation with Tris-buffered saline containing 5% normal goat serum was found to reduce background staining and was also used in the antibody incubation solutions. Primary antibody incubations with $(NH_4)_2SO_4$ fractions of antisera obtained from rabbits B and D were performed at 37°C for 90–120 min in a moist chamber. The coverslips were washed 4 times in Tris-buffered saline and then incubated with the second antibody, an IgG fraction of goat anti-rabbit IgG labeled with FITC, for 90–120 min at 37°C. After washing, the coverslips were mounted on slides in Tris-buffered saline, pH 8.0 containing 20% glycerol and 0.02% NaN_3 and sealed with fingernail polish. Observation was with a Leitz Laborlux 11 microscope using the K2 filter cube for FITC fluorescence. Photographs were taken with Tri-X film (Kodak).

Other methods. Protein determinations were

performed with a modification of the procedure of Lowry et al. [35] designed to remove interfering lipids and reducing agents [44]. SDS-gel electrophoresis was on 7.5–15% polyacrylamide gels according to Laemmli [42]. Gels were stained in 0.1% Coomassie Brilliant Blue R in methanol/acetic acid/H_2O (5 : 1 : 5) and destained in methanol/acetic acid/H_2O (5 : 1 : 5).

Results

Affinity purification of rabbit anti-IFC antibodies

In order to obtain a specific immunological probe for the hapten, IFC, we took advantage of the binding of anti-IFC antibodies to Cibacron Blue F3Ga linked to agarose (Affigel Blue). In a typical experiment, two chromatography columns were used in series. The $(NH_4]_2SO_4$ fraction of antiserum was passed over DEAE-Sephacel (to obtain the IgG fraction) and the eluant was passed directly over Affigel Blue (see Methods). After most of the IgG passed through the linked columns, the DEAE-Sephacel column was disconnected and material was eluted from the Affigel Blue resin by washing with a two step gradient of 200 mM NaCl and 1.4 M NaCl (Fig. 1). By immunoelectrophoresis, fractions I, II, and III of Fig. 1 showed a single precipitation band with either goat anti-rabbit serum or goat anti-rabbit IgG (data not shown). Double diffusion analysis of fractions I–III of Fig. 1 indicated that all of the anti-IFC activity was retained by the Affigel Blue resin and could be recovered in the high salt fraction, free from anti-hemocyanin antibody (Fig. 2). The IFC-specific IgG obtained with this protocol had 2–6.5% of the protein of the original $(NH_4)_2SO_4$ fraction or 6–10% of all the IgG recovered in peaks I, II, and III, values consistent with the amounts of specific antibodies found in immunized animals [48].

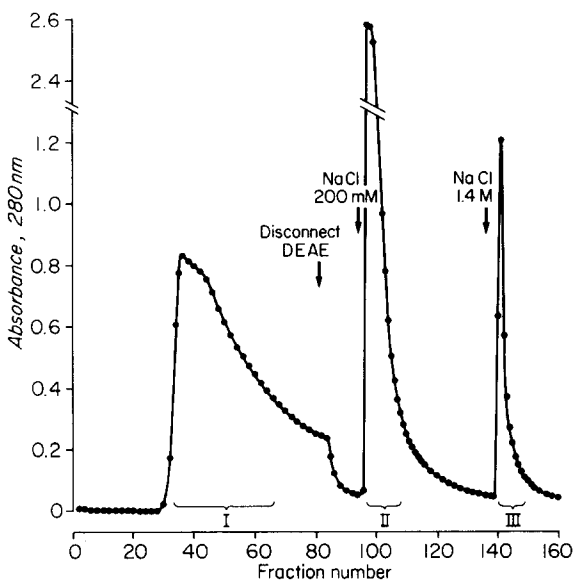

Fig. 1. Affinity purification of anti-IFC antibody using serial column chromatography. 500 mg of an $(NH_4)_2SO_4$ fraction of rabbit antiserum prepared against IFC-hemocyanin was loaded onto a 2.6×40 cm column of DEAE-Sephacel equilibrated in 20 mM sodium phosphate, pH 8.0 at 20°C. The outflow from this column was directly passed over a 1.6×10 cm column of Affigel Blue at approx. 0.5 ml/min. Fractions (6 ml) were collected and their absorbance at 280 nm was monitored during the run. After the majority of the breakthrough fraction had been collected ($A_{280} = 0.2$) the DEAE column was disconnected (Disconnect DEAE) and the Affigel Blue resin washed until $A_{280} \leq 0.050$. At this point 200 mM NaCl was added to the 20 mM sodium phosphate buffer. After the A_{280} had returned to 0.050 the NaCl concentration was increased to 1.4 M. Fractions pooled for Ouchterlony analysis are indicated as I, II, and III.

Characterization of anti-IFC immunoadsorbent

Purified anti-IFC antibody was linked to Sepharose 4B to form an immunoaffinity resin that bound IFC-containing sperm proteins. The ^{125}IFC-labeled sperm protein sample containing radioactive polypeptides of diverse molecular weights (Fig. 3a) provided a good test of the binding properties of the immunoadsorbent. About

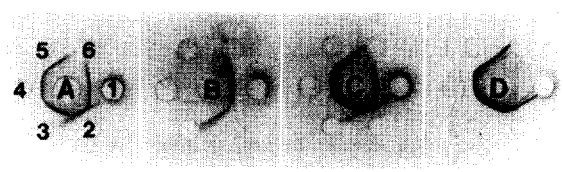

Fig. 2. Ouchterlony double immunodiffusion of fractions from the affinity purification of anti-IFC antibody. The outer wells received 10 µl of the following: (1) 3.0 mg/ml hemocyanin; (2) 3.0 mg/ml IFC-hemocyanin; (3) 6.2 mg/ml IFC-bovine serum albumin; (4) 1.2 mg/ml IFC-bovine serum albumin; (5) 0.62 mg/ml IFC-bovine serum albumin; (6) 1.0 mg/ml bovine serum albumin. The middle wells received 30 µl of the following: (A) $(NH_4)_2SO_4$ fraction of anti-IFC-hemocyanin (starting material); (B) fraction I (3 mg/ml); (C) fraction II (3 mg/ml); and (D) fraction III (3 mg/ml). The gels were stained with Coomassie Brilliant Blue.

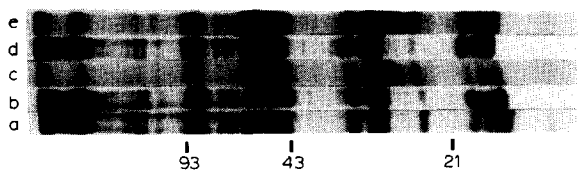

Fig. 3. SDS-gradient gel (7.5–15% polyacrylamide) analysis of ^{125}IFC-labeled sperm proteins. Lanes a–e show autoradiograms after electrophoresis with an equal amount of radioactivity in each lane. Lane a: detergent-solubilized ^{125}IFC-proteins; lane b: nonbinding fraction of the detergent-solubilized ^{125}IFC proteins after incubation with an immunoadsorbent prepared from the $(NH_4)_2SO_4$ fraction of anti-IFC serum; lane c: fraction eluted with SDS sample buffer from the immunoadsorbent prepared from the $(NH_4)_2SO_4$ fraction of anti-IFC serum; lane d: nonbinding fraction of the detergent-solubilized ^{125}IFC-proteins after incubation with an immunoadsorbent prepared from affinity purified anti-IFC; lane e: fraction eluted with SDS sample buffer from the immunoadsorbent prepared from affinity purified anti-IFC. Molecular weight markers in kilodaltons are shown along the bottom.

50% of the ^{125}IFC-labeled sperm protein bound to the resin under the standard conditions (see Methods), and substitution of Nonidet P-40 with equivalent amounts of *n*-octyl glucoside or deoxycholate had little effect on binding. Although all of the experiments reported below were done in buffer 1, we subsequently found that by increasing the concentration of Nonidet P-40 to 0.5% and adding SDS to 0.1%, even higher recoveries could be obtained.

The specificity of the anti-IFC immunoadsorbent was tested in several ways. As shown in Table I, under conditions in which 50% (or greater) of the ^{125}IFC-labeled proteins were bound to an immunoadsorbent made with immune serum, less than 3% binding was observed for a control immunoadsorbent prepared from an $(NH_4)_2SO_4$ fraction of preimmune serum. Nonspecific binding to underivatized Sepharose 4B was negligible (Table I). IFC-lysine and IFC-bovine serum albumin inhibited the binding of ^{125}IFC-labeled proteins; preincubation with IFC-lysine reduced binding over 85%, while preincubation with IFC-bovine serum albumin was less effective, presumably due to the lower effective concentration of IFC obtainable with IFC-bovine serum albumin (Table I).

To determine the extent of nonspecific protein binding to the anti-IFC resin, i.e., the binding of proteins not containing IFC, we incubated the immunoadsorbent with an extract of sperm proteins labeled with [^{3}H]succinimidyl propionate (see Methods), but not with IFC. This preparation had high specific activity (0.1 mCi/mg), and all of the proteins in the extract were labeled (data not shown). When the [^{3}H]propionylated extract was incubated with the anti-IFC immunoadsorbents under conditions where over 50% of the ^{125}IFC-proteins bound, only 0.2% of the ^{3}H-labeled proteins bound. This is nearly the same as the 0.1% binding of ^{3}H-proteins that was observed for underivatized Sepharose 4B, and demonstrates that the anti-IFC immunoadsorbents exhibited little nonspecific protein binding. The determination of ^{3}H-protein (i.e., nonspecific) binding also provided a means to calculate the extent of ^{125}IFC-protein

TABLE I

SPECIFICITY OF DETERGENT-SOLUBILIZED ^{125}IFC-SPERM PROTEIN BINDING TO ANTI-IFC IMMUNOADSORBENTS

Experiments with IFC-lysine and IFC-bovine serum albumin were performed by preincubating the immunoadsorbents for 90min at room temperature and then proceeding with the basic binding experiments as described in the Methods. The IFC-lysine concentration was 7.5 mM and IFC-bovine serum albumin was at 5 mg/ml (equivalent to 42 μM IFC). The immunoadsorbents were prepated to contain 2.0 ± 0.2 mg protein/ml resin. The numbers are averages of duplicate determinations.

Resin	% Bound		
	Control	+ IFC-lysine	+ IFC-bovine serum albumin
Sepharose 4B	0.5 ± 0.0	–	–
Preimmune-Sepharose 4B	2.6 ± 0.1	–	–
Immune-Sepharose 4B	50 ± 2.8	2.8 ± 0.3	11.7 ± 0.5
Affinity purified immune-Sepharose 4B	55 ± 1.6	6.7 ± 1.1	20 ± 0.4

enrichment in the immunoaffinity protocol; thus:

$$\text{fold enrichment} = \frac{\%\ ^{125}\text{IFC-protein bound}}{\%\ ^{3}\text{H-protein bound}}$$

$$= \frac{50\%}{< 0.2\%} \geq 250 \text{ fold}$$

Even higher recoveries (65–70%) of the ^{125}IFC-proteins were obtained if the resin volume was kept above 50% (v/v) in batch incubations or by passing the ^{125}IFC-proteins over an anti-IFC Sepharose resin column.

Although the anti-IFC immunoadsorbents allowed recovery of ^{125}IFC-labeled proteins from crude mixtures, and exhibited little nonspecific binding, elution of the bound ^{125}IFC-proteins was difficult. Many reagents commonly used to dissociate antigen-antibody complexes [36] were not effective in removing the bound ^{125}IFC-labeled species from the immunoadsorbent: 1 M NaCl, pH 10.3; 8 M urea; 2% Nonidet P-40; 2% Nonidet P-40, pH 10.3; 3 M KSCN; 2% SDS, 2% β-mercaptoethanol; and 0.5 M acetic acid each eluted less than 10% of the radioactivity. Guanidine-HCl (6 M) was only partially effective, releasing 30–40% of the radioactivity. However, by boiling the resins in SDS sample buffer (2% SDS, 2% β-mercaptoethanol, 50 mM Tris, pH 6.8, 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride), approx. 80% of the radioactivity was eluted, permitting a direct comparison between the bound material and the original extract on SDS gels (Fig. 3). As can be seen by comparing lane a with lanes c and e, the ^{125}IFC-labeled proteins in the original extract were recovered in the material eluted from both an immunoadsorbent prepared with the $(NH_4)_2SO_4$ fraction of anti-IFC serum and an immunoadsorbent prepared with the affinity purified anti-IFC. Although most major ^{125}IFC-labeled species were recovered from the resin, some labeled polypeptides above 100 kDa were under-represented (Fig. 3, compare c or e with a). The non-binding fraction had an ^{125}IFC-protein composition like that of the original extract (Fig. 3, lanes b and d). Elution of the immunoadsorbents with SDS sample buffer also released IgG, which resulted in some distortion in the ^{125}IFC-labeled protein bands migrating at the same position on SDS gels and thus limited the amount of sample that could be applied to the SDS gel.

Characterization of antisera produced against the isolated sperm proteins

The IFC-labeled sperm proteins isolated by the anti-IFC immunoaffinity technique were used to immunize rabbits in order to produce antisera against the proteins themselves. Immunization was with an IFC-protein: immunoadsorbent complex as in Fig. 3 lanes c and e to minimize the requirement for antigen (less than 100 μg) and increase the immunogenicity [36,45–47].

Antibodies directed against the sperm proteins were detected by an ELISA [38,39]. As shown in Fig. 4a and b, respectively, two rabbits injected with the complexes made high titer antibodies against sperm proteins, with significant activity still evident at dilutions of 1/6250. Since the pre-

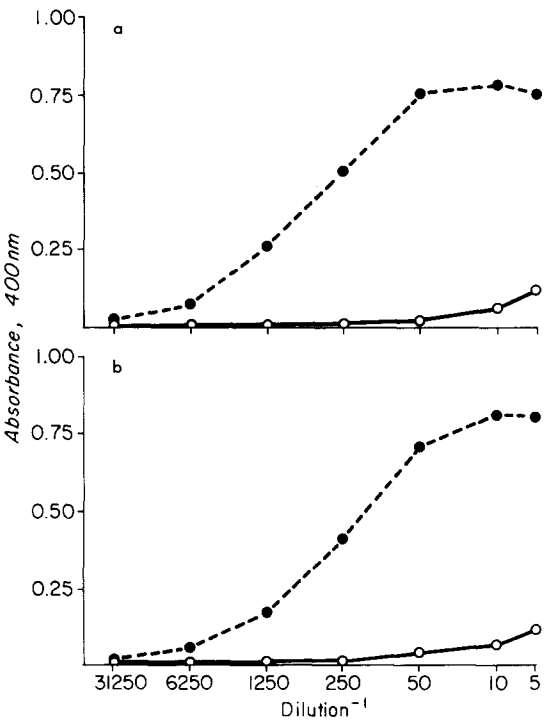

Fig. 4. ELISA of antisera obtained from rabbits B and D. The ordinate shows the absorbance (A_{400}) of the *p*-nitrophenol released from *p*-nitrophenyl phosphate by alkaline phosphatase coupled to goat anti-rabbit IgG. (a) rabbit B: O——O, $(NH_4)_2SO_4$ fraction of preimmune serum; ●- - - - -●, $(NH_4)_2SO_4$ fraction of immune serum; (b) rabbit D. O——O, $(NH_4)_2SO_4$ fraction of preimmune serum; ●- - - - -● $(NH_4)_2SO_4$ fraction of immune serum. Each well was coated with 200 μl (40 μg/ml) of detergent-solubilized proteins from unlabeled sperm.

immune antisera gave significant background values at the lower dilutions, comparison of different antiserum preparations was made by using the ratio of the reactivity (as A_{400}) of immune and preimmune sera at dilutions of 1 to 250, near the midpoint of the ELISA curves. For the antisera shown in Fig. 4, the immune to preimmune ratio was 51 for rabbit B and 68 for rabbit D. Lower titer antisera gave ratios of 20–40.

Both rabbit IgG and IFC were components of the immunoadsorbent complex injected into the rabbits, thus the rabbits might have also responded to these antigens. By double-diffusion analysis, we found that some low titer antibody was produced against IFC, but none to rabbit IgG.

Identification of the individual antigenic species in the unlabeled sperm extract was performed by western blot analysis, in order to compare the polypeptides detected by antisera in unlabeled sperm with those polypeptides originally labeled with ^{125}IFC. Fig. 5 shows a comparison of the detergent-extracted ^{125}IFC-sperm proteins transferred to nitrocellulose (lane c) with the proteins detected by antisera from rabbit B (lanes f, g) or D (lanes d, e), using ^{125}I-protein A. The comparison was made with an unstained blot of detergent-extracted ^{125}IFC-sperm proteins, since shrinkage occurred during staining of the gel for protein (compare lanes a and b with c). Virtually all of the

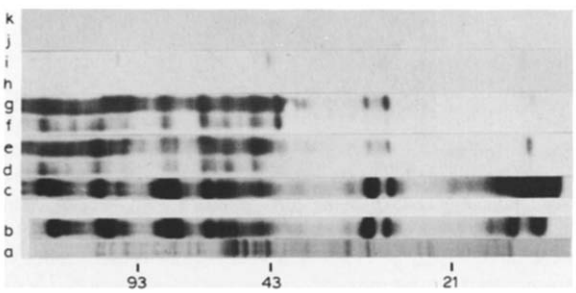

Fig. 5. Analysis of rabbit B and D antisera specificity with ^{125}I-protein A on SDS-gels transferred to nitrocellulose paper. A 7.5–15% gradient SDS-gel was run on the detergent-solubilized proteins from unlabeled sperm (lanes c–k) and ^{125}IFC-labeled sperm (lanes a–c) and then transferred to nitrocellulose paper. Strips of the nitrocellulose, containing material transferred from the SDS-gel, were then incubated with $(NH_4)_2SO_4$ fractions of sera from rabbits B and D and then 0.1 μCi of ^{125}I-protein A. Lanes d, f, h, and j received 3 μg protein and lanes e, g, i, and k received 12 μg protein. Lanes d, e: rabbit D immune serum, 1/1000 dilution; lanes f, g: rabbit B immune serum, 1/2000 dilution; lanes h, i: rabbit D preimmune serum, 1/1000 dilution; lanes j, k: rabbit B preimmune serum, 1/2000 dilution; lane c is the detergent-solubilized proteins from ^{125}IFC-labeled sperm (60000 cpm, 10 μg protein) that have been transferred to nitrocellulose, air-dried, and autoradiographed. For comparison, lane a and b contain the same material (60000 cpm, 10 μg) that has been transferred to nitrocellulose and then stained with amido black (lane a) before autoradiography (lane b). Molecular weight markers in kilodaltons are shown on the bottom of the figure.

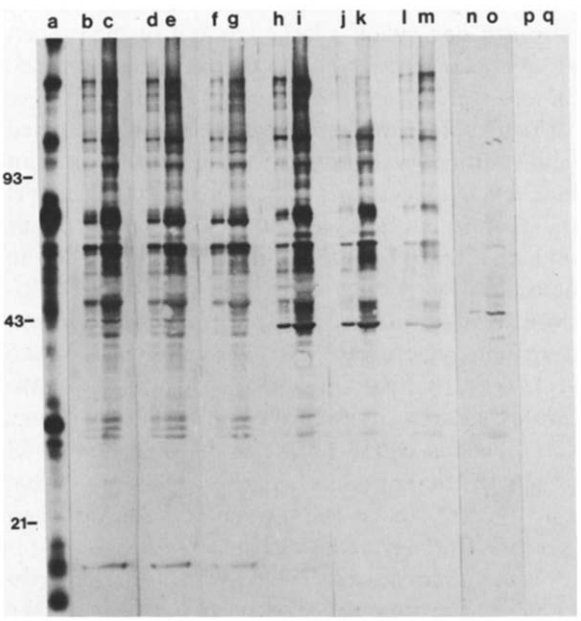

Fig. 6. Analysis of proteins detected by antisera from rabbit B and D with goat anti-rabbit IgG conjugated to peroxidase. A 7.5–15% gradient SDS-gel was run and then transferred to nitrocellulose. Strips of the nitrocellulose were then incubated with $(NH_4)_2SO_4$ fractions of each serum and then with a 1/1000 dilution of goat anti-rabbit IgG conjugated to peroxidase. The bound peroxidase was then identified with 4-chloro-1-naphthol and H_2O_2. Each strip contained 2 lanes of the detergent-solubilized proteins from unlabeled sperm: lanes b, d, f, h, j, l, n, p received 5 μg protein and lanes c, e, g, i, k, m, o, q received 25 μg protein. Lanes b, c: rabbit D immune serum, 1/125 dilution; lanes d, e: rabbit D immune serum, 1/500 dilution; lanes f, g: rabbit D immune serum, 1/1000 dilution; lanes h, i: rabbit B immune serum, 1/125 dilution; lanes j, k: rabbit B immune serum, 1/500 dilution; lanes l, m: rabbit B immune serum, 1/1000 dilution; lanes n, o: rabbit D preimmune serum, 1/1000 dilution; lanes p, q: rabbit B preimmune serum, 1/1000 dilution. Lane a is the detergent-solubilized proteins from ^{125}IFC-labeled sperm transferred to nitrocellulose and then dried and autoradiographed. Size markers in kilodaltons are shown on the left-hand side of the figure.

proteins identified by both antisera with immunoblotting were those originally labeled with IFC in the immunogen (Fig. 5). There was very little reactivity with preimmune serum (lanes h–k). One major band detected by rabbit B (40 kDa) did not seem to align with any major radioactive species found in the ^{125}IFC-sperm proteins; however, slight reactivity toward this species was observed even in preimmune serum from the same rabbit, although not at the dilutions shown. Both antisera detected a species of approx. 85–90 kDa that was not observed in the ^{125}IFC proteins, although only when a large amount of the extract of unlabeled sperm was run on the gel (lanes e and g).

Fig. 6 shows that similar proteins were detected with rabbit B or D antisera when goat anti-rabbit IgG conjugated to peroxidase was used to detect the primary antibody binding; the sharper bands obtained with this technique made it easier to detect minor species that were occasionally difficult to align with species appearing in the autoradiograms, which were of lower resolution. Fig. 6 also shows that with higher concentrations of antisera no new bands were observed, although there was a general enhancement of the detection of all polypeptides.

Reaction of the antisera with intact sperm

Sperm components labeled with fluorescein derivatives have unusual properties when studied after fertilization of eggs. Some of the labeled sperm components enter the egg to persist as a localized patch in the embryo cytoplasm, as detected by fluorescence microscopy or autoradiography [24,31]. Previous work had indicated that FITC or IFC labeled sperm at the cell surface [29,30], so that the internalization after fusion of sperm with eggs was a surprising and interesting finding [31]. The antisera directed against the proteins that were targets for FITC- or IFC-labeling, characterized in Figs. 5 and 6, allowed us to localize these proteins in the sperm, by using immunofluorescence. The pattern of fluorescence exhibited by sperm labeled with antiserum from rabbit B and D is typical of membrane fluorescence, with the whole sperm membrane being labeled (Fig. 7a and e). Controls with preimmune sera showed no staining (Fig. 7c and g). To detect

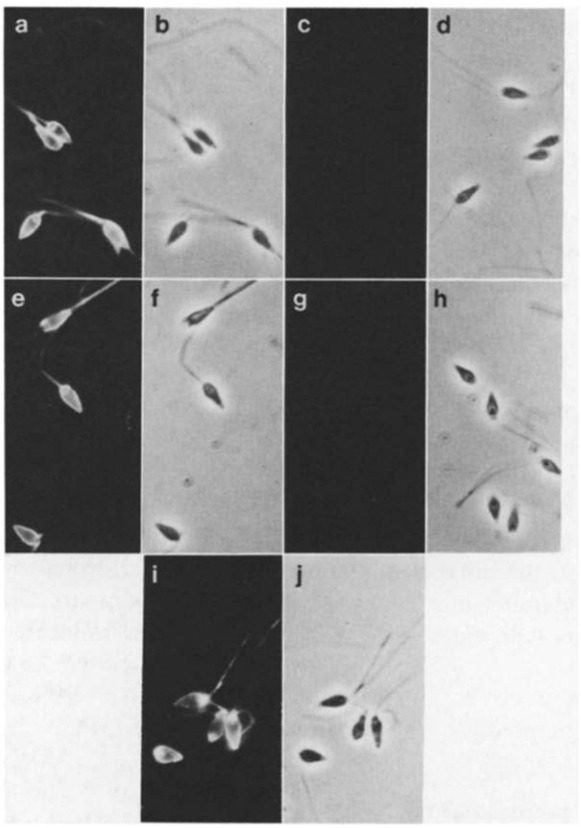

Fig. 7. Indirect immunofluorescence of $(NH_4)_2SO_4$ fractions of rabbit B and D sera on fixed sea urchin sperm. The right hand side of each pair is the corresponding phase micrograph. All sperm were fixed with paraformaldehyde. Sperm in i and j were subsequently permeabilized with acetone. Fluorescent exposures were 5 s for immune sera, except i which was 20 s, and 10 s for preimmune sera; phase exposures were approx. 1 s. (a, b) rabbit B immune serum, 1/40 dilution; (c,'d) rabbit B preimmune serum, 1/40 dilution; (e, f) and (i, j) rabbit D immune serum, 1/40 dilution; (g, h) rabbit D preimmune serum, 1/40 dilution.

antigens at intracellular sites, the fixed sperm were made permeable by treatment with acetone at −20°C. The patterns obtained for these permeable cells indicated that no new sites were found with antiserum from rabbit D (Fig. 7i) or rabbit B (data not shown). Similar results have been observed with sperm made permeable with methanol fixation at −15°C. Therefore, the cellular locus for the antigens detected with the two antisera seems to be exclusively within the plasma membrane, in agreement with other data [29,30].

Discussion

This paper outlines a novel method for the preparation of antiserum to proteins derivatized with a covalent label. By utilizing immunoadsorbents prepared from antisera directed against the label, IFC, we have been able to purify enough of the derivatized proteins to produce antisera against the protein moieties themselves. These antisera provide reagents with which to study the proteins in their native state. In this case, the protein antigens appear to be principally in the plasma membrane.

The purification of the IFC-labeled proteins from sperm was accomplished with immunoadsorbents directed against IFC. The feasibility of such an approach was suggested by a paper in which anti-dinitrophenol and anti-azobenzenearsonate immunoadsorbents were used to purify, in one step, modified peptides generated by limited proteolysis of the modified proteins [33]. IFC has the advantage of being a milder reagent which can be used on living cells [24,30–32] and can also be prepared to high specific activity (using ^{125}I), permitting analysis of small amounts of the labeled species [29,30]. Antibodies that react with IFC can be purified by using mild elution conditions (1.4 M NaCl) and a commercially available resin (Affigel Blue). We do not understand the nature of the interaction between the anti-IFC antibodies and the Affigel Blue, although it is possible that the fused, three-ringed structures of Cibacron Blue F3Ga (the dye on the Affigel Blue) and IFC are similar.

The immunoadsorbents prepared by coupling either an $(NH_4)_2SO_4$ or affinity purified fraction of anti-IFC antiserum were effective in recovering the IFC-labeled proteins from a crude detergent extract of sperm: yields of up to 70% and an estimated purification of over 250-fold were obtained in a single step. All of the major ^{125}IFC-labeled proteins in the detergent extract were retained by the anti-IFC immunoadsorbents, demonstrating a lack of selectivity of the immunoadsorbents for a particular labeled species (see Fig. 3). The specificity of the resins was shown by the absence of binding of ^{125}IFC-sperm proteins to either underivatized Sepharose or an $(NH_4)_2SO_4$ fraction of pre-immune serum coupled to Sepharose, as well as the ability of IFC-lysine and iFC-bovine serum albumin to reduce the binding of the ^{125}IFC-sperm proteins. Nonspecific protein binding (binding of unlabeled proteins) to the immunoadsorbents was negligible as shown by the lack of binding of a detergent extract of sperm proteins labeled with [^{3}H]succinimidyl propionate.

A drawback to using the anti-IFC immunoadsorbents was the difficulty in eluting the bound IFC-proteins; none of the commonly used antigen-antibody dissociation reagents eluted ^{125}IFC-proteins from the immunoadsorbents (see Results), although an analysis of the bound species was possible by boiling the immunoadsorbents in SDS sample buffer. The drastic conditions necessary to elute the ^{125}IFC-proteins from the immunoadsorbents may reflect the high affinity constant of anti-IFC antibodies; antibodies raised against the related hapten, FITC, have been shown to have unusually high affinity constants [49,50].

By injecting the purified IFC-labeled proteins as a complex with the immunoadsorbents we were able to obtain antisera that were reactive against the protein moieties of the IFC-labeled proteins at relatively high dilutions; the midpoint of the dilution curve from an ELISA was 1/250, and significant activity was detected at dilutions up to 1/6250 (see Fig. 4). Similarly, western blot analysis could be performed at antisera dilutions up to 1/2000 (see Fig. 5). The western blot analysis showed that antibodies were elicited to about 80% of the major species derivatized with IFC. Only one major band found by immunoblotting did not align with a radioactive band from the concentrated detergent extract of ^{125}IFC-labeled sperm. Although this reactivity may have resulted from the production of antibody to an unlabeled protein that was nonspecifically bound to the resin, it might also represent antibody produced to an unlabeled subunit of an oligomeric protein that was labeled in another subunit. The high sensitivity of the immunoblotting protocol, estimated at 100 pg [41], may be capable of detecting species that are not resolved in the autoradiogram of the ^{125}IFC-labeled proteins (see Fig. 6).

Thus, the antisera obtained from rabbits B and D seem to be excellent reagents for identification of the proteins that were initially labeled with the fluorescein derivatives. Their reactivity with intact

sperm was tested by immunofluorescence (Fig. 7), where the antigens were detected on the sperm surface. When acetone was used to make the fixed sperm permeable, in a protocol used to demonstrate intracellular actin in marine sperm [43], no further sites of fluorescent labeling were found (Fig. 7). Additionally, in preliminary experiments on absorption of the reactivity of the antisera, essentially all the immunoreactivity was absorbed by fixed, nonpermeabilized sperm (Gundersen, unpublished data). Finally, in a separate study, we have found that almost all the ^{125}IFC-labeled polypeptides in the sperm are sensitive to externally added proteases (unpublished data). These data support our previous findings by immunoelectron microscopy [30] that much, if not all, of the labeling with fluorescein derivatives was on the sperm surface.

The IFC-labeling technique described in this paper provides a direct means of assessing both the purity and recovery of small amounts of surface-labeled material, which can then be used as an immunogenic vehicle. Although we were interested in obtaining antibody against all the species we had labeled, it would be possible to use the IFC-labeled protein: immunoadsorbent complex as an immunogenic vehicle for the production of monoclonal antibodies to isolate individual species. Another means of obtaining monospecific antibody probes is to use bands from nitrocellulose blots to purify single antibody specificities [51], and using this technique we have obtained monospecific antibodies against several of the labeled proteins (Gundersen, unpublished data). With the purification scheme presented in this paper, FITC and ^{125}IFC provide not only a valuable means of following surface-labeled proteins by fluorescence and radioactivity, but permit their isolation and characterization.

Acknowledgments

We are grateful to Dr. C.A. Gabel for much useful advice and insight, to Michael Kalnoski for lively technical assistance, and to Pamela Horbett and Mary Patella for careful typing of the manuscript. This research was supported by NSF Grant PCM 7720472 to B.M.S.

References

1 Zukin, R.S., Hartig, P.R. and Koshland, D.E. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1932–1936
2 Huang, K.H., Fairclough, R.H. and Cantor, C.R. (1975) J. Mol. Biol. 97, 443–470
3 Vanderkooi, J.M., Ierokomas, A., Nakamura, H. and Martonosi, A. (1977) Biochemistry 16, 1262–1267
4 Stryer, L. (1978) Annu. Rev. Biochem. 47, 819–846
5 Pick, U. and Karlish, S.J.D. (1980) Biochim. Biophys. Acta 626, 225–261
6 Muallem, S. and Karlish, S.J.D. (1983) J. Biol. Chem. 258, 169–175
7 Taylor, D.L. and Wang, Y.-L. (1980) Nature 284, 405–410
8 Wang, Y.-L., Heiple, J.M. and Taylor, D.L. (1982) Methods Cell Biol. 25, 1–11
9 Simpson, I., Rose, B. and Loewenstein, W.R. (1977) Science 195, 294–296
10 Feramisco, J.R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3967–3971
11 Kreis, T.E., Winterhalter, K.H. and Birchmeier, W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3814–3818
12 Nairn, R.C. (1976) Fluorescent Protein Tracing, Churchill Livingstone, Edinburgh
13 Yahara, I. and Edelman, G.M. (1973) Exp. Cell Res. 81, 143–155
14 Condeelis, J.R. (1979) J. Cell. Biol. 80, 751–758
15 Schreiner, G.F. and Unanue, E.R. (1976) Adv. Immunol. 24, 38–165
16 Frye, L.D. and Edidin, M. (1970) J. Cell. Sci. 7, 319–335
17 Edidin, M., Zagayansky, Y. and Lardner, T.J. (1976) Science 191, 466–468
18 Peters, R., Peters, J., Tews, K. and Bähr, W. (1974) Biochim. Biophys. Acta 367, 282–294
19 Schlessinger, J., Axelrod, D., Koppel, D.E., Webb, W.W. and Elson E.L. (1977) Science 195, 307–309
20 Cherry, R.J. (1978) Methods Enzymol. 54, 47–61
21 Cherry, R.J., Nigg, E.A. and Beddard, G.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5899–5903
22 Peters, R., Brünger, A. and Schulten, K. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 962–966
23 De Luca, N., Bzik, D., Person, S. and Snipes, W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 912–916
24 Gabel, C.A., Eddy, E.M. and Shapiro, B.M. (1979) Cell 18, 207–215
25 Nigg, E., Kessler, M. and Cherry, R.J. (1979) Biochim. Biophys. Acta 550, 328–340
26 Lepock, J.R., Thompson, J.E., Kruuv, J. and Wallach, D.F.H. (1978) Biochem. Biophys. Res. Commun. 85, 344–347
27 Wolf, D.E., Edidin, M. and Dragsten, P.R. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2043–2046
28 Sheetz, M.P. and Koppel, D.E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3314–3318
29 Gabel, C.A. and Shapiro, B.M. (1978) Anal. Biochem. 86, 396–406
30 Gabel, C.A., Eddy, E.M. and Shapiro, B.M. (1979) J. Cell. Biol. 82, 742–754

31 Gundersen, G.G., Gabel, C.A. and Shapiro, B.M. (1982) Dev. Biol. 74, 86–100
32 Shapiro, B.M., Gundersen, G.G., Gabel, C.A. and Eddy, E.M. (1982) in Cell Recognition (Frazier, W.A., Glaser, L. and Gottlieb, D.I., eds.), pp. 833–844, Alan R. Liss, New York
33 Wilchek, M., Bocchini, V., Becker, M. and Givol, D. (1971) Biochemistry 10, 2828–2834
34 Shapiro, B.M. (1975) Dev. Biol. 46, 88–102
35 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–278
36 Garvey, J.S., Cremer, N.E. and Sussdorf, D.H. (1977) Methods in Immunology, W.A. benjamin, Inc., Reading, MA
37 March, S.C., Parikh, I. and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149–152
38 Engvall, E. (1980) Methods Enzymol. (Van Vanakis, H. and Langone, J.J., eds.), Vol. 70, pp. 419–439, Academic Press, New York
39 Atherton, B.T. and Hynes, R.C. (1981) Cell 25, 133–141
40 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203
41 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354
42 Laemmli, U.K. (1970) Nature 227, 680–685
43 Kleve, M.G. and Clark, W.H., Jr. (1980) J. Cell Biol. 86, 87–95
44 Tornqvist, H. and Belfrage, P. (1976) J. Lipid Res. 17, 542–545
45 Maurer, P.HJ. and Callahan, H.J. (1980) Methods Enzymol (Van Vanakis, H. and Langone, J.J., eds.), Vol. 70, pp. 49–70, Academic Press, New York
46 Crowle, A.H., Revis, G.J. and Jarret, K. (1972) Immunol. Commun. 1, 325–336
47 Kroll, J. and Anderson, M.M. (1976) J. Immunol. Methods 13, 125–130
48 Bigley, M.J. (1975) Immunological Fundamentals, pp. 14–23, Year Book Medical Publishers, Inc., Chicago, IL
49 Watt, R.M. and Voss, E.W., Jr. (1978) Immunochem. 15, 875–882
50 Protmann, A.J., Levison, S.A. and Dandliker, W.B. (1971) Biochem. Biophys. Res. Comm. 43, 207–212
51 Krohne, G., Stick, R., Kleinschmidt, J.A., Moll, R., Franke, W.W. and Hansen, P. (1982) J. Cell Biol. 94, 749–754